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Unraveling umami complexity: From exploring umami peptides in fermented soybean curd to molecular elucidation of taste mechanisms

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ABSTRACT

Sufu is a traditional umami fermented soybean curd (FSC). We isolated and identified 25 potential umami peptides using ethanol precipitation, dialysis desalination, resin adsorption recovery, nano-HPLC-MS/MS and virtual docking prediction. The results of sensory experiments showed that 23 of the synthetic peptides presented a lower umami threshold than that of MSG (0.50 mg/mL), and 10 peptides had obvious effects of increasing umami and saltiness. Seven peptides were quantified in FSC by UPLC-QQQ-MS, and the content of one novel umami tripeptide ADL (0.51 ± 0.03 mg/mL) was four times its umami threshold (0.125 mg/mL). Taste reconstitution experiments revealed that the umami peptides enhanced the umami and saltiness of the simulated system by 24.53% and 24.00%, respectively. Molecular dynamics proved that hydrogen bonding is the main interaction between peptide and receptors. This study is important for understanding and improving the taste quality of FSC.

1. Introduction

Fermented soybean curd (FSC) is a fermented soybean delicacy that originated in China and is gained popularity in Asia. It is known as the "cheese of the East" and is widely enjoyed by the public for its delicate and umami taste (Han et al., 2001). The umami of FSC is generally believed to be derived from umami amino acids (Wang et al., 2021; Wei et al., 2023). NaCl and glutamic acid in FSC have an important synergistic effect on its umami (Lioe et al., 2018). Liao et al. (2017) identified three umami peptides from white FSC by ultrafiltration and gel chromatography. In addition, Chen et al. identified a decapeptide with salt-enhancing effect from FSC by gel chromatography and ion-exchange chromatography, which suggests that peptides in FSC have multiple contributions to the taste (Chen et al., 2021). Moreover, previous studies have not quantified umami peptides to investigate the actual contribution of umami peptides in FSC.

The isolation and purification of umami peptides were mainly performed by chromatography and resin separation. After elution by reversed-phase high-performance liquid chromatography (RP-HPLC), a certain component was selected for mass spectrometric identification to obtain the final peptide sequence. This conventional separation method is not only time-consuming, but may also miss some key umami peptides during the classification of the fractions (Liu et al., 2014). Based on the structure-activity relationship between umami peptides and umami receptors, high throughput and accurate screening of umami peptides can be achieved by virtual docking (Yu et al., 2017; Zhang et al., 2021). Up to date, numerous studies have reported the effective isolation and identification of umami peptides from various foods, such as chicken

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broth (Zhang, Zhang, et al., 2023), sheep bone (Li et al., 2023), and edible mushrooms (Shen et al., 2023). However, the umami peptides of FSC still need be explored.

In this study, peptidomics was used to identify the umami peptide fractions of the aqueous extracts of FSC prepared using a sensory-based method. Subsequently, the candidate synthetic peptides were evaluated by a sensory panel for umami, umami-enhancing, and salt-enhancing effects, and peptides with prominent taste were selected for quantification by the external standard method. The synergistic effects of the umami peptides on the umami taste of amino acids, organic acids, and nucleotides in FSC were further investigated using a taste recombination model. Molecular dynamic (MD) simulations were performed to determine the interaction between the umami peptide and umami receptors T1R1/T1R3 at solvent environment.

2. Materials and methods

2.1. Materials and reagents

The FSC was purchased from Kaiping Guanghe Curd Co., Ltd. Food-grade alcohol (95%) was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). The screened peptides were synthesized by Gill Biochemical Co. (Shanghai, China). Food-grade sodium chloride, citric acid, sucrose, quinine, glutathione, and monosodium glutamate were purchased from Quzhou Geno Biotechnology Co., Ltd. (Quzhou, Zhejiang, China). Purification equipment and filtration membranes were purchased from Shaoxing Haina, Ltd. (Shaoxing, Zhejiang, China). Bicinchoninic Acid Protein Assay Kit and chromatographic organic solvents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Extraction and isolation of umami peptides from FSC

The whole separation process of umami fraction of FSC is shown in Fig. S1.

2.2.1. Preparation of water extract of FSC

The block curd was removed from the bottle, drained, lyophilized, ground into powder, and passed through a 100-mesh sieve. The powdered sample was weighed and n-butane was added at a ratio of 1:10 (g/mL, w/v) and stirred for 2 h to extract fat. The defatted powder was naturally evaporated in a fume hood for 12 h to remove organic solvent. The defatted powder was mixed with deionized water at a ratio of 1:20 (g/mL, w/v) and stirred for 2 h, and then the mixture was centrifuged at 1500g force maximum under 4 °C for 30 min, and the supernatant was filtered through 0.45-μm filter paper, and then filtered through a 0.22-μm filter membrane to collect the filtrate.

2.2.2. Ultrafiltration of water extract

The water extract was subjected to ultrafiltration, and the low molecular weight components were intercepted by a 3-k Da ultrafiltration membrane, concentrated using rotary evaporator at 45 °C and 2000 Pa, lyophilized, and the sample was recorded as UF-3kDa.

2.2.3. Precipitation with different concentrations of ethanol

UF-3kDa was dissolved in ultrapure water and gradually separated into four grades using an aqueous solution containing increasing concentrations of ethanol. For EA, aqueous ethanol was added to the dissolved solution with stirring, such that the final concentration of ethanol was 40%. Subsequently, precipitate and supernatant were obtained by centrifugation at 16000*g* force maximum under 4 °C for 15 min, which was repeated thrice and the precipitate was collected (EA), and the supernatant was retained as well. The above steps were repeated using 60% and 80% aqueous ethanol solutions to treat the supernatant to obtain EB and EC, and the final supernatant was recorded as ED. EA, EB, EC, and ED were lyophilized, preserved, and heir amino acid and molecular weight distributions were determined (Zhu et al., 2020).

