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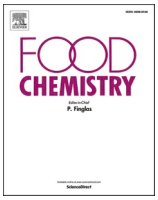

Exploring structural features of sleep-enhancing peptides derived from casein hydrolysates by chemometrics and random forest methodology

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ABSTRACT

Milk casein is regarded as source to release potential sleep-enhancing peptides. Although various casein hydrolysates exhibited sleep-enhancing activity, the underlying reason remains unclear. This study firstly revealed the structural features of potential sleep-enhancing peptides from casein hydrolysates analyzed through peptidomics and multivariate analysis. Additionally, a random forest model and a potential Tyr-based peptide library were established, and then those peptides were quantified to facilitate rapidly-screening. Our findings indicated that YP-, YI/L, and YQ-type peptides with 4–10 amino acids contributed more to higher sleep-enhancing activity of casein hydrolysates, due to their crucial structural features and abundant numbers. Furthermore, three novel strong sleep-enhancing peptides, YQKFPQY, YPFPGPIPN, and YIPIQY were screened, and their activities were validated *in vivo*. Molecular docking results elucidated the importance of the YP/I/L/Q- structure at the N-terminus of casein peptides in forming crucial hydrogen bond and π-alkyl interactions with His-102 and Asn-60, respectively in the $GABA_A$ receptor for activation.

1. Introduction

More than 30% of the global population suffers from insomnia increasing significant risk factors such as emotional disturbance, fatigue, memory impairment, neuroinflammation, brain dysfunction, and decrease in immunity (Morin et al., 2015). The assessment of sleep includes two categories: objective and subjective sleep assessment (Cox & Olatunji, 2016). The mechanisms of sleep disturbance are complex and involves several receptors such as gamma butyric acid A ($GABA_A$) receptors, serotonin and melatonin receptors (Xu Kun, 2019; Kersanté et al., 2023; Xue et al., 2023). Therefore, there are no simple *in vitro* assessments to determine sleep quality. Sleep duration is a key indicator to evaluate insomnia and can be used to evaluate the sleep-enhancing activity of bioactive substances *in vivo*. Therefore, a pentobarbital-induced sleep behavioral test can be used to directly evaluate sleep duration (Vgontzas et al., 2013; Liu et al., 2020). The treatment of sleep disorders primarily relies on psychotherapy and pharmacotherapy. Insomnia drugs are often accompanied by high addiction or side effects. Therefore, the use of food-derived bioactive substances, with bioactive peptides has emerged as safe therapeutic alternatives.

To date, limited studies revealed the structural features of sleep-enhancing and anxiolytic peptides, potentially due to the limited data on potent peptides. The reported potential features of sleep-enhancing peptides included the presence of Tyr (tyrosine)/Trp (tryptophan) residues, as suggested by Lv et al. (2021). The structure of Trp at N-terminus might be associated with sleep-promotion. Food sources that are rich in Trp and Trp oligopeptides have been reported to alleviate anxiety and depression symptoms through Trp metabolism and HPA axis regulation (Dela Peña et al., 2015; Zhu et al., 2020). As Sánchez-Rivera et al. (2020) suggested, the presence of Tyr at N-terminus could be distinctly recognized for activating the opioid receptors. In our previous research, we showed that the structure of two symmetrical Pro at the C and N terminals and Tyr at N-terminus played vital roles in the strong sleep-enhancing effects of YPVEPF (Qian, et al., 2024). However, the structural features of strong sleep-enhancing peptides remain unclear.

