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Exploration of multi-functional peptides with bioactive and flavorful properties in Inner Mongolian cheese by peptidomics and bioinformatics

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ABSTRACT

The naturally fermented Inner Mongolian cheese's flavor and nutritional value make it a popular choice among customers. In this work, to create multi-functional peptides that have taste and biological activity, peptidomics and bioinformatics were used to screen flavor peptides from Inner Mongolian cheese and further assess their antioxidant and angiotensin I-converting enzyme (ACE) inhibitory properties. According to sensory data, YH8 and IL7 had detectable bitter tastes with low thresholds of 0.03 and 0.06 mmol/L, respectively. With an umami threshold range of 0.24–0.81 mmol/L, VQ6, FK13, HP13 and QT14 exhibited a range of flavors dominated by umami, including sweet, bitter, salty, sour and kokumi. Antioxidant activity-wise, YH8, VQ6, HP13 and QT14 were well represented. The above-mentioned peptides all had some ACE inhibitory effect. The bitter peptide IL7 (IC_{50} = 0.08 mmol/L) had the highest level of ACE inhibitory activity, followed by YH8 (IC_{50} = 0.33 mmol/L). These multi-functional peptides, which have been assessed for bioactive and taste features in Inner Mongolian cheese, may have positive impacts on health and harmonize the cheese's overall flavor. These results suggest that some flavor peptides produced in fermented foods might be with bioactivities while providing a basis for the exploration and application of multi-functional peptides.

1. Introduction

The top 5 naturally fermented cheeses in China include Xinjiang Kazakh milk knot, Inner Mongolian cheese, Yunnan milk fan, Rubing, and Tibet's Qula (yak milk cheese)[1]. Inner Mongolians use raw milk in a customary fermentation process to make the traditional cheese, known as hurood, which is a type of fermented milk product. Hurood is abundant in calcium, phosphate, iron, protein, fat and vitamins. It has several unsaturated fatty acids, including linoleic and oleic acid, which can lower cholesterol and prevent heart disease. Therefore, during fermentation, microbial activity, lipid oxidation, protein hydrolysis and carbohydrate metabolism are the main factors that affect how flavor is produced[2-3]. Kuhfeld et al.[4] used peptidomics and statistical tools to identify bitter peptides in aged Cheddar cheese and found 5 bitter peptides. Gu et al.[5] isolated and characterized novel umami peptides from Cheddar cheese by peptidomics and bioinformatics approaches, and the study identified a total of 6 umami/umami-enhancing peptides. Cheese, as a dairy product, is also high in bioactive peptides that have a variety of biological effects, including the inhibition of ACE, antioxidant, antibacterial, antidiabetic, immunomodulatory and anti-inflammatory properties[6]. It has been observed that some of the bioactive peptides

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in cheese exhibit umami, bitter, and kokumi tastes. Recently, research related to cheese is focused on volatile taste molecules[7-8], sensory changes during ripening[9], starters[10] and microbial diversity[8] of commercial cheeses (i.e., Cheddar cheese[7], Parmigiano Reggiano cheese[9], Gouda cheese and *Monascus*-ripened cheese[10]) and microbial diversity, production processes and volatile flavor compounds of traditional naturally fermented cheeses in China (i.e., milk fan[11], milk knots[12] and hurood[2]). However, few researchers are working on the exploration of taste peptides (i.e., umami or bitter peptides) with multiple bioactivities.

Typically, the exploration strategy of taste peptides, especially in umami peptides' exploration, usually includes enzymatic digestion, ultrafiltration, gel filtration chromatography, and reversed-phase high-performance liquid chromatography (HPLC), followed by mass spectrometry for identification and organoleptic evaluation[13]. However, the above-mentioned methods expose certain disadvantages such as being time-consuming, laborious, and even missing some potential taste peptides. Thus, peptidomics based on mass spectrometry combined with bioinformatics techniques offer a powerful way to mine, characterize, and screen peptides. Sebald et al. [14] were able to accurately identify and quantify bitter peptides in cheese using peptidomics technology. Galli et al.[15] investigated the peptide profile and bioactive peptides of Asiago-PDO cheese produced by different fermenters during ripening by peptidomics. A total of 101 bioactive peptides were found in aged Cheddar cheese, of which the main activities were antioxidant activity, ACE inhibitory activity, and antimicrobial activity[15].

In this work, typical hurood cheese products-cheese blocks, cheese strips, and cheese ingredients were studied to excavate their taste peptides (umami and bitter) with bioactivity (antioxidant activity or ACE inhibitory activity) through peptidomics and bioinformatics approaches. Their relationship of bioactive peptides and taste peptides was further reported, which may establish a solid foundation for the development of novel products, health care products of cheese.

2. Material and methods

2.1 Cheese sample

Inner Mongolia Hurood cheese is produced in Xilinhot Muxiangyuan Milk Food Co. Raw milk is filtered through the duplex filter and then heated to 30–35 ℃ for milk fat separation to obtain skimmed milk. The skim milk was fermented in a natural environment at 30 – 32 ℃ for around 16 h until the acidity value became approximately 36 ˚T and pH value is approximately 5.4. The fermented curd was then heated and stirred by adding a quantity of raw milk and stopped at 42 ℃. Stirring occurs to help the cheese mold and to extract whey from the curd. Following the molding process, the cheese block has roughly 2 cm^3 of toughness and elasticity. To create the final product of cheese, the formed cheese blocks are fried at a temperature of between 50 and 60 ℃ to remove extra whey. They are then packaged and sterilized. Two cheese products (cheese blocks B and cheese strips A) with varying milk contents were chosen for this study, as well as ingredient C of cheese after molding. In cheese strips, the proportion of whole milk to skim milk was 1:2, while in cheese blocks, the whole milk content was double that of the skim milk added during warming and mixing. Each sample was gathered, transferred to the lab, refrigerated using dry ice, and kept at –80 ℃ for additional examination.

2.2 Water-soluble peptide extraction

Extraction of water-soluble extracts (WSEs) referred to the method established by ÖZTüRK et al.[16] with slight modifications. Hurood cheese sample (2g) was vortexed by adding 10 g of ultrapure water. The homogenate was shaken in a water bath shaker at 40 ℃ and 200 r/min for 1 h and then centrifuged at 9 000 r/min for 20 min at 4 ℃. The supernatant was taken and its pH was adjusted to 4.6 with 1 mol/L hydrochloric acid to precipitate the soluble casein. The supernatant was centrifuged at 9 000 r/min for 20 min at 4 ℃. The supernatant was filtered through a 0.45 μm membrane filter. The extract obtained was used for Easy nLC1200/Q Exactive plus analysis.

