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Identification and characterization of novel dual-function antioxidant and umami peptides from protein hydrolysates of Huangjiu lees

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Abstract

Objectives: Lees are the major by-product of Huangjiu brewing and are prone to decomposition due to abundant protein residues. This study aims to convert the lees into high-value peptides with umami and antioxidant difunctionals.

Materials and Methods: Herein, a one-step hydrolysis method combined with flavorzyme followed by amylase pretreatment was developed and optimized. The target peptide fraction was collected and evaluated after ultrafiltration and ethanol precipitation, and then identified by nano-high-performance liquid chromatography–tandem mass spectrometry. The target peptides were filtered through virtual screening, sensory, and radical scavenging verification.

Results: The results show that 80 U/g amylase and 3500 U/g flavorzyme synergistic hydrolysis at 50 °C for 3 h performed best for umami intensity and antioxidant activity. A total of 5266 peptides was identified from the 80% ethanol precipitation fraction, finally secreened 7 umami peptides. The umami recognition threshold of the 7 peptides ranged from 0.38 to 0.66 mmol/L in water. Among them, DPDGW and DNPNW exhibited good DPPH antioxidant ability with IC_{50} values of 0.6982 mg/mL and 0.4315 mg/mL, respectively. Additionally, molecular docking studies indicated that all umami peptides tend to interact with the T1R3 receptor through hydrogen bonds and van der Waals forces, involving key residues such as ASN68, SER104, HIS145, SER276, VAL277, GLU301, ALA302, THR305, and HIS387.

Conclusion: This study shows that Huangjiu lees is a potential resource for flavor and bioactive peptide development, which provides a reference for other waste protein recycling.

Keywords: Umami peptides; antioxidant; sensory evaluation; molecular docking; Huangjiu lees.

Introduction

Huangjiu, a traditional fermented alcoholic beverage in China, has been consumed widely for more than 2000 a. This beverage is primarily produced by solid-state fermentation with rice, wheat Qu, and yeast, simultaneously. However, the fermentation process fails to fully utilize the proteins of the raw grain materials, leading to a subsequent generation of solid residues during post solid–liquid separation process, and these residues are by-products known as lees. The yield rate of lees is approximately 20%–30%, and the annual output can reach 3.5 million tons (Zhang *et al.*, 2023a). Because lees are rich in nutritional ingredients such as moisture, proteins, and starch, they are prone to spoilage by the action of environmental microorganisms. Conventionally, lees have been used for animal feed, vinegar production, and as a substrate for cultivating edible fungi, yet these utilization methods have limited economic returns. Considering the abundant protein content (13.70%–41.30%) of lees and the context of emerging health food trends, the derivation of functional peptides from plant-based protein is becoming an effective way to develop high-value products (Zhang *et al.*, 2022a). Functional peptides, with a molecular weight of less than 3000 Da, have been reported with many bioactive functions, including antioxidant, antihypertensive, antidiabetic, and immune-boosting functions (Chai *et al.*, 2020; Yuan *et al.*, 2022; Suryaningtyas and Je, 2023). Besides, low-molecular-weight peptides also act as crucial flavor precursors or enhancers because of their natural, nutritious, and safe qualities (Yu *et al.*, 2018; Le *et al.*, 2022). Some peptides could also improve the umami intensity with glutamate or nucleotide. The origins of taste peptides are varied and predominantly sourced from plants, animals, aquatic products, and edible fungi (Wang *et al.*, 2020; Ning *et al.*, 2021).

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In our previous study, we discovered several umami peptides in Huangjiu, indicating that lees could be a potential source of functional peptides and flavor enhancers. Meanwhile, to date, there are few studies on lees umami peptides. The main steps for taste peptide preparation include production, separation, and identification. The ways to produce umami peptides could include extraction from the food matrix, hydrolysis of raw proteins, or heterogeneous expression *in vivo*. Conventional enzymatic conversion has the advantage of being environmentally friendly and ease to pilot test. It provides an opportunity for sustainable and recyclable use of Huangjiu lees. However, the matrix of enzymatic hydrolysate was complex and may decrease the peptide separation efficiency. Thus, it should be better guided by the sensory or physical properties. Common methods include membrane filtration, precipitation, adsorption chromatography, extraction, and ion exchange (Liu *et al*., 2020a; Liu *et al*., 2020b).

Mass spectrometry is a classical method in peptide identification, but traditional methods such as electron spray ionization–tandem mass spectrometry (ESI-MS/MS) face limitations in sequence detection due to equipment precision and data processing algorithms (Qi *et al*., 2022). Recently, peptidomics based on the nano-liquid chromatography–mass spectrometry (nano-LC-MS) has increased the attainable range of peptide information from samples. For instance, 208 candidate umami peptides were rapidly identified in chicken soup (Zhang *et al*., 2023b).

Umami peptides, due to their intrinsic groups and amino acid sequences, may also benefit other bioactives. For example, peptides under 1000 Da from the hydrolysis products of pig bone protein by flavorzyme and papain exhibited 62.9% 1,1-diphenyl-2-trinitrophenylhydrazine (DPPH) free radical scavenging activity and scored 8.9 out of 10 on sensory umami evaluation (Shen *et al*., 2021). According to the study of Chen *et al*. (2021), umami peptide IPIPATKT could even inhibit the activity of dipeptidyl peptidase-IV and angiotensin-I-converting enzymes in mouse and cell models. Additionally, umami peptides with no bioactivity can become bioactive fragments after *in vivo* metabolism.

Hao *et al*. (2020) digested 12 ham water-soluble extracts of umami peptides *in vivo* and found three novel peptides that manifested antihypertensive and antioxidant activity. Overall, these findings pointed to the potential of umami peptides as a novel source of antioxidants.

In the present study, the enzymatic hydrolysis of lees was developed and fine-tuned to yield better umami taste and antioxidant properties. Target peptide fractions were enriched by ethanol precipitation. Integrative methods including nano-HPLC-MS/MS, virtual screening, along with sensory evaluation and free radical scavenging tests, facilitated the identification of novel umami peptides with antioxidant activity. Furthermore, molecular docking provided insight into the interactions between umami peptides and T1R1/T1R3 receptors, suggesting avenues for the high-value application of lees by-products.

Materials and Methods

Materials

Lees were procured from a traditional Huangjiu manufacturer (Shaoxing, China), and the basic information is listed in Table S1. Enzymatic reagents were obtained from commercial sources: medium-temperature amylase, pepsin, alkaline protease, neutral protease, papain (Solarbio Science & Technology Co., Ltd., Beijing, China), and flavorzyme (Dongheng Huadao Biotechnology Co., Ltd., Nanning, China). These enzyme details are shown in Table S2. Analytical-grade reagents included sodium hydroxide, sulfuric acid, potassium sulfate, anhydrous glucose, ethanol, ethyl ether, DPPH, and reduced glutathione (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Chromatography-grade formic acid and acetonitrile were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Food-grade sodium chloride, citric acid, sucrose, quinine, glutathione, and sodium glutamate were obtained from Shanghai Pengheng Biotechnology Co., Ltd. (Shanghai, China). Candidate synthetic peptides, assured of 98% purity, were synthesized (Shanghai Gill Biochemical Co., Ltd., Shanghai, China).