Milk casein rich in Tyr and Pro, stands out as a promising precursor

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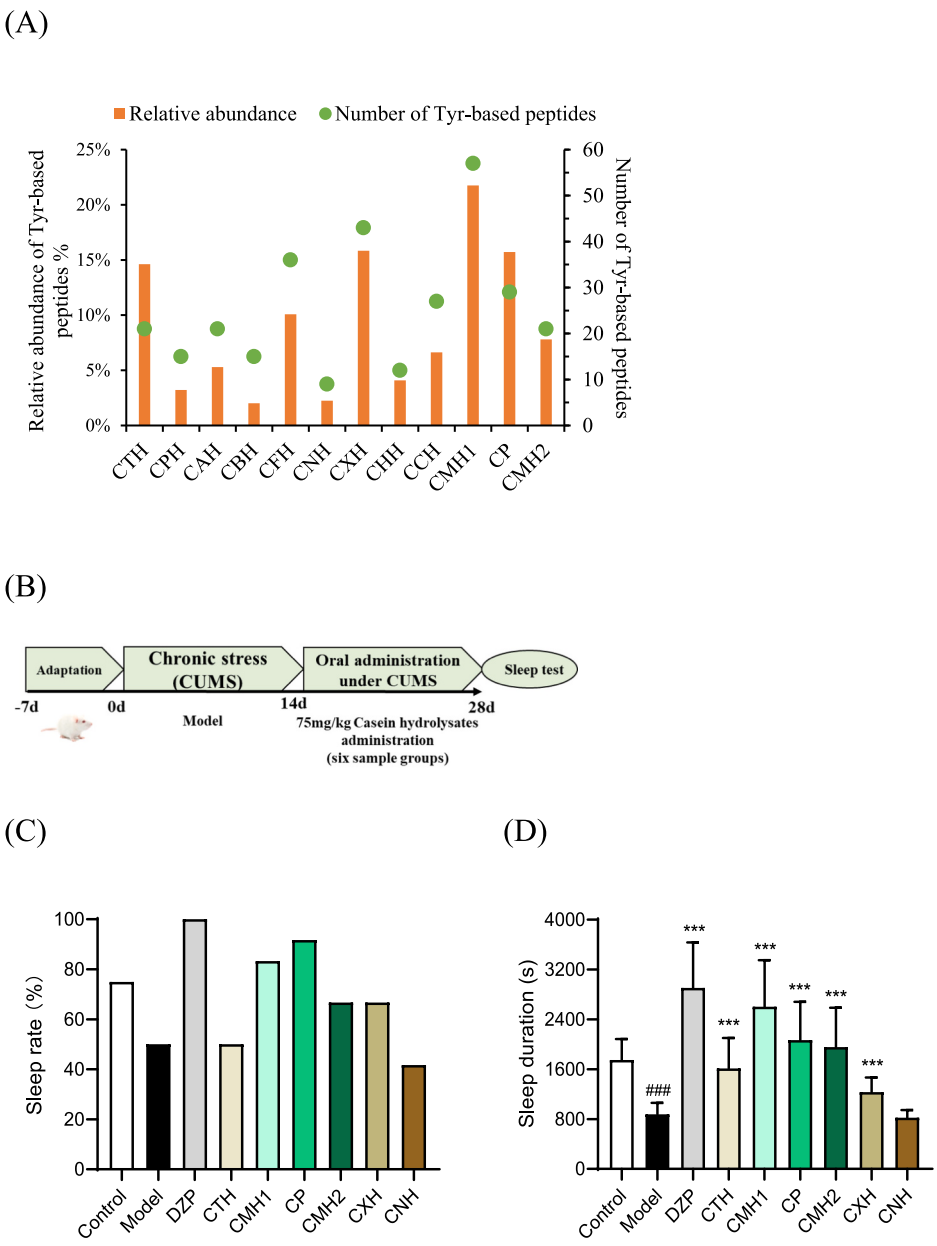

Fig. 1. Relative abundance and number of Tyr-based peptides in different casein hydrolysates (A), the arrangement for experiment (B), sleep rate (C) and duration (D) in pentobarbital-induce mice during the 120 min sleeping test. Each bar represents the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ significantly different from the model group. #p < 0.05, ##p < 0.01, and ###p < 0.001 significantly different from the control group. Abbreviations: Con-control; DZP-diazepam.

protein to produce sleep-enhancing peptides (Dela Peña et al., 2015). Despite the research on casein hydrolysates (CHs) with diverse sleep-enhancing effects the identification and exploration of sleep-enhancing peptides remain limited. Dela Peña et al. (2016) found that αS1-casein tryptic hydrolysate showed sleep-enhancing effects in pentobarbital-induced mice, and then four novel sleep-enhancing peptides were identified by Chen et al. (2023). In our previous study, a novel casein hydrolysate (CP®) showed stronger sleep-enhancing effects than casein itself (Qian, et al., 2024). Furthermore, a novel goat casein hydrolysate alleviated insomnia better than a whey protein hydrolysate (Mo et al., 2024). However, casein hydrolysates obtained by different proteases showed discrepancy in sleep-enhancing effects. The underlying cause remains unclear. There is a need for further investigations to elucidate the detailed structural features of potent sleep-enhancing peptides from casein.