2.3 Identification of peptide sequences by Easy nLC1200/Q Exactive plus

Lyophilized powder of WSEs (2 mg) was dissolved in the sampling buffer (0.1 % formic acid, 100 μL), and then desalted and dried with C_{18} Spin Tips, and then dissolved in 10 μL of sampling buffer to be measured.

NLC conditions: C_{18} reversed-phase column (50 mm × 50 cm monolithic column); mobile phase A: 0.1% formic acid, mobile phase B: 80 % acetonitrile and 0.1 % formic acid mixture. The elution was performed at a flow rate of 500 nL/min with the following liquid-phase elution gradient: 0–2 min, 98%–95% A, 2%–5% B; 2–45 min, 95%–78% A, 5%–22% B; 45–53 min, 78% – 65% A, 22% – 35% B; 53–54 min, 5%–0% A, 35%–100% B; 54–60 min, 0% A, 100% B.

Mass spectrometry conditions: ESI+ mode, data-dependent scanning mode, full-scan acquisition (*m/z* 350–1 800) in an orbital trap with a partitioning rate of 70 000 (AGC 3e6). The first 20 peptide signals (charge state ≥ 1) parent ions were fragmented by high-energy collision (HCD) with a normalized collision energy (NCE) of 28.0 eV. Capillary temperature 275 ℃, spray voltage 1 800 V. Daughter ions were measured in the orbital at a partition rate of 17 500 (AGC 1e5). The maximum fill times for full and MS-MS scans were 50 and 45 ms, respectively, and the dynamic exclusion time was 30 s. Sequence analysis of peptides in the samples was performed using Peaks studio10.0 software and Protein Discoverer 2.4 software.

2.4 Predictive analysis of peptide taste and bioactivity based on an online database

To ensure that the taste peptides and bioactive peptides play a role in flavor presentation properties and bioactivity in Inner Mongolian cheese, the top 500 peptides were selected for analysis. Taste activity was predicted using iUmami-SCM, Umami_YYDS, UMPred-FPL, Tastepeptides_DM, and iBitter-SCM for the prediction of its umami, bitter[17]. Kokumi properties of peptides are predicted by molecular docking. For the kokumi receptor, the crystal structure of the active form of the human CaSR extracellular domain (PDB ID: 5k5s, resolution: 2.6 Å) was utilized[18]. Molecular docking was performed using AutoDock Vina software (v2020.12.10). The center of CASR was selected for docking (center-x = 180, center-y = 45, center-z = 58) and the x/y/z side lengths of the box were as follows: size-x = 60, size-y = 84, and size-z = 112 (the size of the box was large enough to contain the pocket region and hinge region of CASR). Docking parameters were set as 12 exhaustiveness, 10 number of modes, and

10 energy range. Existing kokumi peptides were also selected for docking, and the conservative site estimation method was used to screen potential kokumi peptides.

The toxicity of the peptides was predicted using the online website ToxinPred (http://crdd.osdd.net/raghava/toxinpred/). ACE inhibitory activity according to a manually edited database of experimentally validated antihypertensive peptides developed by Kumar et al.[19] (http://crdd.osdd.net/raghava/ahtpdb/). The information in this database is mainly derived from 3 databases and ACE inhibitory peptides currently reported in the literature, ACEpepDB (http://www.cftri.com/pepdb/), BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep), EROP-Moscow database (http://erop.inbi.ras.ru/) and published literature[19]. Kumar et al.[20] developed a web-based platform based on a database for the prediction, design, and screening of ACE inhibitory peptides (http://crdd.osdd.net/raghava/ahtpin). Antioxidant activity was predicted through 2 online databases, firstly by using a quantitative conformational relationship predictor (AnOxPP) based on a bidirectional long and short-term memory neural network and interpretable amino acid descriptors (http://www.cqudfbp.net/AnOxPP/index.jsp)[21], and also by utilizing the Olsen et al.[22] developed a Prediction of the AnOxPePred tool for antioxidant activity (https://services.healthtech.dtu.dk/services/AnOxPePred-1.0/).

2.5 Peptides synthesis

Based on multi-bioactivities and flavor prediction scores, 6 peptides sequence, VMFPPQ (VQ6), IPYVRYL (IL7), YPFPGPIH (YH8), FVAPFPEVFGKEK (FK13), HKEMPFPKYPVEP (HP13) and QSWMHQPHQPLPPT (QT14) were chemically synthesized by GL Biochem Ltd. (Shanghai, China). The peptides were synthesized by chemical solid phase synthesis and desalination, and the purity of the peptides was greater the 98%.

2.6 Multi-functional peptides activities validation

2.6.1 Determination of flavor peptides threshold

The sensory evaluation team consisted of 10 excellent sensory panelists (6 females and 4 males, age 24 ± 2) recruited by the Muscle Food Flavor Innovation Perception Team of Shanghai Jiaotong University. The sensory evaluation team members were trained by GB/T16291.1–2012, and the synthetic peptide aqueous solution was subjected to the taste descriptive analysis and the determination of the bitter taste threshold.

2.6.1.1 Synthetic peptide flavor profiling

Descriptive analysis was used to evaluate the taste presentation properties of the synthetic peptides. Ultrapure water was used as a solvent and pH was adjusted to 4.6 (pH of WSEs) with 1 mol/L $NaHCO_3$[23]. The synthetic peptide was prepared as a 2 mg/mL aqueous solution and the sensory panelists tasted the samples and described their sensory properties, counted the resulting taste descriptors, and tallied the results.

2.6.1.2 Determination of threshold of synthetic peptides

Referring to the national standard GB/T 33406–2016, the bitter taste recognition threshold of the synthetic peptide was determined by using the three-point optional matching method (3-AFC), taste dilution analysis (TDA), and the lowest concentration at which the sensory panelists were able to judge and recognize the bitter taste. Ultrapure water was used as the solvent, and the pH was adjusted to 4.6 with 1 mol/L $NaHCO_3$ at an initial concentration of 0.125 mg/mL YH8, 0.25 mg/mL IL7 and 2 mg/mL VQ6, FK13, HP13 and QT14, and 8 concentration gradients were diluted. The samples were coded using a three-digit random number, and the samples were presented to the sensory panelists in order of lowest to highest concentration, with 3 samples in each group supplied in random order at an average supply temperature of 25 ℃. The sensory panelists tasted all 5 mL of the samples for 10 s at a time and then spat them out. To avoid sensory fatigue and residual effects, a 2-min break was taken between samples, and the mouth was rinsed thoroughly.

Taste identification thresholds were calculated using the best estimate threshold (BET) method, with the individual thresholds being the geometric mean of the maximum concentration of their incorrect responses and a higher level of concentration. The geometric mean of the individual thresholds of all sensory panelists in the sensory evaluation group was the group threshold, which was the final threshold.