Composition analysis of lees

The moisture, crude protein, and starch contents of lees were quantified. Moisture content was deduced from the mass loss upon drying at 105 °C until a constant weight was achieved (GB5009.3-2016). Crude protein levels were ascertained utilizing the Kjeldahl method (GB5009.5-2016). Starch content assessment adhered to the protocols outlined in the Chinese national standard GB5009.9-2016. Briefly, a measured quantity of lees was pulverized and defatted using 50 mL of ether, and soluble sugars were eliminated through a 100-mL 85% ethanol wash. The ethanol volatilized sample, post-gelatinization, underwent α-amylase hydrolysis, after which the starch content was estimated from the total sugar present post-hydrolysis.

Optimization of amylase pretreatment in lees

To optimize the release conditions of proteins from lees, an amylase pretreatment was implemented, modifying the methodology of Liu *et al*. (2020b). A 20-g sample of lees was subjected to a raw material-to-water ratio of 1:10 and thoroughly mixed. Enzymatic hydrolysis was then initiated across a spectrum of enzyme concentrations (0, 20, 40, 60, 80, and 100 U/g of lees dry weight), adhering to manufacturer-provided optimal conditions. The hydrolysis was conducted at 45 °C for 3 h, and the pH was adjusted to the enzyme's optimal range using a 0.1 mol/L NaOH solution. The hydrolysis reaction was quenched by heating the mixture at 95 °C for 10 min. Then, the mixture was centrifuged (5000×*g*, 10 min), and the clear supernatant was used to measure the total peptide content to screen the most effective amylase concentration. Peptide quantification was performed through a modified Lowry procedure (Markwell *et al*., 1978).

Optimal hydrolase selection for lees

A suite of commercial proteases, specifically pepsin, flavorzyme, neutral protease, alkaline protease, and papain, were selected to process lees. For uniformity across trials, a 20-g portion of the lees was used, maintaining a ratio of 1:10 with water. Adhering to manufacturer-recommended protocols, a dosage of 1500 U/g, relative to the lees' dry weight, was employed for each enzyme. The pH for each protease reaction was fine-tuned using a 0.1 mol/L NaOH solution to achieve optimal enzymatic conditions, followed by hydrolysis

at a steady temperature of 45 °C for 3 h. Enzymatic reactions were ceased by heating the mixture to 95 °C for 10 min. The post-reaction mixture was centrifuged at 5000×*g* for 10 min, after which the supernatant was filtered to yield the hydrolysate. This filtrate was then subjected to determine the total peptide content and DPPH radical scavenging activity. Complementing these analyses, sensory evaluations were applied to compare the taste characteristics inherent in each hydrolysate.

Optimization of dual-enzyme combination for lees treatment

Under the established optimal pretreatment and protease hydrolysis conditions, a dual-enzyme treatment was performed. A serial of parameters including hydrolysis sequence (single-step and two-step), enzymolysis time (1, 2, 3, 4, and 5 h), and protease dosage (750, 1500, 3000, and 4500 U/g) were investigated. For single-step enzymolysis, amylase and protease were simultaneously introduced into the reaction system. Conversely, in the two-step enzymolysis, the protease was applied subsequent to the amylase treatment. Conditions for enzymolysis were maintained as previously described. After the reaction was completed, the supernatant was harvested and subjected to measurement of total peptide content and evaluation of DPPH radical scavenging activity. Optimal conditions for the dual-enzyme combination were determined based on peptide content and antioxidant activity.

Extraction of umami peptides fraction from lees hydrolysate

According to the optimized conditions of the two-enzyme hydrolysis combination for lees, the crude hydrolysate was prepared. The peptide molecular weight of the lees hydrolysis was measured according to Wen *et al*. (2022). The hydrolysate was fractionated using an ultrafiltration membrane with a cut-off of 3000 Da, and fractions smaller than 3000 Da were collected, named HJZUF. The HJZUF fraction was subjected to gradient precipitation at ethanol concentrations of 20%, 40%, 60%, and 80% for 12 h at 4 °C, and the precipitated crude peptide fractions HJZ20, HJZ40, HJZ60, and HJZ80 were obtained sequentially. After freeze-drying, the fractional powders were dissolved in ethanol aqueous solution or suspended in pure water for DPPH radical scavenging ability and sensory evaluation, respectively.

Identification of umami peptides from lees hydrolysate

The umami-dominant peptide fraction, as determined via sensory evaluation, was identified through nano-HPLC-MS/MS referring to our previous study (Chang *et al*., 2023). This sample was dissolved in a solution containing 0.1% formic acid (mobile phase A), followed by analysis using LC-MS/MS outfitted with an online nanoflow electrospray ionization source. The integrated system consisted of a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) connected to an EASY-nanoLC 1200. A total of 6 μL sample was applied to an Acclaim PepMap C18 analytical column (75 μm×25 cm). The separation occurred over a 60-min gradient at a meticulously maintained column flow rate of 300 nL/min. With the column temperature set at 40 °C and the electrospray voltage at 2 kV, the gradient commenced at 2% phase B (comprising 80% acetonitrile and 0.1% trifluoroacetic acid) and increased non-linearly to 35% over 47 min. Subsequently, the gradient increased sharply to 100% within 1 min and was maintained at this level for 12 min. The mass spectrometer was operated in data-dependent acquisition (DDA) mode, automatically switching between MS and MS/MS acquisition phases. The mass spectrometry parameters were as follows: (1) for MS, a scan range of 200–1800 *m*/*z* with a resolution of 70 000; an automatic gain control (AGC) target of 3×10^6; and a maximal injection time of 50 ms; (2) for higher-energy collisional dissociation (HCD) MS/MS, the resolution was set at 17 500, with an AGC target of 1×10^5, a maximal injection time of 45 ms, collision energy of 28, and a dynamic exclusion time of 30 s. The tandem mass spectra were processed utilizing PEAKS Studio (version 10.6; Bioinformatics Solutions Inc., Waterloo, Canada), with a setting for non-enzymatic digestion, facilitating peptide identification. The search was conducted against the UniProt protein database, specifying rice, wheat, and yeast, the primary constituents of Huangjiu, as the species to construct the protein search library. The threshold for peptide confidence was set with a –10lg *P* value exceeding 20, ensuring a robust level of identification stringency.

Umami peptide screening and molecular docking

Umami peptide prediction

Peptides with a sequence length ≤10 amino acids were selected for further analysis, aligning with the characteristic lengths of most umami peptides reported in the literature. These sequences were subjected to umami prediction using the Umami_YYDS computational tool (http://www.tastepeptides-meta.com; Cui *et al*., 2023). Subsequently, peptides exhibiting potential umami properties were assessed for their antioxidant capabilities utilizing the AnOxPePred platform (https://services.healthtech.dtu.dk/services/AnOxPePred-1.0/). Molecular docking was performed with the T1R1/T1R3 umami receptors, and peptides were further filtered based on their docking affinity rankings (Olsen *et al*., 2020).

Homology modeling of umami receptors

Following the methodology outlined by Zhang *et al*. (2023c), homology modeling was performed using the metabotropic glutamate receptor (PDB code: 6N51) as a structural template due to higher sequence coverage. The amino acid sequences for the human umami receptors Q7RTX1 (TAS1R1) and Q7RTX0 (TAS1R3) were retrieved from the UniProt database. The SWISS-MODEL server was employed to construct the homology models, and the conformational validity of the resultant protein structures was evaluated.