To date, the conventional approach of screening bioactive peptides involves fractionation and identification based on physicochemical characteristics such as molecular weight, polarity, or charge (Nongonierma et al., 2018). Alternatively, virtual screening aims to quickly identify potential active peptides, but suffers from false positives (Fitz-Gerald et al., 2019; Zhou et al., 2024). Virtual screening is often combined with the known structure-activity relationship to enhance the screening model accuracy. Currently, chemometric tools such as orthogonal partial least-squares (OPLS) model and Pearson's correlation analysis are rapid-advancing and used to elucidate the structural features of potent active substances applying for classification and searching main bioactive components from complicated samples (Xu et al., 2022; Abdel et al., 2023). Therefore, this study aims to investigate the structural features of potent sleep-enhancing peptides derived from casein by multivariate analysis based on peptidomics of various CHs to elucidate the contributors of strong sleep-enhancing casein hydrolysates. Furthermore, a random forest model and the potential Tyr-based peptides library were established to rapidly screen for novel active peptides. The content of Tyr-based peptides was quantified and three

potential peptides were screened based on structure and releasing contents. We also validated their activity. The study provided new insights into the structural features of potent sleep-enhancing peptides derived from casein, facilitating rapid screening and development.

2. Materials and methods

2.1. Chemicals

Casein from bovine milk (≥ 90% protein) was sourced from Fonterra Co-operative Group Ltd. (Auckland, New Zealand). Sigma Aldrich (St Louis, MO, USA) supplied trypsin (93,615, ≥ 1500 units/mg solid). Two composite proteases, Mixprotease1 and Mixprotease2, and the casein hydrolysate rich in YPVEPF (commercialized as casein Peptides®, CP), were obtained from Huapeptides Biotechnology Co., Ltd. (Guangdong, China). Amano Enzyme (Nagoya, Japan) provided Proteaxh (1400 U/g protein). Neutrase 0.8 L, Flavourzyme and Alcalase were supplied by Novozymes Biotechnology Co., Ltd. (Tianjin, China). Papain and Bromelain were bought from Pangbo Biotechnology Co., Ltd. (Guangxi, China). Thermolysin and Chymotrypsin were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). The peptides Tyr-Pro-Val-Glu-Pro-Phe (YPVEPF), Tyr-Pro-Val-Glu-Pro (YPVEP), Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg (YLGYLEQLLR), Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn (YPFPGPIPN), Tyr-Ile-Pro-Ile-Gln-Tyr (YIPIQY) and Tyr-Gln-Lys-Phe-Pro-Gln-Tyr (YQKFPQY) and other peptides in the Tyr-based peptide library with ≥95% purity were procured from Synpeptide Co. Ltd. (Jiangsu, China). Reagents utilized for UPLC-MS/MS analyses were of MS grade, while all other chemical reagents used were of analytical grade.

2.2. Sample preparation

In our previous study, Tyr at N-terminus showed its significance in the sleep-enhancing effects of YPVEPF and YPVEP (Qian et al., 2022). Twelve different casein hydrolysates (CHs), each containing varying numbers of Tyr-based peptides, were prepared. The casein tryptic hydrolysate (CTH) was prepared following our previous study as a reference (Qian et al., 2021). CP is rich in the content of sleep-enhancing peptide YPVEPF and potential Tyr−/Trp containing peptides as described in our previous study (Qian, et al., 2024). The other ten proteases, including Papain, Alcalase, Bromelain, Flavourzyme, Thermolysin, Chymotrypsin, Mixprotease1, Mixprotease2, Proteaxh (AXH), and Neutrase were employed for the hydrolysis of casein. Briefly, casein was dispersed in distilled water (1:8, *w*/w). The enzymatic conditions were as follows: Papain (55 °C, pH 7.0, CPH), Alcalase (55 °C, pH 8.0, CAH), Bromelain (45 °C, pH 6.5, CBH), Mixprotease1 (55 °C, pH 7.0, CMH1), Mixprotease2 (55 °C, pH 7.0, CMH2), Flavourzyme (50 °C, pH 7.0, CFH), Thermolysin (70 °C, pH 8.0, CHH), Chymotrypsin (37 °C, pH 7.5, CCH), Proteaxh (55 °C, pH 7.0, CXH), Neutral protease (50 °C, pH 7.0, CNH). Subsequently, ten enzymes were added at an enzyme/substrate ratio of 0.5% (*w*/w, protein basis) and the mixture was incubated for 4 h. The hydrolysates were promptly heated in boiling water for 10 min to stop incubation. The mixture was then cooled to room temperature, centrifuged at 8000*g* for 10 min at 4 °C, and the supernatant was freeze-dried, and stored at −20 °C until use.