2.6.2 In vitro antioxidant activity assay

The antioxidant activity was determined according to Wei et al.[24] with slight modification. Free radical scavenging activity was determined by 1,1-diphenyl-2-trinitrohydrazine (DPPH). In brief, 1 mL of 0.1 mmol/L DPPH solution was added to 1 mL WSEs and synthetic peptides in a centrifuge tube. The samples were mixed by shaking and incubated in the dark for 30 min at room temperature. 95% ethanol solution was used as a blank. The absorbance of the incubated samples was measured at 517 nm. DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = [1-(A_1-A_2)/A_3] \times 100 \quad (1)$$

Where A_1 is absorbance of the sample; A_2 is absorbance of the reaction between 95% ethanol and the sample; A_3 is absorbance of the reaction between distilled water and DPPH solution.

The $ABTS^+$ method was determined according to Wei et al.[24] with slight modification. $ABTS^+$ solution was prepared by adding ABTS solution (7.0 mmol/L) to an equal volume of potassium persulfate (2.45 mmol/L) and incubated in the dark for 12–16 h at room temperature. Before use, the $ABTS^+$ solution was diluted with ethanol (95%), and the absorbance at 734 nm was 0.70 ± 0.02. Then 0.5 mL of diluted $ABTS^+$ solution was added to 0.5 mL of the sample, and the absorbance at 734 nm was measured by incubating the sample for 10 min at room temperature. The $ABTS^+$ scavenging activity was calculated as follows:

$$ABTS^+ \text{ radical scavenging activity (\%)} = [1-(A_1-A_2)/A_3] \times 100 \quad (2)$$

Where A_1 is absorbance of the sample; A_2 is absorbance of the reaction between 95 % ethanol and the sample; A_3 is absorbance of the reaction between distilled water and $ABTS^+$ solution.

2.6.3 In vitro ACE inhibitory activity assay

The method of Hanafi et al.[25] for determination of ACE inhibitory activity was slightly modified. Preparation of hippuryl-histidyl-leucine (HHL) solution: 0.107 g HHL and 0.877 g NaCl were accurately weighed, 5 mL of sodium borate buffer (pH 8.3) was accurately measured, and the 3 were fixed with ultrapure water in a 50 mL volumetric flask and set aside.

Totally, 20 μL of the sample was pre-incubated with 10 μL of ACE (100 mU/mL) for 10 min at 37 ℃. The mixture was then incubated with 50 μL of substrate solution containing 5 mmol/L HHL buffer (pH 8.3) and 300 mmol/L NaCl for 60 min at 37 ℃. The reaction was terminated by the addition of 75 μL of HCl (1 mol/L) to all samples except the blank control (75 μL of 1 mol/L HCl was added before preincubation). Hippuric acid was extracted with 1 mL of ethyl acetate, vortexed for 30 s, centrifuged (4 000 r/min, 10 min, 4 ℃), and left to separate the aqueous phase from the organic phase, the entire ethyl acetate was collected, and the ethyl acetate was evaporated using a 110 ℃ oven for 30 min. The residue was dissolved in 1.0 mL of ultrapure water and the absorbance at 228 nm was measured by UV spectrophotometer. ACE inhibitory activity was determined by the following equation:

$$\text{ACE inhibition (\%)} = (A_c - A_s)/(A_c - A_b) \times 100 \quad (3)$$

Where A_c is absorbance of the control; A_s is absorbance of the sample; A_b is absorbance of the blank control (75 μL of hydrochloric acid was added before the addition of ACE).

2.7 Molecular docking of peptides with ACE

The 3D structure of the ACE from human (PDB: 1O8A) was obtained on the Protein Data Bank website (https://www.rcsb.org/)[26]. The structure was conducted to remove water and add hydrogen atoms before docking using PyMOL (2.5.7), and prepared using AutoDock Vina software. Water and ligands were removed from the ACE model before docking, whereas the cofactors zinc atoms were retained in the active site of the crystal structure of ACE. The center of ACE was selected for docking (center-x = –36.639, center-y = 32.667, center-z = 45.475) and the x/y/z side lengths of the box were as follows: size-x = 110, size-y = 122, and size-z = 112 (the size of the box was large enough to contain the pocket region and hinge region of ACE). Docking parameters were set as 20 exhaustiveness, 20 number of modes and 10 energy range. In addition, to realize the visual presentation of docking results, 3D docking results are displayed by PyMOL.

2.8 Statistical analysis

Mass spectrometry data were processed using Compass Data Analysis 4.3 software, and peptide sequences were identified using Peaks Studio 10.0 software, Protein Discoverer 2.4 software and the Uniprot library (http://www.uniprot.org/). To ensure the accuracy of the results, the peptides that appeared in all triplicate experiments were considered as the results. The IC_{50} values for antioxidant activities and ACE inhibitory activities were calculated using GraphPad Prism 8.0. Graphing was performed using Origin 2024 and Excel. Statistical Analysis Software IBM SPSS Statistics 24 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Differences were evaluated using a one-way analysis of variance (ANOVA) and Tukey's test, and the results were considered significant if $P < 0.05$. Except for molecular docking results and hydrophobicity calculations, all experiments were performed in triplicate, and the data are presented as mean ± standard deviation.

3. Results and discussion

3.1 Peptide profiles of Inner Mongolian cheese

The water extraction method was employed to extract the cheese samples from Inner Mongolia. Their basic taste profile was evaluated (Fig. 1A), the sensory evaluation revealed that the cheese strips and the ingredients presented similar scores in sour, kokumi and umami taste, while cheese strips presented low sweetness and bitterness compared with cheese ingredients. The cheese blocks had a higher umami and kokumi taste, which enriched the overall flavor profile of the cheese blocks and reduced their bitterness. Studies have demonstrated that by boosting the kokumi sensation of plant protein hydrolysates through lactic acid and heat treatment, bitterness could be decreased. Kokumi compounds, particularly kokumi peptides, have been proven to increase umami, salty, sweet and fatty texture[27].

In addition, the peptides' sequence and abundance of WSEs were identified by Nano-LC/MS. There are 2 712, 2 383 and 2 580 peptides were identified in cheese blocks, cheese strips and ingredients, respectively, including 1 385 communal peptides (Fig. 1B and Table S1). And most of the peptides dominated by 6–14 amino acid residues in length (Fig. 1C). Among them, the number of peptides was more in cheese blocks than in cheese strips, probably due to more whole milk adding in cheese blocks and accelerating microbial activities. That is consistent with the report that milk fat in whole milk may enhance the activity of *Pseudomonas* species protease in cheese, affect the metabolism of *Pseudomonas* species, stimulate the secretion of protease, promote protein hydrolysis, and therefore produce more peptides[28]. Moreover, the classification of the peptides according to their Q-values (Fig. 1D) showed that the cheese blocks contained more hydrophilic peptides compared with the cheese strips and ingredients, suggesting that the cheese blocks may contain more potentially umami peptides, which is consistent with the results of the sensory evaluation of WSEs. Since it is difficult for peptides with low content to exert the corresponding taste attributes and bioactivities in cheese, the top 500 peptides were selected for taste and bioactivity prediction.