Molecular docking of peptides to umami receptors

The peptide structure was built by ChemDraw software and energetically optimized under the MMFF94 force field. AutoDock Tools-1.5.6 software (https://autodock.scripps.edu/) was facilitated for the preparation of peptides and receptors (T1R1–T1R3) by adding polar hydrogens, Gasteiger charges, and assigning AD4 atom types, saving them as pdbqt files. Active sites for docking were identified by aligning the model proteins over the template protein (PDB: 6N51), and the docking region was at the dimensions of 25 Å×25 Å×25 Å. The active sites for T1R3 were set at *x*,*y*,*z*=120.01, 142.94, 171.88 and for T1R1 at *x*,*y*,*z*=151.42, 128.41, 171.88.

Docking was conducted using AutoDock Vina 1.2.3 (https://vina.scripps.edu/), specifying nine conformational modes.

Sensory evaluation of lees hydrolysate and umami peptides

Descriptive sensory analysis

Freeze-dried samples of lees enzymatic hydrolysate and ethanol precipitation fractions were reconstituted to a 10 mg/mL solution using deionized water. A panel of 10 professionally trained sensory analysts (comprising 5 males and 5 females, aged 22–30) performed evaluations on these samples, assigning scores based on reference standard solutions. Synthetic peptides were similarly prepared at a 2 mg/mL concentration. All sensory assessments adhered strictly to the ISO-8586-1, 2012 guidelines, were conducted within a controlled environment at 24 °C, and were approved by the Ethical Committee of Jiangnan University Medical School. For calibration purposes, standard taste solutions were used to represent the basic tastes: sour (0.4 g/L citric acid), sweet (10 g/L sucrose), bitter (0.05 g/L quinine), salty (3.5 g/L salt), umami (3.5 g/L MSG), and astringent (0.5 g/L tannic acid). Each sample was scored from 0 to 9 within 10 points, where 0 corresponded to the taste intensity of pure water, and 5 referred to the intensity of standard taste solutions. During the tasting sessions, the panelists sampled 2 mL of each solution, swirled it in their mouths for 10 s, and subsequently expectorated it. A rinse with pure water followed each tasting to cleanse the palate. Data on taste attributes and intensity scores were systematically collected for further analytical processing.

Umami recognition threshold

The recognition threshold of synthetic peptides was determined by triangle test and taste dilution analysis (TDA) referring to Zhang *et al.* (2022b). The initial concentration for peptide tasting is referred to in the descriptions of sensory analysis results. The peptides with 0.25 mg/mL were serial 1:1 dilution with ultrapure water five times to 0.0125 mg/mL, which was below the two levels of the threshold. The peptides were evaluated as the concentration step increased, and the panelists should taste and distinguish between two cups of water and one cup of peptides. When the panelist chose the lowest peptide samples, one more sample with the same concentration was used for confirmation. The individual threshold was defined as the mean of the last two concentrations at which the panelists could correctly identify peptides and water, and the final threshold was calculated as the mean of the sensory panel in the three sessions.

Umami-enhancement assessment of synthetic peptides

Umami intensities for MSG solutions at concentrations of 2, 3.5, and 6 g/L were assigned scores of 3, 5, and 9, respectively. Evaluations of umami intensity for a blend of synthetic peptide solution (1 mg/mL) with 3.5 g/L MSG spanned a scoring range from 3 to 9. Using the TDA triangle test, peptides were incrementally diluted with MSG solution at a 1:1 volume ratio to create a gradient of concentrations paired with 0.35% MSG. The sensory panel was asked to discern the taste variations between two samples of 0.35% MSG solution and one mixed sample of peptide with 0.35% MSG. The mean of the individual umami-enhancing thresholds established the final threshold value.

Evaluation of the antioxidant activity of lees hydrolysates and target peptides

The antioxidant evaluation protocol was adapted from Fang *et al.* (2021), with slight modifications. This evaluation revealed that DPPH free radical scavenging encapsulates both hydrogen atom transfer and single electron transfer mechanisms, as detailed in the comprehensive statistical assessment of various antioxidant capacity assays for lignins (Rumpf *et al.*, 2023). Employing this refined approach allowed for swift comparative analysis of the antioxidant effects manifested by hydrolysates and synthetic peptides, which were processed using distinct proteolytic enzymes. As a benchmark, reduced glutathione (GSH), at a standard concentration of 0.5 mg/mL, served as the control. Following initial screenings that identified peptides with potent radical scavenging capabilities, the DPPH free radical scavenging IC_{50} values were ascertained across a concentration spectrum ranging from 0.4 to 2.0 mg/mL. To ensure reliability and reproducibility, each assay iteration was conducted in triplicate.

Data processing

SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA) was used for variance analysis (ANOVA) and Duncan's test, with a significance level set at 0.05. Data processing and graphing were conducted by Origin 2016 (OriginLab, Northampton, MA, USA). The residue docking interactions between the peptide and umami receptors were determined at https://plip-tool.biotec.tu-dresden.de/plip-web/. Heatmap plotting was performed using the R program (version 4.3).

Results and Discussion

Single enzymatic treatment of lees with amylase and protease

Upon amylase pretreatment, the starch in lees was effectively hydrolyzed into soluble sugars, which subsequently enhanced the solubility of starch-associated proteins. The study observed a proportional increase in soluble protein content with increasing amylase additions (Figure 1). Stability in soluble protein levels was attained at an amylase concentration of 80 U/g, with no significant changes observed upon increasing the concentration to 100 U/g. Consequently, the amylase pretreatment was optimized at an additional level of 80 U/g.

In general, enzymatic treatment with various proteases yielded differential peptide contents, illustrating the distinctiveness of each enzymatic hydrolysis action. The total peptide concentrations of the five investigated enzyme groups ranged from 8.02 to 11.47 g/100 g. Among them, the hydrolysates from pepsin and flavorzyme exhibited the highest peptide content, significantly greater than the yields from alkaline protease, neutral protease, and papain treatments. The better performance of flavorzyme may be due to its good hydrolysis ability on grain-derived protein. In an enzymatic conversion of brewers' spent grain proteins to antioxidant peptide study, the flavorzyme had the highest degree of hydrolysis (24%), followed by alkaline protease (17.7%) (Abeynayake *et al.*, 2022). The DPPH radical scavenging rates of the five enzyme hydrolysis ranged between 5.98% and 16.33%, which were greater than those of the untreated raw groups (4.88%). The significantly increased peptide content and antioxidant activity both revealed that the proteins in lees were converted to low-molecular-weight peptides during the enzymatic

process. Despite observing similar peptide yields resulting from pepsin and flavorzyme treatments, the latter's product antioxidant ability was much better, because pepsin was a single endo enzyme, while the flavorzyme was a mixture of several enzymes fermented by *Aspergillus oryzae* (Merz *et al*., 2015). Sensory analysis further indicated that the hydrolysates from flavorzyme treatment could enhance umami (4.37) and saltiness (3.25) much better than other enzymatic treatments (Figure 2). In contrast, hydrolysates from neutral and papain treatments were characterized by stronger bitterness and astringency. This may be due to both the endo and exo peptidase activities of flavorzyme, which can reduce the generation of bitter peptides from the hydrophobic C-terminal regions of cereal proteins such as those from rice and wheat (Zhou *et al*., 2021). Thus, the flavorzyme was chosen for hydrolysis because of its ability to enhance both the antioxidant properties and the umami profile of the peptides (Xie *et al*., 2023).