2.2.4. Isolation and purification of umami peptides (peptide length >4)

The highest umami fraction after ethanol grading was selected for the next step of separation. Based on sensory evaluation, the ED fraction had a distinct salty taste, but the salt content was high. Desalination of the ED fraction was performed using a 500-Da dialysis bag, which was done by fully dissolving the ED lyophilized powder in distilled water, placing the beaker in a refrigerator at 4 °C, and the dialysis was performed for 24 h. After dialysis, the retained liquid was lyophilized and labelled as ED-I, and the exudate was recovered and labelled as ED-II. Fraction ED-I was used for peptides identification (Peptide length >4).

2.2.5. Isolation and purification of short umami peptides (Peptide≤4)

ED-II was passed through an XAD-16 resin for enrichment of the short umami peptides. The XAD-16 resin was pre-treated with anhydrous ethanol and washed with distilled water before loading onto a chromatography column (2.6 × 60 cm). The column was compacted with distilled water at a flow rate of 1 mL/min. After an equilibrium was reached, distilled water was replaced with dialyzed exudate at a flow rate of 0.4 mL/min. After the exudate was adsorbed onto the resin, the chromatography column was sequentially eluted with water, 20% ethanol, and 40% ethanol at a flow rate of 0.4 mL/min. The eluates with different ethanol concentrations were collected and sequentially labelled as ED–II–I, ED–II–II, ED–II–III, and the eluates were concentrated to remove the ethanol by evaporator, followed by lyophilization and storage.

2.3. Identification and screening of umami peptides

2.3.1. Identification

The most enriched umami fractions (ED-I and ED–II–I) were identified using nano-HPLC-MS/MS according to our previous method (Chang et al., 2023). The samples were desalted using a Pierce C18 rotary pipette tip, re-dissolved in solvent A (0.1% formic acid aqueous solution), and analyzed using a Q-Exactive Plus coupled to an EASY-nanoLC 1200 system (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The sample (6 μL) was loaded into a 25-cm analytical column with a resin (Dr Maisch) with an inner diameter of 75 and 1.9 μm, and then the separation was initiated with a gradient of 2% buffer B (80% acetonitrile with 0.1% formic acid) in a 60-min gradient, which was progressively increased to 35% in 47 min, and then to 100% in 1 min, and sustained for 12 min. The column temperature was 40 °C and the electrospray voltage was set to 2 kV. The mass spectrometer was operated in data-dependent acquisition (DDA) mode. Full-scan MS spectra (m/z 200–1800) were recorded at a resolution of 7×10^4. The automatic gain control (AGC) target was 3×10^6 with a maximum injection time of 50 ms. Next, the parent ions were selected in the collision cell and fragmented using high-energy collisional dissociation (HCD). MS/MS resolution was set to 1.75×10^4, the AGC target to 1×10^5, the maximum injection time to 45 ms, and the dynamic exclusion to 30 s. The MS spectra were acquired using PEAKS Studio version 10.6 (Bioinformatics Solutions, Waterloo, Ontario, Canada) to process the tandem mass spectra, which were then matched to *Glycine* max *(Soybean) (Glycine hispida)*.

2.3.2. Screening

From the classification ED-I and ED–II–I, 1315 and 479 peptides, respectively, with confidence −10lgP greater than 20 were identified, then predicted through online prediction tools iUmami-SCM, UMPred-FRL, and Umami_YYDS respectively (Charoenkwan et al., 2020, 2021; Cui, Li, et al., 2023). The retained peptide sequences were sequentially judged by machine learning model prediction to filter out the peptide sequences without umami. Based on the predicted results, 231 and 73 umami peptides were retained, respectively. The filtered peptides were screened for molecular docking using the umami receptors, T1R1/T1R3.

For umami receptor homology modeling, the metabotropic glutamate receptor (PBD: 1EWK) was used as a template according to the method of Zhang et al. (2022) The protein sequences of human umami receptors Q7RTX1 (TAS1R1) and Q7RTX0 (TAS1R3) were retrieved from the UniProt database, and homology modeling was performed using the SIWSS-MODEL online server. The models were also evaluated for protein structural rationality using the SAVES 6.0 tool (https://saves.mbi.ucla.edu/) for ERRAT, VERIFY 3D, and PROCHECK.

Molecular docking was done using Auto Dock Vina1.2.3 (The Scripps Research Institute, La Jolla, USA) program. The structures of the peptides were built using ChemDraw 20.0 software (PerkinElmer, USA), and the energy was minimized at the MMFF94 force field. Umami receptors and peptide ligands were processed using the Auto Dock Tools-1.5.6(The Scripps Research Institute, La Jolla, USA) program with polar hydrogen, charge Gasteiger, and atom type AD4 and exported as pdbqt files. The active site of protein T1R1/T1R3 were defined from the receptor cavities refer to the previous works (Dong et al., 2023; Zhao et al., 2022), and the T1R1 site x,y,z = 10.94, 10.16,169 15.41,T1R3 site x,y,z = 32.66, −3.61, 33.63. The maximum number of output conformational docking modes for each peptide was set to default nine. After docking, the peptide and receptor 2D residue interactions were analyzed using the Ligplot + v.2.2.8 (EMBL-EBI, Hinxton, Cambridgeshire, UK.) program. The docking contact heat maps were drawn using the Hiplot platform (https://hiplot.com.cn/cloud-tool).