3.2 Screening of potential taste peptide and analysis

Machine learning methods help to make more evidence-based decisions, and as more and more umami peptides/bitter peptides were discovered, several machine prediction models have been developed for rapid screening of umami or bitter peptides. Charoenkwan et al.[29] developed iUmami-SCM to predict umami peptides using a newly developed scoring card method (SCM) in conjunction with the propensity scores of amino acids and dipeptides. Cui et al.[17] established the system TastePeptides-Meta, containing a taste peptide database TastePeptidesDB an umami/bitter taste prediction model Umami_YYDS and an open-source machine learning package Auto_Taste_ML, which are helpful for rapid screening of umami or bitter peptides.

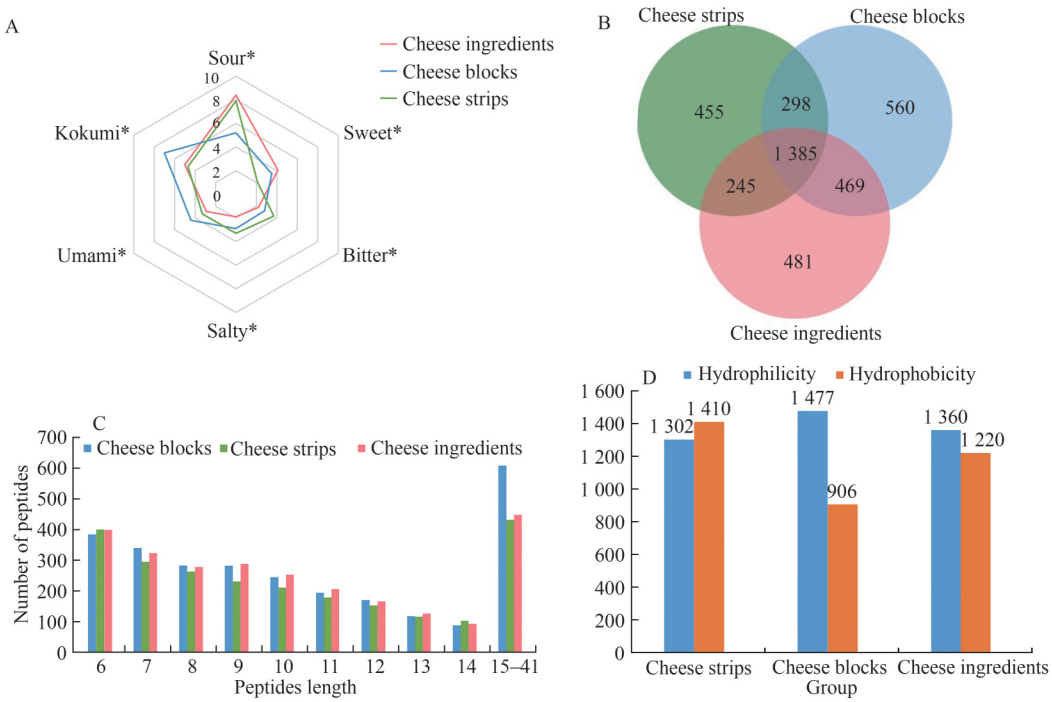

Fig. 1 The profile of peptides identified in cheese strips, cheese blocks and cheese ingredients. (A) Sensory evaluation of WSEs, taste attributes of cheese strips and cheese blocks were compared to cheese ingredients *via* Tukey's HSD to analyze significance levels. *$P < 0.05$; (B) Venn diagram of the number of peptides identified from cheese strips, cheese blocks and ingredients by Nano-LC/MS; (C) number of peptides categorized by length; (D) classification of hydrophilic and hydrophobicity of identified peptides.

Umami peptides are important components of umami substances that impart umami to foods and are widely found in various foods, such as puffer fish[30], mushrooms[31] and chicken[32]. Umami peptides were predicted using Umami_YYDS, iUmami-SCM, and TastePeptides DM online prediction sites, and potential umami peptides were judged based on prediction scores. In this study, 121, 138 and 126 potential umami peptides were predicted from cheese strips, cheese blocks and ingredients, respectively (Fig. 2A). These potential umami peptides could be coordinated with other flavor substances and at the same time serve to inhibit the bitter in cheese and improve the overall taste of Inner Mongolian cheese products.

The bitter peptides in cheese are mainly related to the decomposition of casein to produce peptides with different physicochemical properties and are also related to the content of raw milk, pH, fat and salt during cheese production[33]. Studies have shown that bitter peptides in cheese are mainly derived from hydrolysis of β-casein and α_{s1}-casein, with lesser sources of α_{s2}-casein, κ-casein and whey proteins[4]. TastePeptides DM and iBitter-SCM online websites were used to predict whether the bitter taste was present based on the scores. In this study, 114, 94 and 84 potential bitter peptides were predicted for cheese strips, cheese blocks and cheese ingredients (Fig. 2A), which was consistent with the sensory results, with cheese strips being more bitter than cheese blocks.

Kokumi peptides in cheese could improve the complexity, continuity and mouthfulness of cheese, such as γ-Glu-Phe, γ-Glu-Tyr, γ-Glu-Leu, γ-Glu-Met[34]. Hillmann et al.[35] found that γ-glutamyl dipeptides are a key factor in the presentation of kokumi flavor in Parmesan cheese, imparting a sense of persistence and complexity to the cheese texture. In this study, 147, 166 and 191 potential kokumi peptides were predicted in cheese strips, cheese blocks and cheese ingredients (Fig. 2A), based on the conservative site estimation method for predicting the presence or absence of a sense of thickening in conjunction with the reported docking scores of kokumi peptides.