Optimization of dual-enzyme hydrolysis conditions for lees

In alignment with the optimization results, a dual-enzyme system comprising amylase and flavorzyme was utilized (Figure 3). The comparative analysis between single-step and two-step hydrolysis revealed no substantial difference in the total peptide yield or DPPH radical scavenging activity. This observation was in agreement with the study of Liu *et al*. (2023) on dual-enzyme processes applied to Baijiu lees. Consequently, the single-step method was chosen for its procedural simplicity. The incremental addition of flavorzyme led to a corresponding increase in both total peptide concentration and DPPH scavenging efficacy. As the flavorzyme concentration increased to 3000 U/g, the peptide content tended to peak, beyond which no significant further improvements were noted, suggesting a balance in the enzymatic reaction dynamics. Thus, the ideal quantities for amylase and flavorzyme were ascertained to be 80 U/g and 3000 U/g,

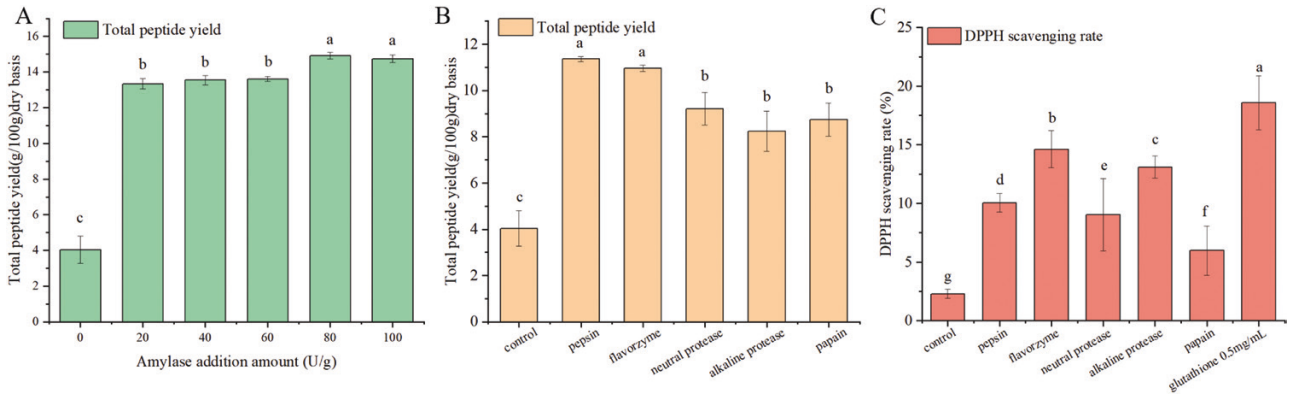

Figure 1. Optimization of single enzyme hydrolysis conditions for Huangjiu lees.

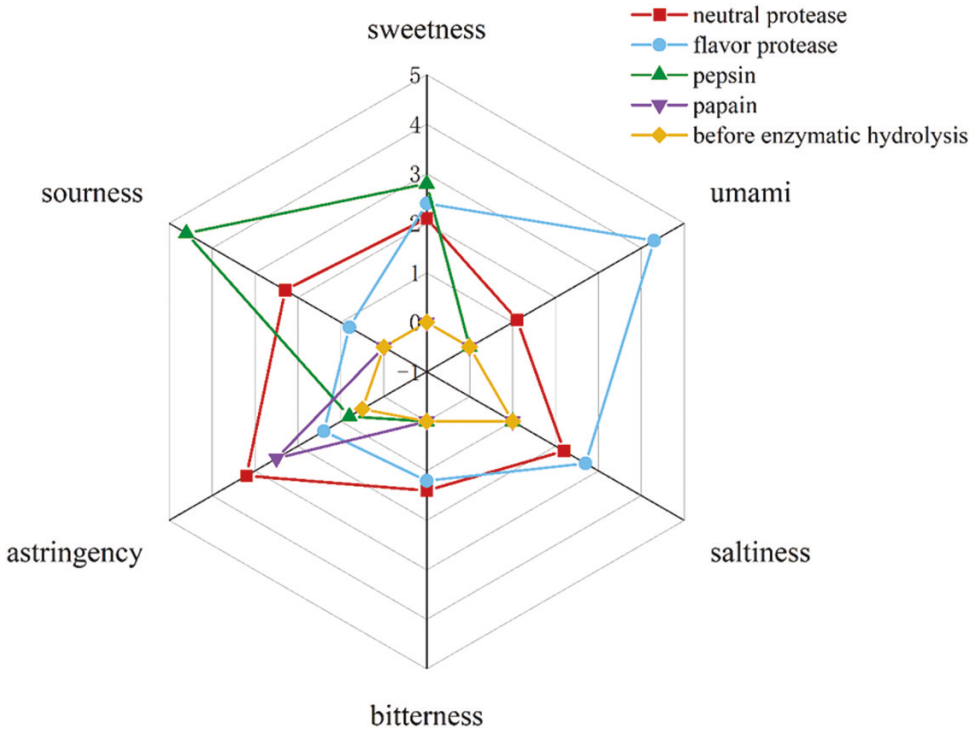

Figure 2. Sensory profile of Huangjiu lees treated with a single enzyme.