2.4. Quantitative analysis of FSC

2.4.1. Quantification of free amino acids

Referring to a previous method (He & Chung, 2020), a sample of lyophilized curd powder was mixed with 5% sulfosalicylic acid (1:4, w/v), extracted for 5 min, and homogenized at 4000 rpm for 1 min to mix well. The extract was centrifuged at 16000g force maximum under 4 °C for 10 min and the supernatant was filtered through a 0.22-μm aqueous membrane filter for liquid phase analysis. Detection conditions were as follows: the supernatant was analyzed using an Agilent 1100 series instrument equipped with an ODS HYPERSIL column (250 × 4.6 mm, 5 μm) and a UV detector (Agilent Technologies, Inc., Palo Alto, CA, USA). Buffer A was crystallized sodium acetate: triethylamine:water ratio of 6.5 g:200 μL:1000 mL, and buffer B was crystallized sodium acetate:acetonitrile:methanol:water = 6.5 g:400 mL:400 mL:200 mL, and pH was adjusted to 7.2 ± 0.05. The column temperature was maintained at 40 °C, the detection wavelengths were 338 and 262 nm, and the flow rate was set to 1.0 mL/min.

2.4.2. Quantification of 5′-nucleotides

The curd powder was mixed with ultrapure water (1:5, w/v) and sonicated for 30 min, then centrifuged at 15000g force maximum under 4 °C for 20min. The supernatant was filtered through a 0.22 μm aqueous membrane. An appropriate amount of the supernatant was mixed with an equal volume of 10% trichloroacetic acid, allowed to stand for 1 h, followed by centrifugation at 15000*g* force maximum under 4 °C for 20 min, and the supernatant was filtered through a 0.22 μm aqueous membrane for liquid phase analysis. All analyses were performed in triplicate and quantified by retention time and peak area against each 5′-nucleotide standard solution (IMP and GMP separately, 2.5, 5, 10, 25, 50, 100, 200 and 400 μg/mL). Detection conditions were as follows: a column equipped with an ODS HYPERSIL column (250 × 4.6 mm, 5 μm) and a UV detector on an Agilent 1100 series instrument (Agilent Technologies, Palo Alto, CA, USA) to analyze the supernatant. Buffer A (methanol):Buffer B (aqueous solution of potassium dihydrogen phosphate containing 2 mmol/L tetrabutylammonium hydrogensulfate, adjusted to pH 3.0 with K_2HPO_4) = 1:99; flow rate of 0.8 mL/min, detection wavelength of 254 nm, injection volume of 10 μL.

2.4.3. Quantification of organic acids

The curd powder was mixed with ultrapure water (1:5, w/v) and sonicated for 30 min, and then centrifuged at 15000g force maximum for 15min under 4 °C, 1 mL of the supernatant was added with 0.2 mL of potassium ferricyanide (10.6%, w/w) and 0.2 mL of zinc sulfate (30%, w/w), and centrifuged at 18000g force maximum under 4 °C for 15 min, and the supernatant was filtered through a 0.22 μm aqueous membrane for liquid chromatographic analysis. All analyses were performed in triplicate and quantified using the retention time and peak area against standard solutions of each organic acid (0.101, 0.203, 0.507, 1.014, 2.535 and 5.069 g/L for succinic acid and 0.204, 0.409, 1.023, 2.047, 5.117 and 10.234 g/L for lactic acid). The detection conditions were as follows: the column temperature was 30 °C, flow rate was 0.8 mL/min, detection wavelengths were 338 and 262 nm, mobile phase was phosphate buffer (0.01 M KH_2PO_4), and pH was adjusted to 2.3 ± 0.05 with 5% acetic acid (w/v).

2.4.4. Quantification of NaCl

For the determination of NaCl content, refer to the Chinese standard 5009.44–2016 Determination of Chloride in Food.

2.4.5. Quantification of peptides

Peptide quantification was performed according to a previously described method (Li et al., 2021). The umami peptides in the FSC were quantified using the UPLC-QQQ-MS method in multiple reaction monitoring (MRM) mode by establishing a five-point external standard curve. When quantifying short peptides in complex matrices, non-specific fragment ions interfere with MS profiles. Therefore, the MS parameters of the ion pair must be optimized to improve the accuracy, as shown in Table S1. Under ESI source positive mode, 10 μL of each standard peptide solution (0.2 mg/mL) was injected to automatically optimizing parameters, including precursor ion (m/z), product ions (m/z), cone voltage (V), and collision energy (eV). The capillary voltage of 2 kV, desolvation temperature of 400 °C, and desolvation gas flow 800 L/h was applied. Separation was performed using a C18 column (ACQUITY UPLC BEN C18 Column, 2.1 × 100 mm, 1.7 μm), with mobile phases 0.1% (v/v) formic acid in water as solvent A, and 100% ACN as solvent B, flow rate 0.2 mL/min. The FSC samples (n = 3) were analyzed using the optimized parameters described above. MassLynx software (version 4.0) was used for data processing.

2.5. Sensory evaluation analysis

2.5.1. Sensory panel training

The sensory panel consisted of eight laboratory members (three males and five females, aged 18–28 years). The panelists were in good physical condition and had no history of smoking or taste disorders. All panelists provided informed consent before each sensory session, which was approved by the Medical Ethics Committee of JNU (JNU20230601IRB21). Panel members were able to accurately identify six taste sensations after 3 months of sensory training: sourness (0.08% citric acid solution, w/v), sweetness (1% sucrose solution, w/v), saltiness (0.35% table salt solution, w/v), bitterness (0.25% quinine solution, w/v), umami (0.35% monosodium glutamate solution, w/v), and richness (5 mM glutathione solution)(Li et al., 2020). Each tasting was 15 mL, and to avoid taste fatigue and sample residual effects, the mouth was rinsed with 50 mL of purified water between the two groups of samples and at 1-min intervals. In addition, they were trained on the intensity of taste standards in a series of graded concentrations (0.1%, 0.35%, and 0.6% of MSG or NaCl solution correspond to intensity of 1, 5 and 9, respectively), and were trained to accurately provide sensory ratings at different taste intensities.