3.3 *Screening of potential bioactivity and analysis*

Recently, more and more researchers have unearthed food-borne active peptides, among which the most common bioactive peptides are antioxidant peptides and ACE inhibitory peptides. Antioxidant peptides could effectively inhibit the deterioration of food, and play a certain antioxidant effect after human intake to prevent heart disease, diabetes, cancer and other diseases[36]. Currently, cardiovascular diseases such as coronary heart disease, stroke and heart failure are a major problem plaguing human health, as well as a major research hotspot. Angiotensin I-converting enzyme is a dipeptide carboxypeptidase that is involved in the regulation of blood pressure, fluid and electrolyte homeostasis, and plays a dual role in the renin-angiotensin-aldosterone system[37]. Furthermore, studies have shown that hypertension is usually associated with oxidative stress[37], therefore in this work, peptides were predicted and screened for antioxidant activity and ACE inhibitory activity. Firstly, to ensure the screening of novel multiple active peptides, the reported active peptides were firstly screened by 3 databases, including BIOPEP Active Peptide Database (http://mbpdb. nws. oregonstate. edu/), MBPDB Milk-derived Peptide Database (http://mbpdb.nws.oregonstate.edu/) and DFBP database (http://www.cqudfbp.net/). After screening the first 500 peptides, 81, 94 and 87 reported active peptides were identified from cheese sticks, cheese blocks and ingredients, with a total of 128 active peptides excluding duplicates. These 128 active peptides originated from β-casein, α_{s1}-casein, α_{s2}-casein and κ-casein (Fig. 2B), of which,

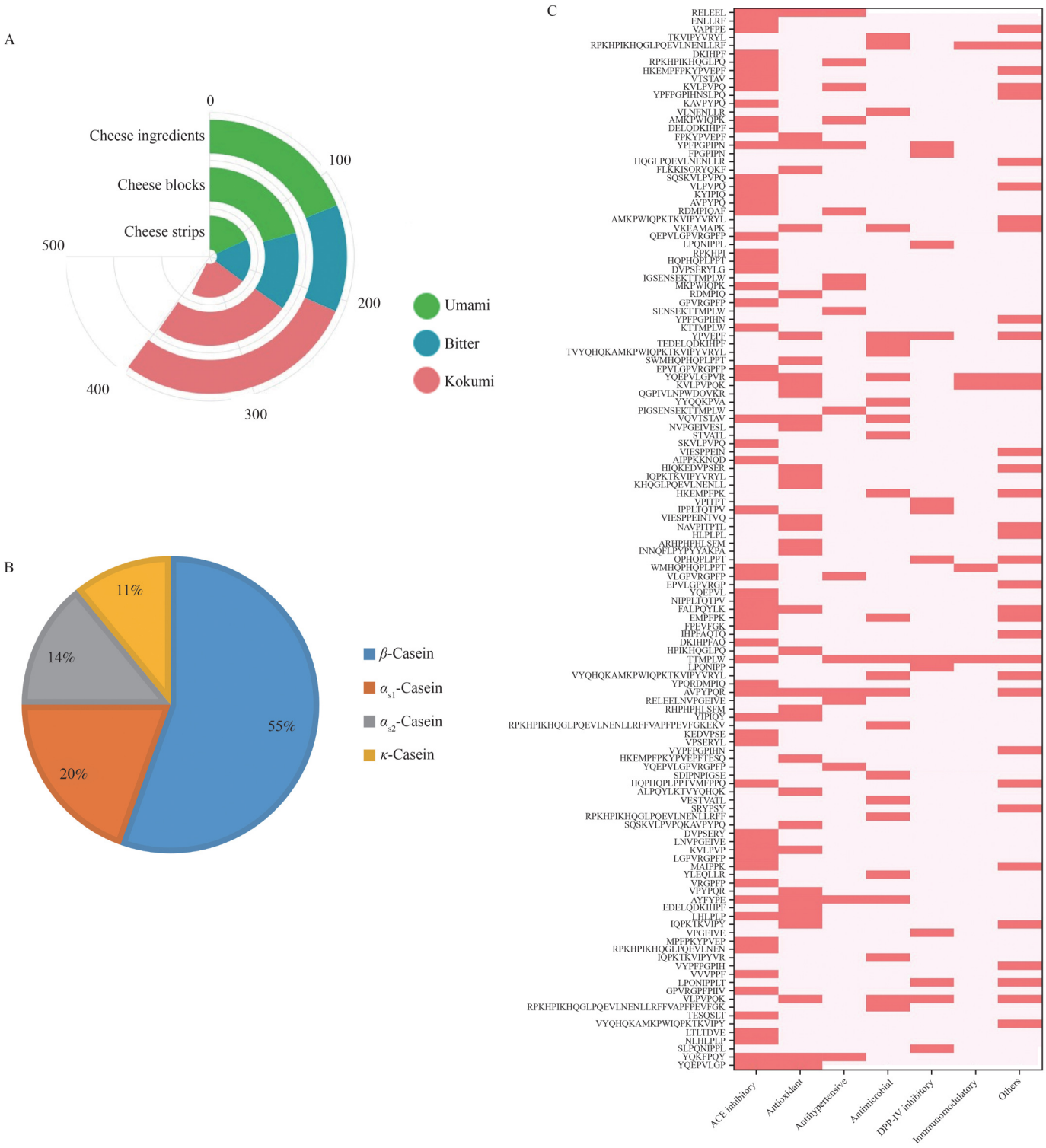

Fig. 2 Cheese peptide screening and prediction results. (A) Results of umami, bitter and kokumi prediction; (B) sources of reported bioactive peptides screened in the database; (C) main bioactivities of reported bioactive peptides.

55% were derived from *β*-casein. The active peptides are mainly derived from *β*-casein, α_{s1}-casein and α_{s2}-casein, as these are the main components of casein. During fermentation, lactic acid bacteria can extracellular proteases and peptidases, such as endopeptidases, dipeptidases, and aminopeptidases[38], which can hydrolyze the more structurally rigid *κ*-casein and release more active peptides. Statistical analysis of the 128 active peptides showed that most of them possessed ACE inhibitory and antioxidant activities, as well as antihypertensive, antimicrobial, DPP-IV inhibitory, immunomodulatory and other activities (Fig. 2C). Therefore, antioxidant activity and ACE inhibitory activity were predicted for 419, 405 and 414 peptides from cheese strips, cheese blocks and cheese ingredients.

3.4 Six potential multi-functional peptides

Peptides common to the 3 samples, cheese sticks, cheese blocks and ingredients, were screened by predicting taste properties and biological activities (175 peptides in total). First, the ACE inhibitory activity was screened and the predicted score > 1 was selected as the potential ACE inhibitory peptide. The antioxidant activity of the peptides was combined with 2 indicators: antioxidant indicator 1 to directly determine the antioxidant properties, and antioxidant indicator 2 to select FRS scores (> 0.5) and CHEL scores (> 0.2) for screening. The combined ACE inhibitory activity and antioxidant activity screen yielded a total of 10 potential multi-functional peptides (Table S2). Finally, 6 potential multi-functional peptides were selected based on the peptide content in the cheese ingredients, including IL7, YH8, VQ6, FK13, HP13 and QT14. These 6 peptides possess not only taste properties, but also antioxidant activity and ACE inhibitory activity, and are expected to be added to food as functional ingredients.