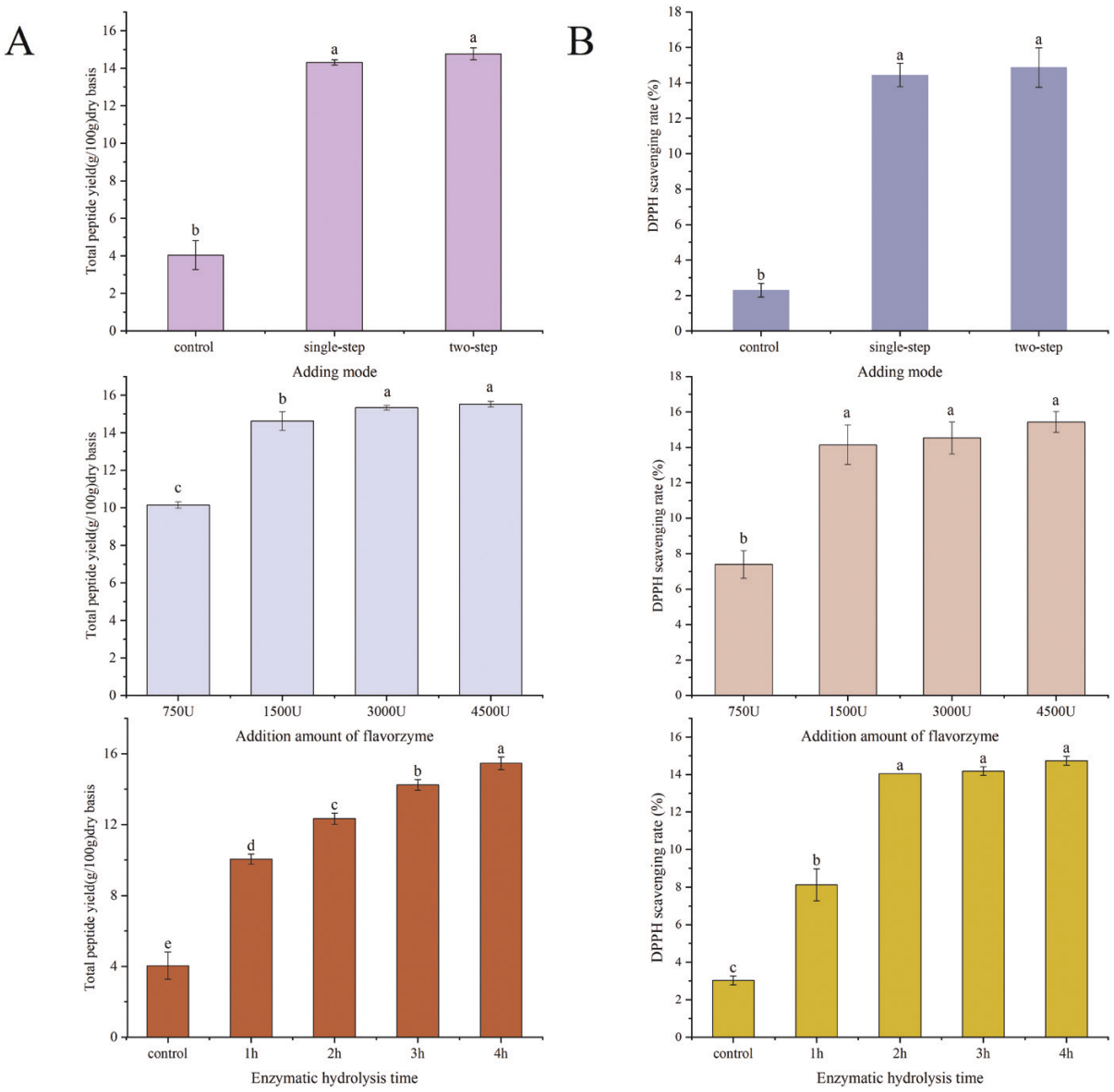

Figure 3. Optimization of double-enzyme hydrolysis conditions for Huangjiu lees.

respectively. Further optimization regarding hydrolysis time indicated a positive correlation between extended enzymatic reaction periods and increased yields of total peptides and scavenging rates. However, prolonging the hydrolysis beyond 3–4 h did not further augment the antioxidant activity, although the peptide concentration continued to increase. Thus, the optimal hydrolysis duration is 3 h, and the peptide concentration can reach 14.23 g/100 g (dry basis), which is approximately 2.55 times that of raw lees. Although these results were lower than those of the study of Liu *et al.* (2023), who applied alkaline protease on Huangjiu lees and increased the amino acid nitrogen by four times, but peptides had larger molecular weights than those of amino acids and more potential activity were produced (Liu *et al.*, 2019).

Sensory evaluation of umami peptide fractions in hydrolysates from lees

Based on the optimal parameters for enzymatic hydrolysis described above, a hydrolysate was prepared by treating lees with 80 U/g of amylase and 3000 U/g of protease, maintaining a material-to-liquid ratio of 1:10, and performing the hydrolysis at 45 °C for 3 h. The molecular weight result of hydrolysate showed that over 95.67% was lower than 3000 Da. Due to the predominance of active peptides with molecular weights below 3000 Da, the hydrolysate was subjected to ultrafiltration for basic enrichment. Then, the permeate that was lower than 3000 Da was concentrated and precipitated sequentially at ethanol final concentrations of 20%, 40%, 60%, and 80% based on the hydrophobic characteristics. The yield ratios of the fractions were 7.35%, 2.29%, 43.43%, and 47.00%, respectively. Sensory analysis revealed a progressive increase in both astringency and umami flavor intensity with precipitation fractions derived from increasing ethanol concentrations (Figure 4). The pronounced astringency perceived in all fractions may be due to the polyphenolic compounds in the lees, which could also partially disperse into the product wine (Cai *et al.*, 2019). On the other hand, the DPPH radical scavenging ability showed that the 80% precipitation was the highest

(68.42%) at 5 mg/mL, which was significantly larger than the 60% precipitation (60.37%) and the other two fractions (52.90%–61.79%). This was consistent with the peptides obtained by 80% ethanol precipitation of abalone muscle hot water extract, which also exhibited better antioxidant activity than the supernatant and 40% ethanol precipitation (Wei and Weng, 2020). Therefore, the 80% ethanol precipitate fraction exhibited the highest umami intensity and antioxidant potential, and was thus selected as the target fraction.

Identification and screening of umami peptides

Among the highest umami fraction precipitated by 80% ethanol, 5266 peptides were identified, which were derived from the primary raw materials of Huangjiu, such as rice, wheat, and yeast. The initial screening focused on 3078 peptides with fewer than 10 amino acids. Utilizing the predictive tool Umami_YYDS, 2228 peptides were determined to exhibit umami potential without associated bitterness. Subsequently, 166 peptides with a peak area intensity surpassing 1×10^8 were selected for further analysis, involving antioxidant activity prediction and the binding energy with umami receptors (Table S3). The results revealed a little higher affinity of peptides for the T1R1 receptor than for the T1R3 receptor, indicating that peptides tend to interact with T1R3. By applying the filter parameters involving binding energy to the T1R3 receptor below –9.2 kcal/mol, radical scavenging activity above 0.4, and chelating activity over 0.25, 10 target candidate synthetic peptides were further screened (Table 1).

The proteomic database match method was used for accession of the protein origins of the candidate synthetic peptides,

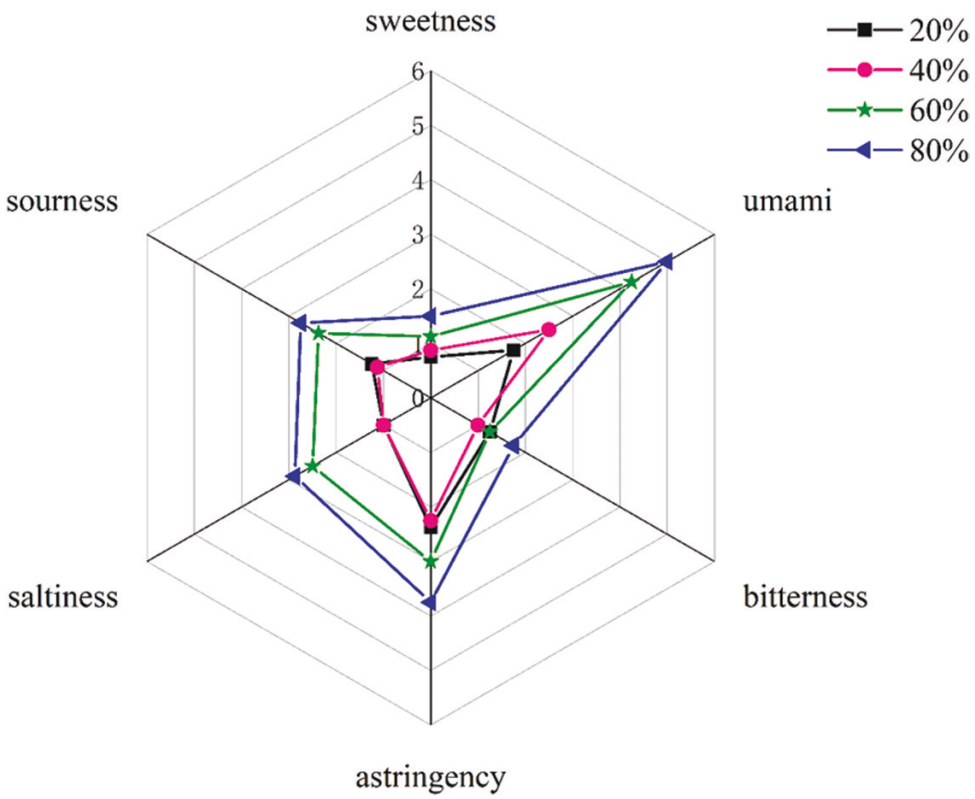

Figure 4. Sensory descriptive analysis of the grade fractions from Huangjiu lees double-enzyme hydrolysate.