2.5.2. Sensory evaluation of separated components

The fractions EA, EB, EC, and ED graded with different ethanol concentrations were dissolved in ultrapure water (10 mg/mL), and the panelists scored the separated fractions using the 5-point intensity values corresponding to the concentration of the taste standards in

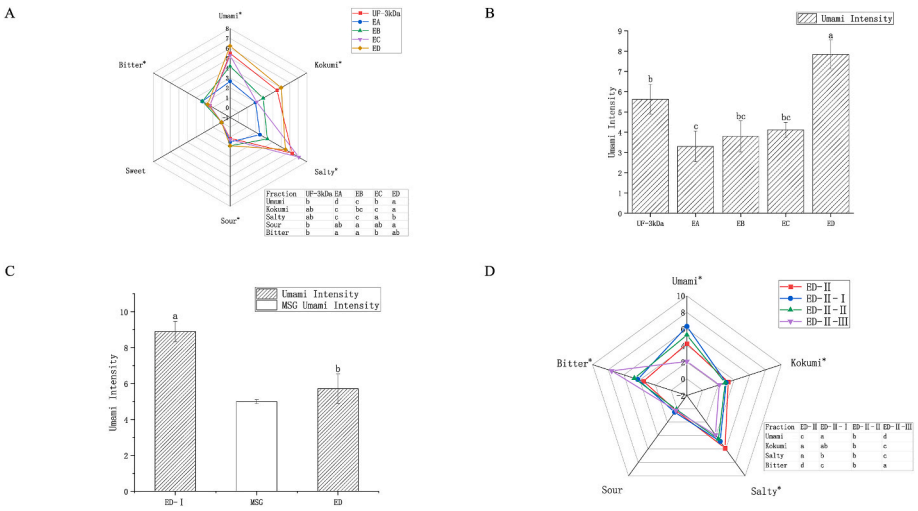

Fig. 1. (A) Sensory evaluation of precipitated fractions with different ethanol concentration gradients; (B) Sensory evaluation of precipitated fractions with different ethanol concentration gradients at the same salt concentration; (C) Differences in umami intensity before and after desalination of ED fractions (D) Sensory scores of fraction of ED-II-I after resin enrichment.

Section 2.5.1, as a reference (0–10 points, with 0 representing very weak and 10 representing very strong). To avoid the influence of salt on umami perception, the salt content of each component solution was adjusted to be consistent with another umami and salt intensity assessment. The desalted component ED-I and the macroporous resin-enriched components ED–II–I ~ III were dissolved in ultrapure water (10 mg/mL), and their taste characteristics and intensity were assessed.

2.5.3. Sensory evaluation of synthetic peptides

Taste characteristics and umami intensity analysis of synthetic peptides. The synthetic peptides were dissolved in ultrapure water (1 mg/mL), and their taste characteristics were descriptively analyzed and scored for taste intensity according to the tasting method described in Section 2.5.1.

Determination of the umami threshold, umami enhancement, and salt-enhancing threshold of synthetic peptides. Thresholds were determined using the taste dilution method. Synthetic peptide solutions of 1, 0.5, 0.25, 0.125, 0.0625 mg/mL were prepared in water, 0.35% MSG solution, 0.35% NaCl solution, respectively, and the synthetic peptide solutions of each group of samples were triangulated by the panelists according to the order of increasing concentration (one sample, two blank control groups), and the concentration of synthetic peptides that could clearly distinguish between the samples and the blank control groups was recorded. The corresponding concentration was recorded and considered as the threshold value (Cui, Li, et al., 2023).

Evaluation of umami-enhancing and salt-enhancing effects. Each peptide was dissolved in 0.35% MSG and 0.35% NaCl solutions at a concentration of 1 mg/mL, and three points corresponding to the taste intensities of 1 (0.1% MSG solution, 0.1% NaCl solution), 5 (0.35% MSG solution, 0.35% NaCl solution), and 9 (0.6% MSG solution, 0.6% NaCl solution) were used as reference.

2.5.4. Evaluation of the contribution of synthetic peptides to the taste of FSC

According to the results of the quantitative experiments described in Section 2.4, the detected amino acids, nucleotides, organic acids, NaCl, and umami peptides (peptides higher than 0.001 mg/g) were dissolved in deionized water to prepare different groups of simulated solutions according to the dry basis content of the FSC and were compared with the extracts of actual FSC samples. The different groups of simulated solutions were scored for umami, saltiness, and richness according to the tasting method described in 2.5.3.

2.6. Molecular dynamic simulation of ADL-T1R1/T1R3 complex

To investigate the dynamic interactions of the umami peptide ADL and receptors T1R1/T1R3 in the water environment, a 50 ns molecular dynamics simulation in an explicit solvent was performed using the Gromacs program (2018.3). The ADL-T1R1/T1R3 complex with the lowest docking mode was center into a cubic water box (9.7 × 9.7 × 9.7 Å). The system was relaxed with 5000 steps of conjugate gradient minimization and then related to a 20 ns restraint pre-equilibrium stage at the NPT ensemble with an Amber99SB-Ildn force field and Berendsen temperature coupling. In the production simulation, the step was 0.002 ps, the V-rescale method was used for temperature (298.15 K) control, and the Parrinello–Rahman method was applied for pressure coupling. The Coulomb interaction was handled using the PME (particle mesh Ewald) method with a van der Waals threshold of 1.0 nm. Finally, the analysis was based on the gmx tools and system index file, including the root-mean-square deviation (RMSD), hydrogen bond counts, and solvent-accessibility surface area (SASA). The DSSP method was used to determine protein secondary structure.