3.5 Identification of taste characteristics of synthetic peptides by sensory evaluation

Water solubility of peptides is a prerequisite for peptides to exert their activity, while toxicity is a key consideration in the development of flavor peptides. The synthesized peptides were found to be well water-soluble and non-toxic by online prediction. Based on sensory evaluation, 2 peptides, YH8 and IL7, presented a single bitter taste with low bitter taste thresholds of 0.03 and 0.06 mmol/L. The taste of the remaining 4 peptides was complex and diverse, with umami as the main taste characteristic and slightly bitter, sour, sweet and kokumi tastes (Table 1). These 4 umami peptide thresholds were, from low to high, QT14 (0.24 mmol/L) < HP13 (0.31 mmol/L) < FK13 (0.43 mmol/L) < VQ6 (0.81 mmol/L). Among them, compared with the other 3 umami peptides, VQ6 had a higher umami threshold, which may be due to its shorter peptide chain[39]. This correlation between peptide chain length and binding affinity may be attributed to the unique interactions that occur between the peptide and the binding cavity and thus may result in a higher threshold for shorter peptides[39]. The results of the sensory evaluation indicated that VQ6, FK13, HP13 and QT14 have great potential for the development of novel flavor additives in the food industry.

3.6 Analysis of the antioxidant activity of synthetic peptides in vitro

Lipid peroxidation is a very serious problem in the food industry, where free radicals produced by oxidation lead to the breakdown of fatty acids, reducing the quality, and acceptability of the food. In this study, DPPH and $ABTS^+$ free radical scavenging assays were used to evaluate the *in vitro* antioxidant activity of the 6 synthesized taste peptides and their dose-response relationship. The results are shown in Fig. 3. The DPPH free radical scavenging method is suitable for assessing the antioxidant activity of hydrophobic peptides, whereas the $ABTS^+$ method is suitable for hydrophilic and lipophilic antioxidant systems[40]. The DPPH radical scavenging ability of all 6 peptides showed an increasing trend with concentration over the tested concentration range of 0.5 to 20 mg/mL (Fig. 3A). In contrast, the DPPH radical scavenging rates of VQ6, QT14, YH8 and HP13 varied relatively strongly over the concentration range, suggesting that they have better antioxidant activity. The IC_{50} value was used to assess the free

Table 1
Taste properties and thresholds of synthetic peptides.

Peptides	Acronyms	Mass (Da)	Source	Taste description	Threshold value (mmol/L)	Water solubility	Toxicity
YPFPGPIH	YH8	927.063 3	*β*-Casein	Bitter	0.03	Good	Non-toxin
IPYVRYL	IL7	923.108 6	α_{s2}-Casein	Bitter	0.06	Good	Non-toxin
VMFPPQ	VQ6	717.883 8	*β*-Casein	Umami, bitter and kokumi	0.81	Good	Non-toxin
FVAPFPEVFGKEK	FK13	1 494.747 1	α_{s1}-Casein	Umami, bitter, sweet, sour and kokumi	0.43	Good	Non-toxin
HKEMPFPKYPVEP	HP13	1 598.877 1	*β*-Casein	Umami, salty, sour, bitter and kokumi	0.31	Good	Non-toxin
QSWMHQPHQPLPPT	QT14	1 683.911 9	*β*-Casein	Umami, bitter sour and kokumi	0.24	Good	Non-toxin

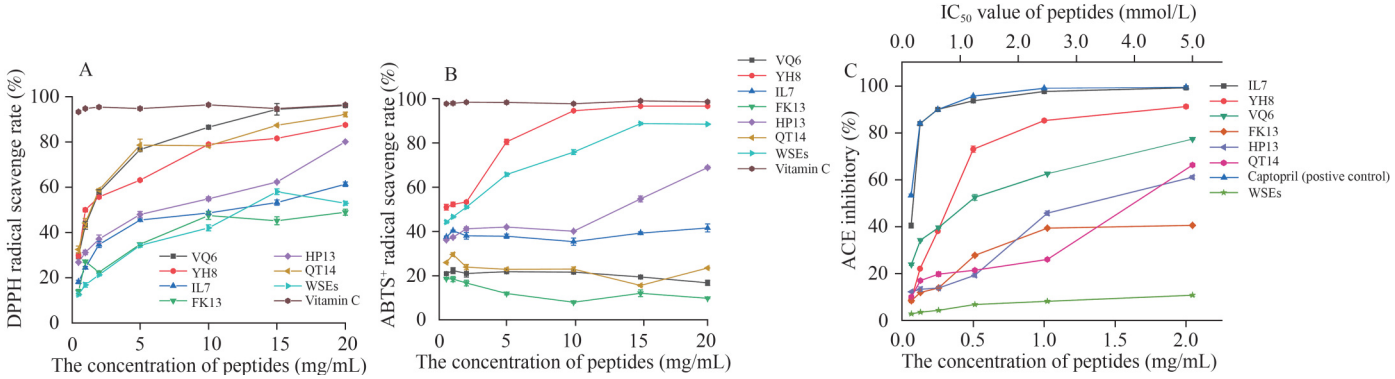

Fig. 3 Dose-response curves of 6 synthesized peptides for scavenging (A) DPPH and (B) $ABTS^+$ free radical scavenging, (C) dose-response curves of 6 synthesized peptides for ACE inhibitory activity.

radical scavenging ability, with smaller IC_{50} values indicating greater antioxidant capacity.

As shown in Table 2, the IC_{50} values of QT14, YH8 and VQ6 were significantly smaller than those of HP13, IL7, FK13 and WSEs. The 6 peptides isolated and screened in this study were all hydrophobic peptides except QT14, but QT14 had a lower ability to scavenge $ABTS^+$ radicals. Hydrophobic amino acids (His, Trp, Phe, Pro, Gly, Lys, Ile and Val) have high $ABTS^+$ radical scavenging capacity, but high ratio hydrophobic amino acids show a low solubility and thereby the $ABTS^+$ radical scavenging capacity of peptide[40]. QT14 contains a large amount of hydrophobic amino acids (Try, His and Phe), resulting in its poor $ABTS^+$ radical scavenging activity. In contrast, YH8 and WSEs had a stronger ability to scavenge $ABTS^+$ free radicals, with YH8 having a strong antioxidant capacity with an IC_{50} value of 0.84 mmol/L. The antioxidant activity of peptides is mainly affected by amino acid composition, amino acid position, amino acid sequence, peptide chain length, spatial conformation and hydrophobicity[41]. It has been shown that amino acid residues such as Phe, Pro, Tyr, His, Ala, Asp and Leu could act as effective proton/hydrogen donors playing an important role in the antioxidant activity of peptides, while the C-terminal end of the peptide chain containing aromatic amino acids, amphiphilic and polar amino acids as well as N-terminal end containing hydrophobic amino acids have a strong antioxidant activity[42]. Besides, there are 2 mechanisms of free radical inactivation: hydrogen atom transfer (HAT) and single electron transfer (SET). The HAT mechanism is associated with the amino acid Tyr, while SET is associated with amino acid Cys, Trp and His[40]. YH8 has the amino acid Tyr at the N-terminus and the amino acid His at the C-terminus, so YH8's radical scavenging ability may be related to the SET mechanism. Meanwhile, YH8 contains a large amount of Pro and Phe, which can act as an effective proton/hydrogen donor and improve the overall antioxidant activity of YH8. Cui et al.[43] released antioxidant peptides by hydrolyzing milk proteins by lactic acid bacteria and 3 peptides with antioxidant activity were found in the study. Among them, VKEAMAPK showed excellent free radical scavenging ability, and the IC_{50} values of DPPH and ABTS free radical scavenging activities were 0.63 and 0.84 mg/mL, respectively[43].