2.3. Simulated gastrointestinal digestion in vitro

The digests of the selected six CHs and casein were acquired through simulated gastrointestinal digestion following a harmonized INFOGEST procedure with some modifications (Brodkorb et al., 2019). The simulated gastric (SGF) and intestinal fluids (SIF) were prepared strictly according to the procedure. Six CHs and casein (100 mg/mL) were mixed with the pre-made SGF (1:1, *v*/v), along with $CaCl_2$ (10 mM of total digesta). Subsequently, the mixture underwent incubation with pepsin (2000 U/mL of mixture) for 2 h and was adjusted to pH 7. The gastric digesta were mixed with pre-made SIF (1:1, v/v), bile (10 mM), $CaCl_2$ (10 mM), and pre-heated into 37 °C for digestion with pancreatin (100 U trypsin activity/mL of digesta) for 2 h. Finally, the gastrointestinal digestion was terminated by boiling for 15 min. Seven digests were collected, standardized to a constant concentration of 10 mg/mL protein, and stored at −20 °C until analysis.

2.4. Pentobarbital-induced sleep test

One hundred and eight male Kunming mice (8 weeks old), weighing 19–23 g, were provided by Zhuhai BesTest Bio-Tech Co., Ltd. (permit number: SCXK (yue) 2020–0051). The animal experiments were approved by the Animal Care and Use Ethics Committee of Hua Teng Biomedical Technology Co., Ltd. (SYXK (yue) 2020–0237, HTSW221013). Mice were randomly classified into nine groups ($n = 12$ in each group): control (normal mice), model (under chronic unpredicted mild stress, CUMS), diazepam (DZP, 1 mg/kg, i.p., under CUMS), CTH, CMH1, CP, CMH2, CXH and CNH (75 mg/kg, p.o., under CUMS) groups (temperature 22 ± 2 °C and humidity 55 ± 5%, 12 h light/dark cycle). The detail time arrangement is shown in Fig. 1B. CUMS was given to all mice except control mice as detailed in Table S1 (supplementary data). Sleep duration was determined after oral administration for 7 days, as our previously described (Qian et al., 2021). The pentobarbital-induced sleep behavior test started 30 min after the last oral administration (p.o.), with mice receiving an intraperitoneal injection of pentobarbital sodium (42 mg/kg, i.p.). The numbers of mice falling asleep in each group were recorded. The sleep duration was recorded by stopwatch. Finally, the mice were sacrificed by cervical dislocation after being anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection.

2.5. Degree of hydrolysis (DH)

The previous o-phthalaldehyde (OPA) assay was used to determine the DH of six CHs with some modifications (Nielsen et al., 2001). Briefly, 180 μL of the OPA reagent was mixed with 24 μL of the sample, ultrapure water (control) or 0.97 mM serine (standard) and measured absorbance at 340 nm by ReadMax 1900-Light absorption full-wavelength microplate reader (Shanghai Flash Spectrum Biotechnology Co., Ltd., China) after 2 min.

2.6. Molecular weight distribution

The molecular weight (MW) distribution of six CHs was analyzed using our previous method, employing a TSK Gel G2000 SWXL analytical column (7.8 mm × 300 mm, Tosoh Bioscience, Shanghai, China) (Zheng et al., 2013). The mobile phases were comprised of 20% acetonitrile and 0.1% trifluoroacetic acid in water. Samples were eluted at a flow rate of 0.5 mL/min, and the absorbance was monitored at 280 nm. Six standard molecular markers, bovine serum albumin (66,463 Da), cytochrome C (12,384 Da), aprotinin (6512 Da), bacitracin (1423 Da), GGYR (451 Da), and GGG (189 Da) were used to establish a calibration curve, which was received from the log (MW) of the markers and their respective retention time ($R^2 = 0.995$).