Table 1. Umami and antioxidant activity screening of candidate synthetic peptides

Serial	6N51	T1R1	T1R3	Scavenging activity	Chelating ability	Umami prediction	Protein source
1	EQFPGAND	–8.475	–10.3	0.4222	0.2867	0.894	P15280
2	EDPFAED	–8.582	–10.17	0.4004	0.2823	0.894	P00925
3	EPAGW	–8.93	–10.05	0.486	0.2675	0.613	Q7G065
4	ASHLGRPN	–8.668	–10.02	0.4587	0.2696	0.894	P00560
5	DPDGW	–9.542	–9.74	0.4494	0.263	0.894	Q948T6
6	IDEQHPR	–7.812	–9.592	0.4683	0.2823	0.894	P06169
7	RADSYNPR	–7.494	–9.388	0.4428	0.2536	0.894	P14323
8	DNPNW	–9.09	–9.355	0.4374	0.2887	0.894	P15280
9	FDGF	–9.582	–9.242	0.4168	0.2598	0.613	Q01401
10	GFGPE	–7.853	–9.21	0.4987	0.2633	0.653	Q948T6

and the results are displayed in Table S4. EQFPGAND, DNPNW, and EPAGW were traced back to glucose-1-phosphate adenylyltransferase small subunit 2 from Japanese rice (*Oryza sativa* subsp. japonica). Peptides DPDGW and GFGPE were linked to lactoylglutathione lyase from the same rice variety. Furthermore, peptides EDPFAED, IDEQHPR, ASHLGRPN, RADSYNPR, and FDGF were attributed to enolase 2, pyruvate decarboxylase isozyme 1, phosphoglycerate kinase from brewing yeast (*Saccharomyces cerevisiae*, strain ATCC 204508/S288c), glutelin type-B 1 subunit, and 1,4-alpha-glucan-branching enzyme from Japanese rice, respectively. These findings underscore that a significant proportion of candidate umami peptides in lees are products of enzymatic activity from rice and yeast sources.

Sensory evaluation of umami peptides

The sensory roperties of the synthesized peptides were evaluated to ascertain their umami flavor profiles. Sensory panel assessments revealed that the peptides DPDGW, IDEQHPR, and GFGPE displayed the most pronounced umami intensities, followed by peptides EDPFAED, EQFPGAND, RADSYNPR, and DNPNW with lower umami intensity (Figure 5). The umami recognition threshold for these 7 synthetic peptides in water ranged from 0.3825±0.0896 to 0.6650±0.2471 mmol/L, which was significantly lower than the recognition threshold of 1.6 mmol/L of MSG in water (Table 2). Notably, DPDGW showed the lowest umami threshold in water, while RADSYNPR exhibited the highest. When evaluated in a 0.35% MSG solution, peptide concentrations of 1 mg/mL for EQFPGAND, EDPFAED, DPDGW, IDEQHPR, FDGF, and GFGPE exhibited substantial umami-enhancing effects, with recognition threshold values of 0.6196±0.2177, 0.1101±0.0258, 0.2762±0.1026, 0.2238±0.0722, 0.6196±0.2177, and 0.5938±0.2086 mmol/L, respectively. DPDGW was identified as having the most ability to enhance the umami intensity. This indicates that the lees hydrolysates derived from the enzymatic treatment could act as a flavor enhancer due to these peptides.

Evaluation of antioxidant activity of umami peptides

Hydrophobic and bitter amino acids are intrinsic characteristics of umami peptides, contributing significantly to their flavor profile and functional properties (Dutta *et al.*, 2022). The presence of such amino acids is also crucial for the antioxidant capacity of peptides. Antioxidant peptides commonly encompass hydrophobic amino acids such as HIS, TRP, PHE, PRO, GLY, LYS, ILE, and VAL. In a study by Hao *et al.* (2020), the sequence analysis of 148 umami peptides revealed that 48 contained antioxidant-promoting amino acids including PRO, TYR, TRP, and PHE. These amino acids not only impart a bitter taste but are also recognized for their

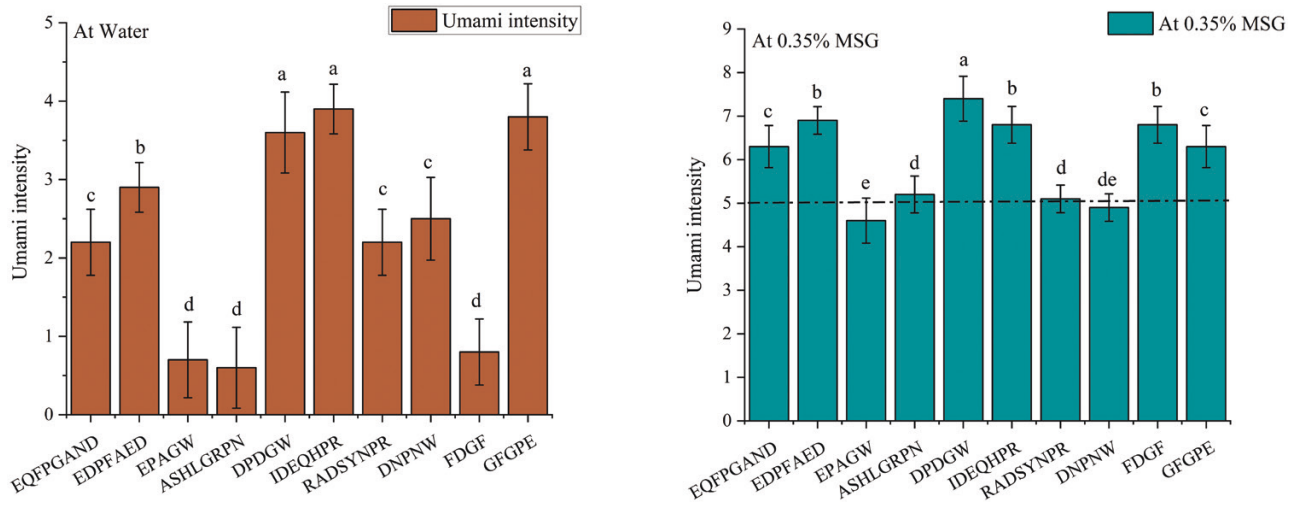

Figure 5. Sensory descriptive analysis of candidate umami peptides identified from Huangjiu lees.