Thus, the antioxidant activities of YH8, VQ6, QT14 and HP13 were relatively strong among the 6 peptides, but weaker compared to the activities of antioxidant peptides already reported in the literature.

3.7 Analysis of ACE inhibitory activity of synthetic peptides in vitro

Angiotensin-converting enzyme inhibitory peptides have been shown to play an important role in the regulation of blood pressure. The results of ACE inhibitory activity of the 6 peptides with IC_{50} values are shown in Fig. 3C and Table 2, IL7 (IC_{50} = 0.08 mmol/L), YH8 (IC_{50} = 0.33 mmol/L) and VQ6 (IC_{50} = 1.40 mmol/L) had better ACE inhibitory activity. In addition, the other 3 umami peptides had relatively weak ACE inhibitory activities. Among them, HP13 (IC_{50} = 2.11 mmol/L) and QT14 (IC_{50} = 2.40 mmol/L) presented lower IC_{50} than VQ6 (IC_{50} = 1.40 mmol/L). FK13 had the lowest ACE inhibitory activity (IC_{50} = 4.79 mmol/L). It has been shown that the amino acid residues of peptides have a vital effect on the ACE inhibitory activity of the peptide, especially the amino acid position of the peptide chain. Sonklin et al.[44] isolated peptides with ACE inhibitory activity from mung bean all contained leucine, suggesting that leucine in the peptide sequence may contribute to ACE inhibitory activity.

It was found that hydrophobic amino acids, aromatic amino acids and negatively charged amino acids were positively correlated with ACE inhibitory activity[42]. The N- and C-terminal amino acid residues of the peptide chain play key roles in ACE inhibitory activity, with the most potent structure for lowering hypertension being a tripeptide residue composed of hydrophobic amino acids (Phe, Tyr, Pro, Ala, Val and Leu) and aromatic amino acids (Phe and Tyr) at the C-terminus[42]. Consistent with the results of the present study, IL7 (hydrophobic:hydrophilic = 6:1) containing a leucine residue at the end had the strongest ACE inhibitory activity. It has been found that due to the sequence, amino acid composition and molecular weight, bitter peptides can exert a variety of physiological effects, such as antihypertensive, antibacterial, antithrombotic, hypocholesterolemic and antioxidant effects[36]. Yang et al.[45] isolated the bitter peptide RPKHPIK with antioxidant activity and ACE inhibitory activity from yak milk hard cheese. Combined with the results of sensory evaluation, bitter peptides IL7 and YH8, had strong ACE inhibitory activity among the 6 peptides. Bitter is considered an unpleasant flavor that affects its acceptability, so in recent years more and more research has tended to focus on how to modify or mask bitter flavor. In addition, the other 4 umami peptides also have certain ACE inhibitory activity, among which VQ6 has a good ACE inhibitory activity with an IC_{50} value of 1.41 mmol/L. Besides, umami peptides (HP13 and QT14) also have certain ACE inhibitory activity, which can act as synergistic enhancement of biological activities and have good application prospects in the field of new product development such as delicious functional foods.

3.8 Molecular interactions between ACE inhibitory peptides and ACE

Based on molecular docking results, the molecular mechanisms underlying the ACE inhibitory activities of peptides were further

Table 2
Hydrophobicity, antioxidant activity (DPPH and ABTS assays) and ACE inhibitory activity of 6 synthetic peptides.

Peptides	*Q* value	IC_{50} of DPPH radical scavenging (mmol/L)	IC_{50} of $ABTS^+$ radical scavenging (mmol/L)	IC_{50} of ACE inhibitory activity (mmol/L)
YH8	2 110.681	1.55 ± 0.19^{d}	0.84 ± 0.04^{b}	0.33 ± 0.02^{d}
IL7	2 313.917	9.95 ± 0.93^{b}	–	0.08 ± 0.01^{d}
VQ6	1 797.324	1.85 ± 0.11^{cd}	–	1.40 ± 0.10^{c}
FK13	1 638.854	13.13 ± 0.64^{a}	–	4.79 ± 0.30^{a}
HP13	1 819.499	2.87 ± 0.51^{c}	4.79 ± 0.52^{a}	2.11 ± 0.35^{b}
QT14	1 127.779	0.76 ± 0.06^{d}	–	2.40 ± 0.52^{b}

Note: "–" indicates that the IC_{50} value of the peptide was not measured in the concentration range. Different letters represent significant differences in the activities of peptides ($P < 0.05$).

analyzed. The best conformation and key forces involved in the docking of 6 peptides with ACE were shown in Fig. 4, including hydrogen bonds, electrostatic interaction (salt bridge) and hydrophobic interaction in the ACE complex. Generally, ligand and receptor can spontaneously bind when the binding energy is lower than 0 kcal/mol, and the lower the binding energy, the more stable the conformational structure of the ligand-receptor complex[46]. The main active site of ACE consists of 3 pockets, the S1 pocket (Gln281, His353, Lys511 and His513), the S2 pocket (Ala354, Glu384 and Tyr523), and the S1′ pocket (Glu162), which are thought to be the possible sites of contact between the competitive inhibitor and ACE[47].