2.7. Peptide profiles analysis by UPLC-ESI-QTOF-MS/MS

The peptidome analysis of CHs and their digests, and the quantification of these identified Tyr-based potential peptides were conducted using our previous method (Zheng et al., 2019). Briefly, an Acquity UPLC system equipped with a HSS T3 column (2.1 × 50 mm, 1.8 μm, 130 Å) (Waters, Milford, MA, United States) coupled with an IMPACT II Q-TOF mass spectrometer (UPLC-MS/MS; Bruker Daltonics, Germany) was used. The elution rate remained 0.2 mL/min, and a constant volume of 2.0 μL was injected. Mobile phase A was 0.1% formic acid in water, and mobile phase B was acetonitrile. The gradient elution procedure was

0–10 min, 5%–40% B; 10–12 min, 40%–90% B; 12–14 min, 90% B; 14–15 min, 90%–5% B; 15–18 min, 5% B. The nebulizer gas pressure, nitrogen drying gas flow rate and temperature were set at 1.5 bar, 8.0 L/min, and 200 °C, respectively. Mass spectra were acquired in the range of 50 to 1500 *m/z*, with an acquisition rate of 5 Hz, operating in positive mode. The calibration curve was generated by plotting the peak area against peptides concentrations (0.1, 0.25, 0.5, 1, 2, 5, and 10 μg/mL, $R^2 > 0.995$), employing to quantify the potential peptides.

2.8. Data processing and multivariate analysis

The peptides with >6 amino acids were identified using the database search using αS1-casein (P02662), αS2-casein (P02663), β-casein (P02666), and κ-casein (P02668) of Bovine from UniProtKB (https://www.uniprot.org) by Mascot software (Matrix Sciences, London, UK). The peptides with 2–6 amino acids were identified using manual *de novo* sequencing by Data Analysis 4.4 software (Bruker Daltonics). The base peak chromatography with MS (BPC-MS) of six casein hydrolysates and seven casein digests were displayed in Fig. S1. Subsequently, datasets containing all peptides and Tyr-Xaa-type peptides (including sample information, peptide sequences and their peak areas (from data analysis software), peptide type, and peptide length) of each casein hydrolysates and digests were imported into SIMCA 14.1 software (Umetrics AB, Umea, Sweden). Orthogonal partial least-squares analysis (OPLS) was performed after unit variance scaling, following our previous method with some modifications (Xu et al., 2022). Additionally, based on peptide characteristics (type and length), the peak area sum of peptides with the same characteristics in each hydrolysate or digest were calculated. The correlations between sleep durations and peptide characteristics of CHs were analyzed by Pearson's correlation analysis, and Pearson's correlation coefficients (PCC) were calculated.

2.9. Random forest model

We employed the random-forest method in R 4.2.2 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria) with the Random Forest package (V. 4.7–1.1) to meticulously screen peptides that significantly contributed to variations in peptide profiles. The importance of each peptide was assessed according to the mean decrease in accuracy. Tenfold cross-validation was conducted using the rfcv function to select appropriate features.

2.10. Validation of potential sleep-enhancing activity using a mice model

The four screened potential Tyr-based peptides (YPVEPF, YPFPGPIPN, YIPIQY and YQKFPQY, 1.8 mg/kg) were orally administered, while diazepam (1 mg/kg) was intraperitoneally injected twice daily for 7 consecutive days before the test ($n = 12$). Eighty-four male Kunming mice (8-week-old), weighing 22–28 g were purchased from Zhuhai BesTest Bio-Tech Co., Ltd. (production licenses: SCXK (yue) 2020–0051). All animal experiments received approval from the Animal Care and Use Ethics Committee of Hua Teng Biomedical Technology Co., Ltd. (licenses of use: SYXK (yue) 2023–0307, IACUC No.: B202309–18). The specific dosages of these groups were shown in Fig. 5D. The sleep test using pentobarbital-induced sleep behavioral method and the euthanasia of mice were both conducted as above part 2.4 described.

2.11. Molecular docking

The "sleep switch" $GABA_A$ receptor ($GABA_AR$) was utilized to investigate potential binding interactions with selected peptides (Saper et al., 2010). The interaction between receptor and peptide ligands were studied and visualized by Auto Dock, Pymol 1.5 and Discovery studio 3.5, following our previous procedure (Qian et al., 2022). The crystal structure of the $GABA_AR$ was received from the Protein Data Bank database (PDB ID: 6HUO). The agonist ALP and the water molecules from the 6HUO structure were removed, leaving only the BZD sites of the α1γ2 subunits. The docking between ALP and $GABA_AR$ was performed as the template, as well as YPVEPF was used as a reference. Details of the docking procedure are provided in the Supplementary data.