Table 2. Recognition threshold of candidate synthetic peptides

Serial	Sequence	Sensory description	Threshold in water (mmol/L)	Threshold of 0.35% MSG (mmol/L)
1	EQFPGAND	Umami, sweetness, weak sourness	0.6276±0.1804^{b}	0.6196±0.2177
2	EDPFAED	Umami, sweetness, weak sourness	0.4161±0.1182^{c}	0.1101±0.0258^{c}
3	EPAGW	Bitterness, weak sourness	0.9852±0.2832^{a}	/
4	ASHLGRPN	Weak sourness, astringency	0.7055±0.2479^{b}	/
5	DPDGW	Obvious umami, weak sourness	0.3825±0.0896^{c}	0.2762±0.1026^{b}
6	IDEQHPR	Obvious umami, sweetness, bitterness	0.3917±0.1445^{c}	0.2238±0.0722^{bc}
7	RADSYNPR	Umami, sweetness, weak bitterness	0.6650±0.2471^{b}	/
8	DNPNW	Umami, sweetness, astringency	0.4268±0.1227^{c}	/
9	FDGF	Weak bitterness, astringency	0.2840±0.0816^{d}	0.6196±0.2177^{a}
10	GFGPE	Obvious umami, week sourness, astringency	0.3464±0.1278^{c}	0.5938±0.2086^{a}

potent radical-scavenging activities. The imidazole ring in histidine, in particular, serves as a proton donor, contributing to the antioxidant function of the peptide (Zou *et al.*, 2016). A preliminary evaluation of the antioxidant activities of the selected umami peptides was performed to assess their DPPH radical scavenging abilities (Figure 6). Among the 10 peptides, EPAGW, DPDGW, and DNPNW exhibited good DPPH scavenging ability with rates of 47.11%±0.95%, 33.07%±1.23%, and 61.09%±1.26%, respectively.

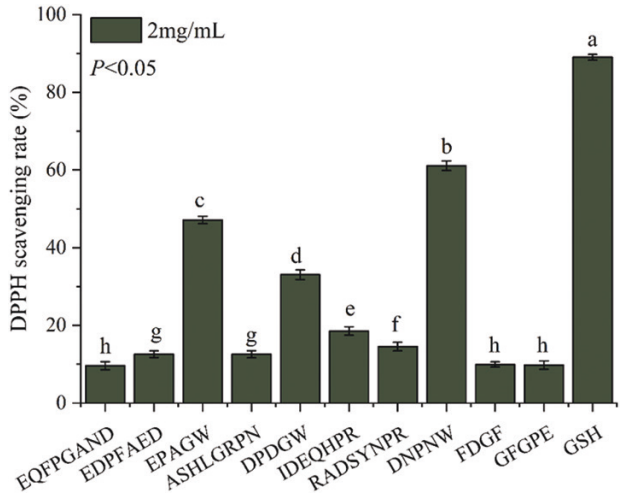

Figure 6. Antioxidant activity of candidate synthetic peptides.

Comparison to the reference (reduced glutathione) scavenging rate of 89.01%±0.73% at a similar concentration (2 mg/mL) confirmed that these three peptides did have certain antioxidant potential. Nevertheless, EPAGW was not considered further due to its bitter taste. Consequently, peptides DPDGW and DNPNW, which exhibit both umami flavor and antioxidant properties, achieved IC_{50} values for DPPH radical scavenging of 0.6982 mg/mL and 0.4315 mg/mL, respectively. These values are significantly lower than those observed for the antioxidant peptide LLPF derived from maize protein hydrolysis using flavorzyme (IC_{50}=1.08 mg/mL; Wang *et al.*, 2014), as well as the peptide KAPDPGPGPM obtained from tilapia skin through basic protease hydrolysis (IC_{50}=2.56 mg/mL; Ma *et al.*, 2022). Remarkably, the IC_{50} value of DNPNW was also significantly lower than those of the antioxidant peptides YENGGGT (IC_{50}=0.66 mg/mL) and YIVYPG (IC_{50}=0.45 mg/mL) isolated from tuna by-products using neutral protease (Saidi *et al.*, 2018), underscoring the notable antioxidant potential of Lees-derived umami peptides.

Interaction of umami peptides with T1R1–T1R3 receptor residues

The interaction of umami peptides with the umami receptor T1R1–T1R3 is a critical event to activate the umami characteristic. To elucidate the specific residue interactions,

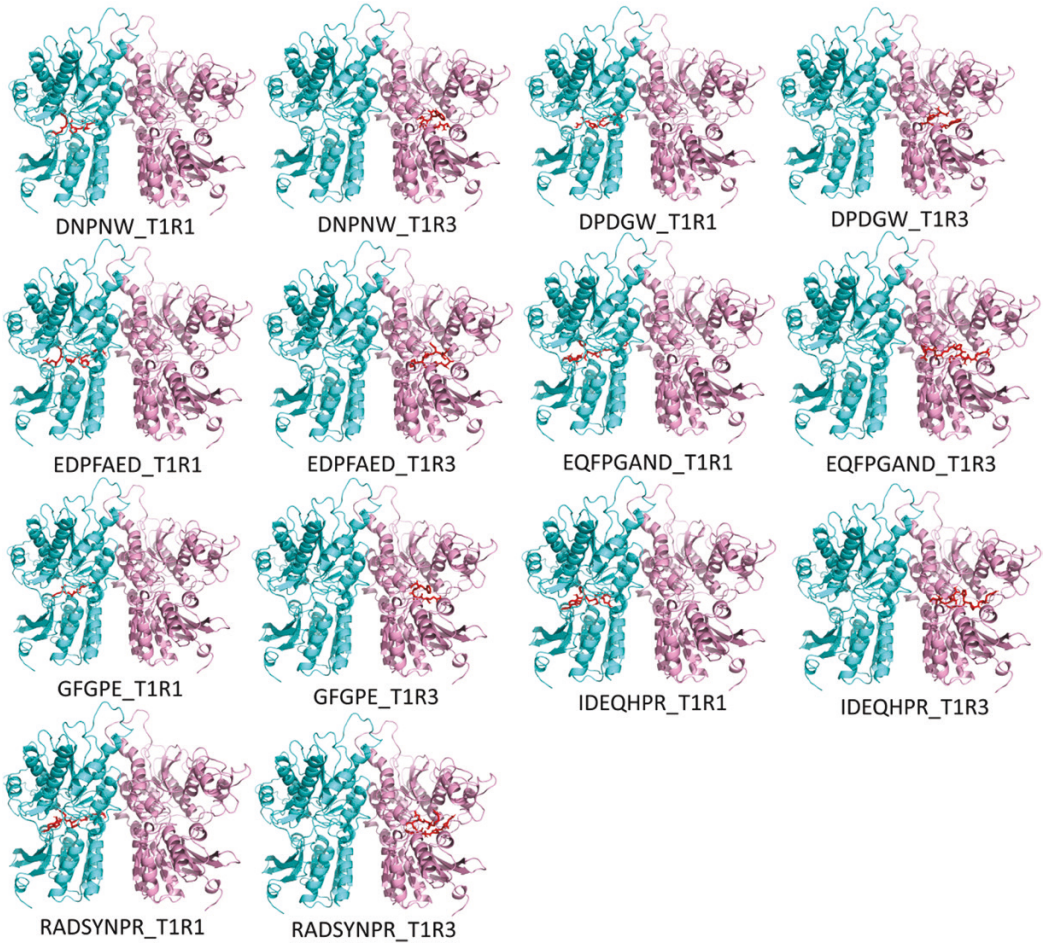

Figure 7. Binding mode of umami peptides with T1R1–T1R3 receptor.