2.7. Statistical analysis

All experiments were conducted in triplicates. The results of the experiments were analyzed using the statistical package SPSS 19.0 (SPSS Inc., Chicago, IL, USA), and significant differences ($p < 0.05$) were determined using Duncan's multiple range test with one-way analysis of variance. Data plots were constructed using origin2019.

3. Results and discussion

3.1. Sensory-guided separation of umami peptide fractions in FSC

After defatting for ultrafiltration, water-soluble low-molecular-weight fractions (UF-3kDa) of FSC were obtained. The umami component was more likely to be present in the low molecular weight fraction; therefore, fractions above 3000 Da were not considered (Amin et al., 2020; Chen et al., 2021).

The UF-3kDa was graded into four fractions by ethanol gradient precipitation, and the umami intensity of the fractions generally increased with increasing ethanol concentration. The umami fraction was gradually enriched in the low-molecular-weight ED fraction. Among these, fractions EC and ED had high umami scores of 5.25 and 6.22, respectively (Fig. 1A and B), which were higher than the original UF-

Table 1
Docking energy of synthetic peptides with T1R1 and T1R3.

Peptides	Docking energy with T1R1/T1R3 (kcal/mol)	
	T1R1	T1R3
Peptides in ED-II-I fraction		
TD	−6.617	−5.218
AD	−6.061	−4.938
GE	−6.226	−4.937
VD	−6.447	−5.885
LE	−6.78	−5.575
TEF	−7.861	−6.813
AGE	−7.589	−5.925
ADL	−7.564	−6.104
TSE	−7.464	−6.231
AGN	−7.415	−5.948
DSQ	−7.397	−6.172
GFAE	−8.598	−7.122
TYET	−8.21	−6.983
THEA	−9.378	−6.982
Peptides in ED-I fraction		
DGRGHL	−9.452	−8.528
EGELPR	−8.933	−8.104
FREGDL	−8.921	−7.659
RFESF	−8.862	−7.635
FREGDI	−8.782	−8.206
DPRVI	−8.566	−7.105
RDNPHW	−8.332	−7.562
HYKGSSF	−8.883	−8.039
GSNRFET	−8.763	−7.993
TFEKP	−8.223	−7.854
HEWQH	−8.889	−7.586

3kDa fraction. This was attributed to the fact that the dielectric constant of the aqueous solution decreased with increasing ethanol concentration, and the weakly polar peptides were gradually separated due to dehydration, which has been reported previously (An et al., 2023). In addition, the results of free amino acid determination of each fraction obtained by ethanol precipitation (Table S2) showed that the content of umami amino acids (glutamic acid and aspartic acid) in the ED fraction was significantly lower than that in the EC fraction; accordingly, we hypothesized that ED was enriched in umami peptides (Fig. S2). To facilitate subsequent identification, we desalted the ED fraction using a 500 Da dialysis bag. The results showed (Fig. 1C) that after desalting, the umami of ED-I fraction was significantly improved and there was no perceptible salty taste. The peptide concentration was as high as 78.33% (w/w). Therefore, numerous umami peptides may exist in this fraction and were selected for the next step of umami peptide identification and screening.

For the permeate ED-II fraction, there were still many short peptides lost, which were not very different from the molecular weight of the sodium salt and were difficult to separate based on molecular weight differences. In this study, a nonpolar macroporous resin was selected to recover ED-II. The elution results using different concentrations of ethanol (Fig. 1D) showed that the water-eluted fraction ED–II–I had the highest umami score, and its saltiness was lower than that of the original fraction. Therefore, this fraction was selected for the next step in identification and screening.

3.2. Identification and screening of umami peptides from ED-I and ED–II–I based on peptidomics

3.2.1. Identification and online prediction of umami peptides

Peptidomics is a proteomics-based approach that allows for the enrichment of plausible peptides from purified fractions (Agyei et al., 2018). This study identified 1315 peptides with chain lengths of 5–10 from ED-I and 479 peptides with chain lengths of 2–4 from ED–II–I, and then screened 231 and 73 potential umami peptide sequences using machine learning models.

Table 2
Sensory descriptions of synthetic peptide aqueous solutions and recognition threshold values of synthetic peptides in the presence of water/MSG/NaCl.

Peptides	Taste attribute	Recognition threshold value (mg/mL) In the presence of water/MSG/NaCl		
		Water	MSG	NaCl
MSG		0.5		
Peptides in ED-II-I fraction				
TD	Umami, sour,kokumi	0.25	0.5	0.125
AD	Umami, sour,kokumi	0.25	0.25	0.125
GE	Umami, sour	0.5	0.5	0.125
VD	Umami, sour	0.5	0.5	0.5
LE	Bitter, slight umami	0.25	–	0.125
TEF	Umami, kokumi	0.5	0.25	–
AGE	Umami, sour	0.25	0.5	0.125
ADL	Strong umami, astringent	0.125	0.25	0.125
TSE	Umami, slight sour	0.5	0.5	0.125
AGN	Umami, sour	0.5	0.125	0.5
DSQ	Umami, sour,kokumi	0.25	–	0.5
GFAE	Umami, kokumi	0.25	0.5	0.5
TYET	Umami, kokumi	0.5	0.25	0.125
THEA	Strong umami, sour	0.25	0.125	0.25
Peptides in ED-I fraction				
DGRGHL	Slight umami, sour	0.25	0.25	0.25
EGELPR	Umami, kokumi,sour	0.5	0.25	–
FREGDL	Umami, kokumi,sour	0.5	0.125	0.25
RFESF	Umami, kokumi,sour, bitter	0.25	0.5	–
FREGDI	Strong bitter,sour	–	–	0.25
DPRVI	Umami, sour	0.125	0.25	0.25
RDNPHW	Sour, kokumi	–	0.5	0.5
HYKGSSF	Slight umami, bitter	0.25	–	–
GSNRFET	Umami, bitter	0.25	–	0.5
TFEKP	Umami, kokumi	0.5	0.125	0.5
HEWQH	Umami, sour	0.5	0.125	0.25