2.12. Statistical analysis

Three or four isolated batches of casein hydrolysates or digests were prepared and analyzed. Each determination was performed in triplicate, and the results were presented as mean ± standard deviation (SD). Statistical analysis and chart visualization were conducted using GraphPad Prism 8.0 Software (GraphPad Prism Software Inc., San Diego, CA, USA). The statistical method used was the *t*-test, where a *p*-value <0.05 denoted statistical significance ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Result and discussion

3.1. Biochemical characteristics of different casein hydrolysates

The casein tryptic hydrolysate was proved to alleviate anxiety-like behavior and improve sleep (Chen et al., 2023; Guesdon et al., 2006). However, various enzymatic hydrolysis patterns of casein releasing different sleep-enhancing peptides with diverse activities are still unclear. As our previous study described, Tyr at N-terminus plays a vital role in YPVEPF and YPVEP to exert strong sleep-enhancing effects (Qian et al., 2022). Therefore, the number and sum peak area of Tyr-based peptides from twelve casein hydrolysates (CHs), which included a product CP and eleven hydrolysates by different commercial proteases, were determined. Fig. 1A shows the highest number and relative peak area of Tyr-based peptides that were detected in CMH1 and CXH among the twelve different CHs, followed by CMH2 and CFH at a medium level, while the least number and relative peak area were detected in CNH. Hence, six CHs including two commercial CHs (CTH and CP) and four CHs (CMH1, CMH2, CXH and CNH) were selected for further studies.

The distinct DH, MW distribution, and contents of representative sleep-enhancing peptides from six CHs are shown in Table S2. Among them, CMH1 and CXH showed the highest DH value (22.17% and 22.51%, respectively), while CTH showed the lowest DH value (6.79%) followed by those medium DH value of CP, CMH2 and CNH (13.12%, 11.30% and 10.65%, respectively). Additionally, the proportion of components <1 kDa ranked in the order of CMH1 (83.56%) > CXH (78.96%) > CP (77.69%) > CMH2 (73.42%) > CNH (56.94%) > CTH (42.05%). The low molecular weight peptides might be potent to exert sleep-enhancing and anxiolytic effects (Yi et al., 2020). It was shown that fish hydrolysate Peptidyss® could reduce acute mild stress-induced corticosterone secretion to exert anxiolytic activity, which is specific for >74% of peptides in which 82.4% have a molecular weight under 1 kDa (Dinel et al., 2021). It was noted that CTH, CMH1 and CP contained representative sleep-enhancing peptides including YLGYLEQLLR (11.83 ± 0.30 mg/g), YPVEP (6.90 ± 0.69 mg/g) and YPVEPF (12.04 ± 0.39 mg/g) (Table S2). Based on these characteristics, the sleep-enhancing effects of these six CHs were further determined. Fig. 1C shows CP, CMH1, CMH2, and CXH treatments increased the sleep rate compared with the model group, while CTH and CNH treatments did not increase the sleep rate. As shown in Fig. 1C, the sleep duration of the CHs ranked in the order CMH1 > CP > CMH2 > CTH > CXH > CNH. It should be noted that CTH had a proportion of 42.05% for components <1 kDa and a low DH value with 6.79%. In contrast, CTH displayed 1.3-fold longer sleep duration than CXH (high DH value and proportion of components <1 kDa). The ranking order of sleep duration did not completely correlate with the DH value, the proportion of small molecular peptides, or the number or relative peak area of Tyr-based peptides. Therefore, it was necessary to investigate the vital structural features of casein-derived sleep-enhancing peptides to reveal their structure-activity