Hydrogen bonding is an important non-covalent interaction in the binding process between inhibitors and ACEs and contributes to the formation of stable docking complexes. None of the 6 peptides with ACE inhibitory activity in this study bound to the 3 pockets, probably as non-competitive inhibitors that can interact with the inactive site of ACE to produce ACE inhibitory activity[48]. In addition, 6 ACE inhibitory peptides did not bind to zinc ions in ACE, which was hypothesized to be possibly related to the composition and position of amino acid, hydrophobicity, peptide chain lengths and the conformation of peptides[26,49]. The binding energies of IL7 and YH8 with ACE were the lowest, –6.8 and –7.9 kcal/mol, respectively. The complexes formed by IL7 and YH8 with ACE were the most conformationally stable, and at the same time were consistent with the results of ACE inhibitory activity *in vitro*. As shown in Fig. 4, IL7, YH8, FK13, HP13 and QT14 bind in similarly localized domains and have the same binding sites. IL7, YH8, HP13 and QT14 form hydrogen bonds with Thr-153, Lys-155 and Asn-194 of the ACE, and IL7 and YH8 form a hydrogen bond with Cys-157 of the ACE, which is probably the reason why this region is more accessible.

Although none of the 6 peptides isolated from Inner Mongolian cheese bound to the reported active site of ACE, they all exhibited ACE inhibitory activity. Two possible reasons were hypothesized: 1) the spatial conformation and size of the ACE inhibitory peptide leads to difficulties in accessing the inside of the active pocket; 2) it is possible that in addition to the 3 reported active sites, the outside of the ACE may also have a potential active site, but further validation is needed.

4. Conclusion

Taken together, 6 multifunctional peptides with flavor characteristics and biological activities from Inner Mongolian cheese have been identified by virtual screening and *in vitro* activity testing. The strongest ACE inhibitory activity was exhibited by bitter peptide IL7, whereas excellent antioxidant and ACE inhibitory activity was exhibited by bitter peptide YH8 among these peptides. Furthermore, 4 umami peptides exhibit strong antioxidant and ACE inhibitory properties, making them promising candidates for application in the functional food industry for both the creation of functional meals and enhancing consumer acceptance in general. Among them, FK13 has a low umami threshold but comparatively modest bioactive activity. Consequently, FK13 may function as a synergistic enhancer of

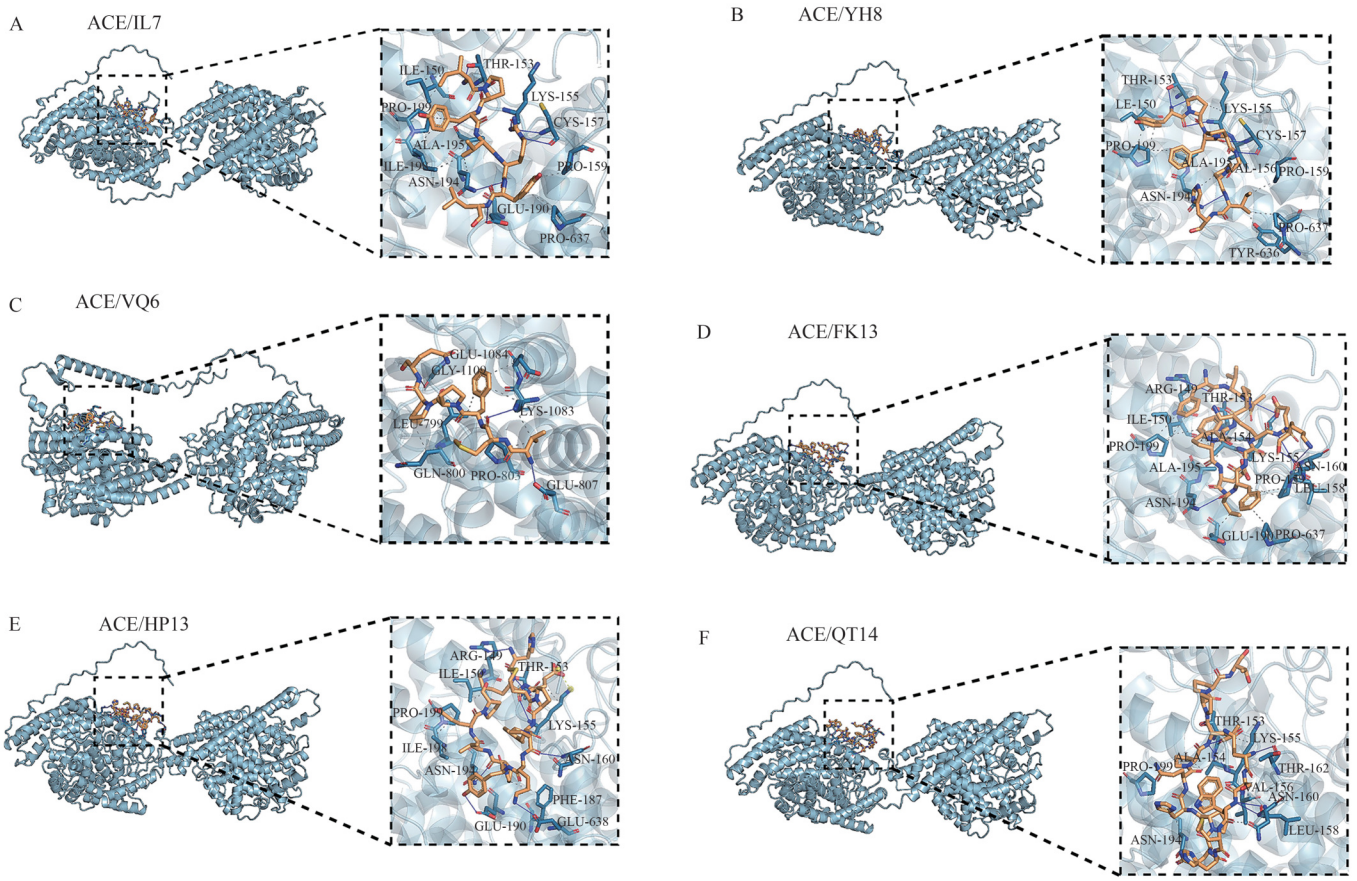

Fig. 4 Molecular docking results of 6 peptides to angiotensin-I-converting enzyme (ACE; PDB ID:1O8A). Three-dimensional binding mode of peptides (A) IL7, (B) YH8, (C) VQ6, (D) FK13, (E) HP13, (F) QT14 to ACE.

bioactivities in addition to balancing the taste of cheese to lessen bitterness and enhancing umami when coupled with other foods. Our research demonstrated that in summary, Inner Mongolian cheese is an outstanding source of flavor and bioactive peptides. Furthermore, these multifunctional peptides possessing bioactive and taste characteristics hold promise for use in the creation of innovative functional foods, aspects of health promotion and nutritional supplements. The comprehensive screening strategy established in this study provides an efficient and convenient method for rapid screening multi-dimensional peptides from fermented dairy food. It also provides a theoretical basis for multi-functional peptides and the development of high-value-added products.

Conflict of interest

Wenli Wang is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250377.

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