3.2.2. Screening of umami peptide based on molecular docking

Molecular docking and molecular dynamics simulation are effective tools for screening bioactive and umami peptides (Dang et al., 2019; Liu et al., 2019). Since the human umami receptor has not been resolved, homology modelling methods have been used to construct structural models of umami receptors. Fig. S3 shows the Ramachandran map of the calculations used to evaluate the model. The results show that 99.6% of the amino acids are in a reasonable range, with 87.3% of the optimal region, 10.4% of the acceptable region, 1.9% of the general region, and 0.4% of the amino acid residues in the impermissible region. This indicates that the homology model is structurally reliable and can be used for virtual screening of peptides. After molecular docking simulation, 11 and 14 peptide sequences with potential umami were screened from ED-I (231) and ED–II–I (73), respectively, and their docking scores are shown in Table S3. A smaller binding energy indicates better affinity and higher umami potential. The screened peptides were then subjected to sensory validation (see Table 1).

3.3. Sensory evaluation of synthetic peptides

3.3.1. Taste characteristics and threshold analysis of synthetic peptides

To study the taste characteristics of the peptides more comprehensively, 25 umami peptides obtained from the above screening were synthesized by solid-phase synthesis (Gill Biochemistry Co., Ltd., Shanghai, China), and the synthesized peptides with a purity higher than 95% were subjected to a quantitative description of taste and determination of thresholds (umami, umami-enhancing, and salt-enhancing thresholds), as shown in Table 2. Except for RDNPHW and FREGDI, the 23 synthetic peptides were perceived as umami. The perception of umami was enhanced by kokumi of some peptides, such as TD and AD. The umami thresholds of these 23 peptides were not greater than that of monosodium glutamate (0.5 mg/mL). Among them, four dipeptides, TD, AD, GE, and VD, were reported to be umami peptides, and quantitative descriptive analysis showed that these four dipeptides

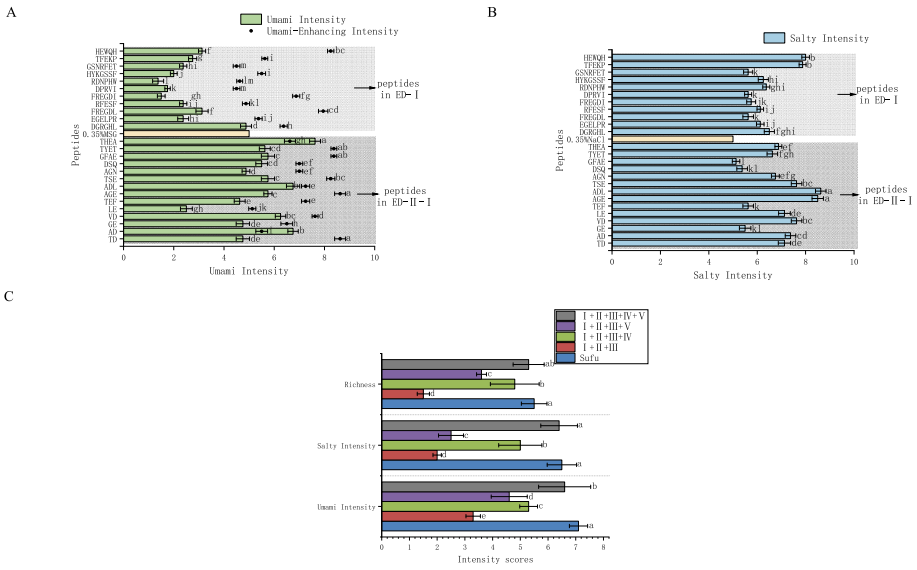

Fig. 2. (A) Umami intensity of synthetic peptides in aqueous and monosodium glutamate (MSG) solutions; (B) salty intensity of synthetic peptides in saline solutions; (C) umami and salty intensity and richness of taste recombination models..

Table 3
Compositions and Dry Contents of Nonvolatile Taste-Active Compounds in sufu (n = 3).

Component	Content (mg/g)	Component	Content (mg/g)
Group I: free amino acids			
aspartic acid (Asp)	7.43 ± 0.11	glutamic acid (Glu)	6.49 ± 0.02
isoleucine (Ile)	3.59 ± 0.06	threonine (Thr)	1.02 ± 0.11
leucine (Leu)	0.31 ± 0.01	serine (Ser)	2.61 ± 0.02
tyrosine (Tyr)	7.25 ± 0.5	aspartic acid (Asn)	1.23 ± 0.05
phenylalanine (Phe)	5.09 ± 0.02	lysine (Lys)	5.00 ± 0.67
glycine (Gly)	2.95 ± 0.11	histidine (His)	0.61 ± 0.19
alanine (Ala)	3.80 ± 0.04	arginine (Arg)	0.09 ± 0.01
valine (Val)	3.26 ± 0.06	proline (Pro)	2.91 ± 0.52
cystine (Cys)	2.95 ± 0.94	methionine (Met)	4.20 ± 0.17
Group II: 5′-nucleotides			
IMP	0.70 ± 0.02	GMP	ND
Group III: organic acids			
lactic acid	10.29 ± 0.33	succinic acid	2.72 ± 0.08
Group IV: NaCl			
NaCl	65.4 ± 8.80		
Group V: umami peptides			
ADL	0.51 ± 0.03	DGRGHL	0.25 ± 0.01
FREGDL	0.42 ± 0.02	AGE	0.10 ± 0.01
HEWQH	0.09 ± 0.01	GFAE	0.05 ± 0.00
TYET	< 0.001	TSE	0.02 ± 0.00
TFEKP	< 0.001	TD	< 0.001