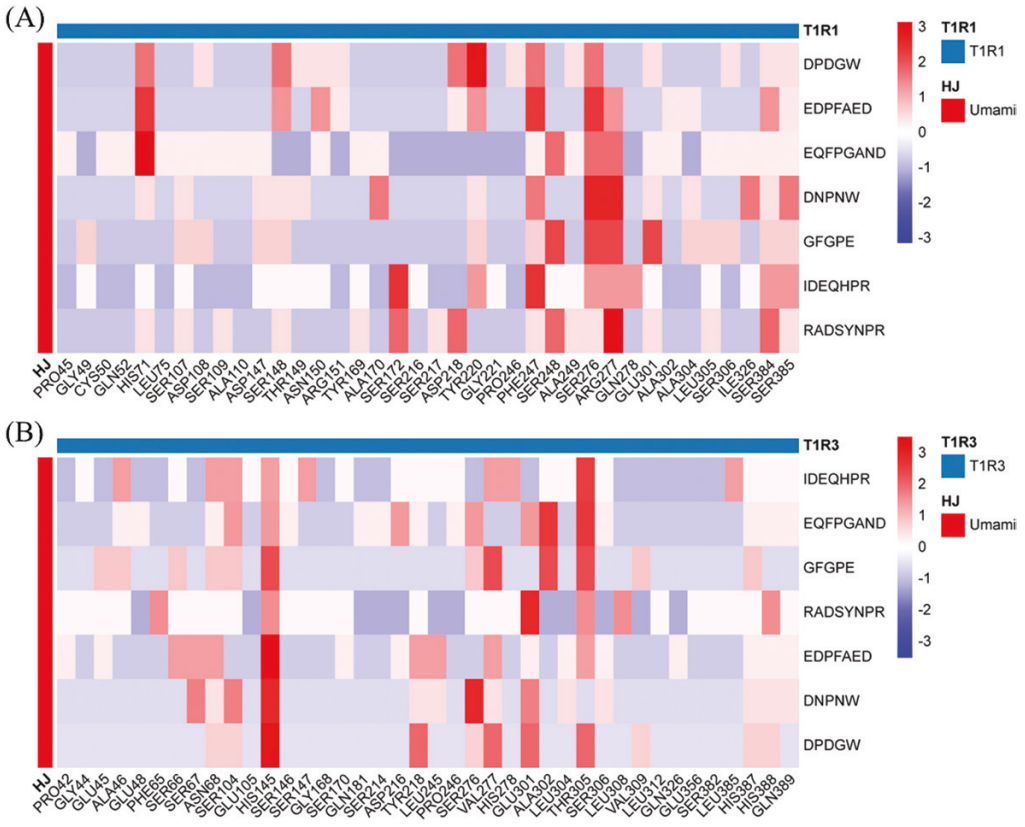

Figure 8. Heatmap of interaction between umami peptide and T1R1–T1R3 receptor residues in Huangjiu lees.

molecular docking was conducted with seven umami peptides against the T1R1–T1R3 receptor. The binding mode is shown in Figure 7, the heatmap of residue contact number distribution is given in Figure 8, and the two-dimensional residue interaction results are shown in Figure S1.

For T1R1, peptides DNPNW, DPDGW, EDPFAED, EQFPGAND, GFGPE, IDEQHPR, and RADSYNPR established 16, 13, 17, 23, 15, 27, and 21 hydrogen bonds, and engaged in 6, 9, 5, 6, 4, 5, and 9 hydrophobic interactions, correspondingly. Key residues with frequent contact were HIS71, SER148, TYR220, PHE247, SER248, SER276, ARG277, GLU301, SER384, and SER385. In prior research, umami peptides derived from *Hypsizygus marmoreus* bound to T1R1 with ARG277, TYR220, and GLU301 as the most prevalent contact residues, and residues HIS71 and SER384 were found to contribute to hydrophobic interactions (Dong *et al.*, 2023). The distribution of these residues suggests that SER residues are crucial for the interaction between umami peptides and their receptor. Similar analysis of six umami peptides from chicken breast soup by Zhang *et al.* (2023c) demonstrated that hydrogen bonds involving SER accounted for 40% (T1R1) and 21% (T1R3) of all hydrogen bonds, with SER276 being the most contacted residue in T1R1 interactions, followed by HIS71, ASP147, SER148, SER276, ARG277, GLN278, SER306, and SER385, which is consistent with the findings of the present study.

For T1R3, the same peptides formed 14, 10, 16, 21, 9, 25, and 23 hydrogen bonds, respectively, along with 5, 5, 10, 6, 5, 7, and 8 hydrophobic interactions each. The high-frequency contact residues implicated in these interactions included ASN68, SER104, HIS145, SER276, VAL277, GLU301, ALA302, THR305, and HIS387. These findings are consistent with those from *Lentinula edodes* umami peptides, where ASN68, HIS145, VAL277, and SER306 interacted with LPDEAR, and SER104, GLU301, and ALA302 engaged with LDELEK (Chen *et al.*, 2023). Similarly, SER104, HIS145, VAL277, and THR305 were identified as key residues in interactions with umami peptides from fermented broad bean sauce (Zhao *et al.*, 2023) and stinky mandarin fish (Yang *et al.*, 2022). The predominance of SER residues in these interactions emphasizes its role in the binding of lees-derived peptides to the T1R3 receptor. Given the lower binding energies observed for the peptides with T1R3, this receptor may constitute the primary binding site for umami perception in lees.

Conclusions

This study employed enzymatic preparation to produce protein hydrolysates from lees, characterized by flavor (umami) and functional (antioxidant activity) properties, thereby enhancing its utilization value. An optimized one-step dual-enzyme treatment process was achieved by employing amylase for pretreatment followed by flavorzyme for hydrolysis. The target peptide fractions in the hydrolysate were extracted using ethanol, with the 80% precipitate showing the best umami and antioxidant activity. By combining virtual screening, sensory evaluation, and *in vitro* activity assessment, seven novel umami peptides were identified from the target fractions. Among them, five peptides also demonstrated umami-enhancing

effects, with DNPNW and DPDGW identified as dual-function peptides offering both umami and antioxidant properties. Molecular docking analysis revealed that umami peptides tend to bind to the T1R3 receptor with a lower binding energy, involving key residues ASN68, SER104, HIS145, SER276, VAL277, GLU301, ALA302, THR305, and HIS387. These findings offer new insights into the high-value transformation of lees and other distillery waste products.

Supplementary Material

Supplementary material is available at *Food Quality and Safety* online.

Author Contributions

Rongbin Zhang: Investigation, formal analysis, writing original draft, and review & editing. Zhilei Zhou: Investigation, formal analysis, and review & editing. Zhongwei Ji: Formal analysis and review & editing. Qingxi Ren: Review & editing. Shuangping Liu: Conceptualization, supervision, and review & editing. Yuezheng Xu: Resources. Jian Mao: Methodology, funding acquisition, and review & editing.

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Conflict of Interest

The authors declare no conflict of interest for the content published in the manuscript.

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