presented obvious umami characteristics (Fig. 2A). Compared with the four reported umami dipeptides, the ADL and DPRVI peptides obtained in this study had a lower umami threshold (0.125 mg/mL). Overall, the umami peptide taste intensity identified in the ED–II–I fractions was generally higher than that of ED-I at the same concentration, indicating that umami peptides were still present in the effluent after desalination, further suggesting the important contribution of low molecular weight umami peptides in fermented foods. Among them, three peptides, THEA, ADL, and AD, showed significant umami. All of the synthetic peptides displayed sourness and astringency, as trifluoroacetate residues were introduced during the synthesis (Zhang et al., 2021). The taste of short umami peptides is significantly influenced by the amino acid composition, and often peptide sequences containing aspartic and glutamic acids have a greater probability of umami taste. For long peptides, the taste characteristics are not only related to the amino acid composition, but are also closely related to the geometric structure, hydrophilicity, and hydrophobicity of the amino acids. In the case of FREGDL and FREGDI screened in this study, the N-terminal amino acids are leucine and isoleucine, respectively, and leucine is more hydrophobic than isoleucine; therefore, FREGDI exhibits a strong bitter taste.

3.3.2. *Evaluation of umami- and salt-enhancing effects of synthetic peptides*

The umami peptides not only have umami characteristics by themselves, but some of them also have umami and saltiness enhancing effects (Yu et al., 2018; Zhang et al., 2019). The umami and salt enhancement evaluations of these 25 synthetic peptides are shown in Fig. 2A and B. TYET, GFAE, TSE, AGE, TD, HEWQH, FREGDL, and DGRGHL possessed significant umami enhancement, and HEWQH, TFEKP, ADL, and AGE possessed prominent salt enhancement. These 10 peptides had umami and salt enhancing abilities in single MSG or NaCl solutions, and we speculate that these 10 peptides may contribute to umami and salt taste in FSC. Therefore, the contents of these 10 peptides in FSC were determined to further evaluate the contribution of the umami peptides in FSC.

3.3.3. *Evaluation of the contribution of umami peptides to the taste of FSC*

Taste reconstitution experiments were conducted based on the quantitative results presented in Table 3. The sensory results of the reconstitution model showed that in the absence of NaCl and umami peptides, the umami, saltiness, and richness indices of the model system were significantly different from those of the original sample (Fig. 2C). After the addition of NaCl, there was a significant increase in the umami and saltiness scores (umami: 3.3 → 5.3, saltiness: 2.0 → 5.0), which showed that the main source of saltiness in the FSC system was NaCl, while the contribution of amino acids, nucleotides and organic acids to umami was only prominent in the presence of NaCl. However, there were still significant differences in umami, salty taste, and richness compared to the original curd sample. Both umami and salty perceptions increased significantly after the addition of umami peptides (umami: 5.3 → 6.6, salty: 5.0 → 6.2). This indicates that umami peptides can enhance the umami and salty tastes of the model system by 24.53% and 24.00%, respectively. However, the contribution of umami peptides to umami and saltiness was slightly lower in the model lacking the NaCl system. The synergistic effect of umami peptides and other taste substances provides a theoretical basis for the development of low-sodium salts.

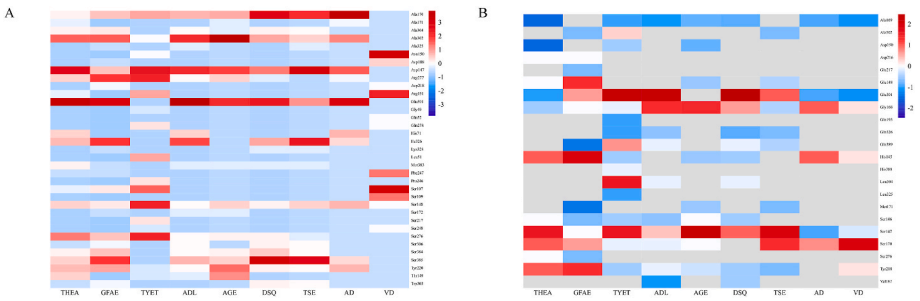

Fig. 3. (A) Active site statistics of nine umami synthetic peptides interacting with the VFTD structural domain of the 1EWK receptor T1R1; (B) Active site statistics of nine umami synthetic peptides interacting with the VFTD structural domain of the 1EWK receptor T1R3 (the redder the color, the greater the number of active sites formed between the umami peptide and umami receptor). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

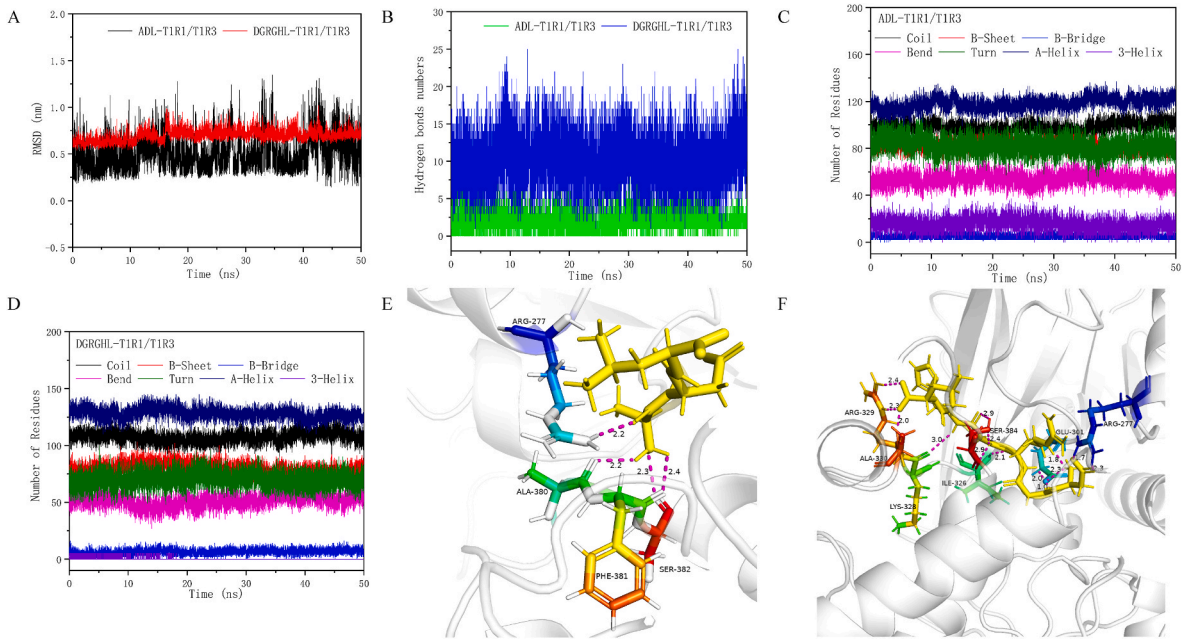

Fig. 4. The results of molecular dynamics simulations. (A) RMSD. (B) Hydrogen bonds. (C) and (D) Secondary structure. (E) and (F)The binding mode of peptide ADL and DGRGHL with VFTD at the last snapshot of the 50 ns MD simulations, and the pink dotted line indicates hydrogen bonds. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Molecular docking results of umami peptides with umami receptors

Based on the sensory results of the umami intensity of the synthetic peptides (Fig. 2A), nine umami peptides with scores >5 were selected to study their umami mechanisms with taste receptors. The origin data of the heat maps are listed in Tables S4 and S5. The docking results showed that these nine peptides mainly interacted with 35 residues of T1R1 and 22 residues of T1R3 through hydrogen bonding and hydrophobic interaction forces, respectively (Fig. 3A–B). Among them, Asp147, Glu301, Ala170, Ala302, and Ser385 were the most frequently contacted residues of T1R1, whereas Glu301, Gly168, Ser147, and Ser170 were the most frequently contacted residues of T1R3. This is consistent with the conclusion that Glu and Ser residues are the key residues in the active site as previously reported by Zhang, Zhang, et al. (2023). However, the energy of molecular docking is only used to screen potential umami peptides and cannot be used as a criterion to judge umami intensity (Li et al., 2023). Notably, we found that GFAE, ADL, and AD were the three peptides with the strongest umami taste, and the docking residues of these three peptides showed that their active sites were concentrated in two amino acid residues, Glu301 and Asp147. This may provide a new reference for screening umami peptides by selecting the umami peptide sequences corresponding to the more frequently contacted residues using the active-site statistics of the docking residues of umami peptides.

3.5. MD simulation

The interaction of the selected represent complex ADL-T1R1 and DGRGHL-T1R1 was further investigated by molecular dynamics simulation to explore the binding stability under explicit water environment. The results were show at Fig. 4. In general, root-mean-square deviation (RMSD) could reflect the relative movement of whole protein complex. The lower RMSD values, the more stable of the complex during dynamic simulation (Acevedo et al., 2016; Sun et al., 2021). Fig. 4A indicated that the complex of ADL-T1R1/T1R3 and DGRGHL-T1R1/T1R3 both reached equilibrium state within 50 ns dynamic simulation time, and their average RMSD were approximately 0.5 and 0.6, belong to small deviation. The main driving force between peptide and receptor was intermolecular hydrogen bonds (Gu et al., 2019). During whole simulation stage, the average hydrogen bonds in ADL-T1R1/T1R3 and DGRGHL-T1R1/T1R3 was about 2 and 10 (Fig. 4B), respectively. This revealed that DGRGHL binding more stable than ALD with T1R1, which was consistent with the docking affinity energy results, DGRGHL to

T1R1 (−9.452 kcal/mol) was much lower than ADL (−7.564 kcal/mol). Meanwhile, the binding actions not affected the receptor pocket regions too much. As shown in Fig. 4C–D, no significant fluctuations in the secondary structure of the complex protein were observed. The slight changes of α-helix content of the receptor T1R1/T1R3 have little impact on the structure stability during simulation. Fig. 4E–F depicted the binding hydrogen bonds related residues of two complex at the equilibrium snapshot. ADL formed hydrogen bonds with residues Arg277, Ala380, Phe381 and Ser382. DGRGHL formed hydrogen bonds with residues Arg329, Ala380, Lys328, Ile326, Ser384, Glu301 and Arg277. From Fig. 4E, the hydrophobic amino acid Ala in ADL plays an important role in the generation of hydrogen bonds, which may contribute to its umami (Song et al., 2023).

4. Conclusion

In this study, 25 umami peptides were identified and screened from curd using nano-HPLC-MS/MS, of which 19 were novel unreported umami peptides. Seven umami peptides with prominent tastes were quantified from sufu samples using UPLC-QQQ-MS, and taste reconstitution experiments demonstrated that these umami peptides contributed significantly to the umami, saltiness, and taste richness of the curd. Molecular dynamics simulations were performed to further investigate the conformational changes of complex ADL-T1R1/T1R3 and DGRGHL-T1R1/T1R3. This study advances the understanding of umami production in sufu and enriches the umami peptide database.

CRediT authorship contribution statement

Yong Dong: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Rui Chang:** Visualization, Methodology. **Zhongwei Ji:** Resources, Methodology. **Yuezheng Xu:** Resources. **Qingxi Ren:** Supervision, Methodology. **Zhilei Zhou:** Supervision, Project administration, Funding acquisition, Conceptualization. **Jian Mao:** Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.103951.

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