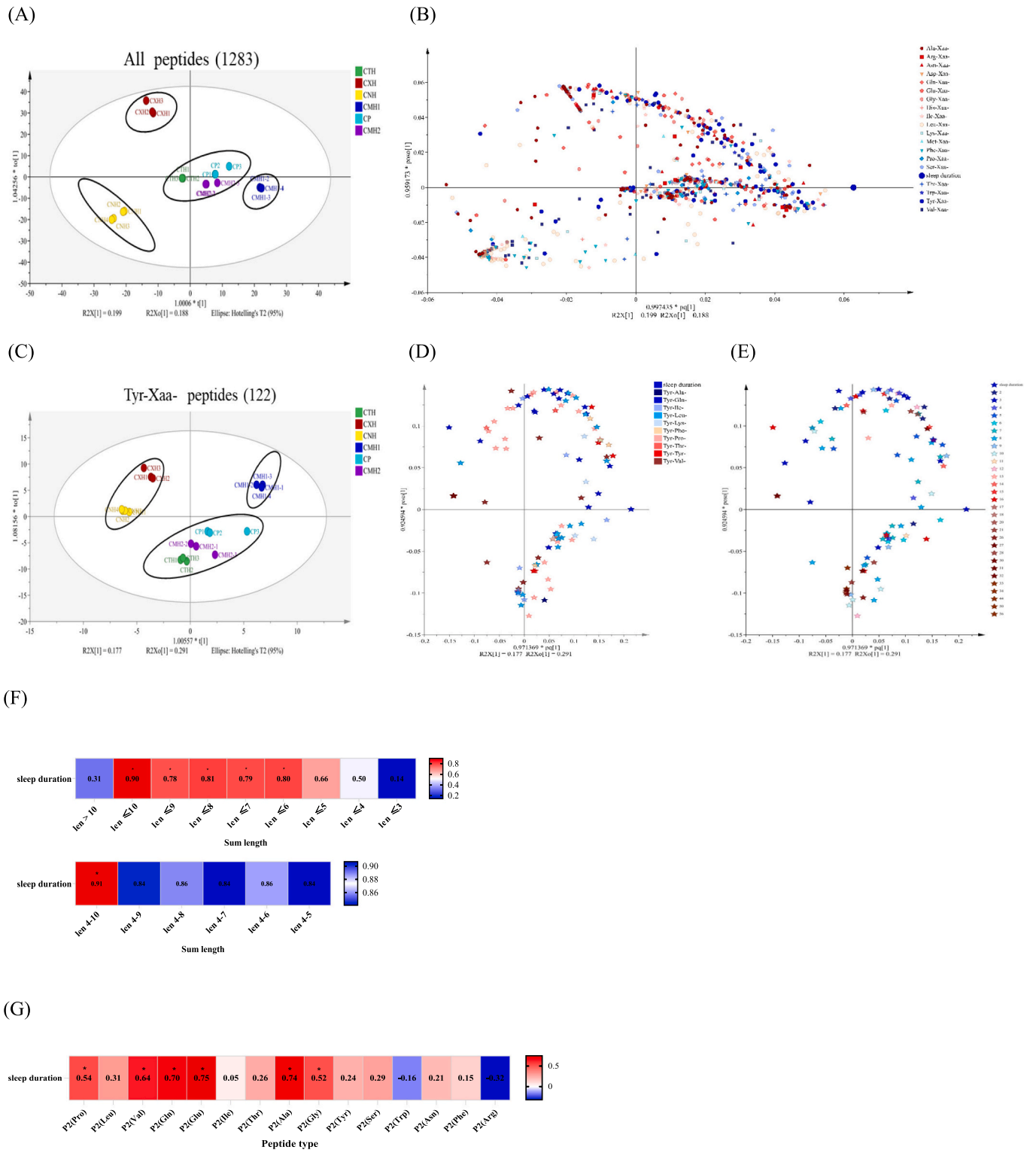

Fig. 2. Score plot (A), and loading plot of different type (B) of orthogonal partial least squares (OPLS) model of all peptides (1283) identified from six different casein hydrolysates (CHs). Score plot (C) and loading plot including colored by peptide types (D) and length (E) of OPLS model of 122 Tyr-based peptides identified from six CHs. Correlation analysis among sleep duration with peptide length (F) and peptide type (G). 3–4 batches of hydrolysates were prepared and analyzed. In the loading plot (Fig. 2B, D and E), different types or length of peptides were shown with different shape and/or different colors and named after peptide type or peptide length classified. Pearson correlation analysis was performed using the relative peak area sum of identified peptides (%) in a specific length or type range of Tyr-based peptides in casein hydrolysates. Correlation (R^2) was presented using Pearson's correlation coefficient (PCC). Xaa represent any amino acids.

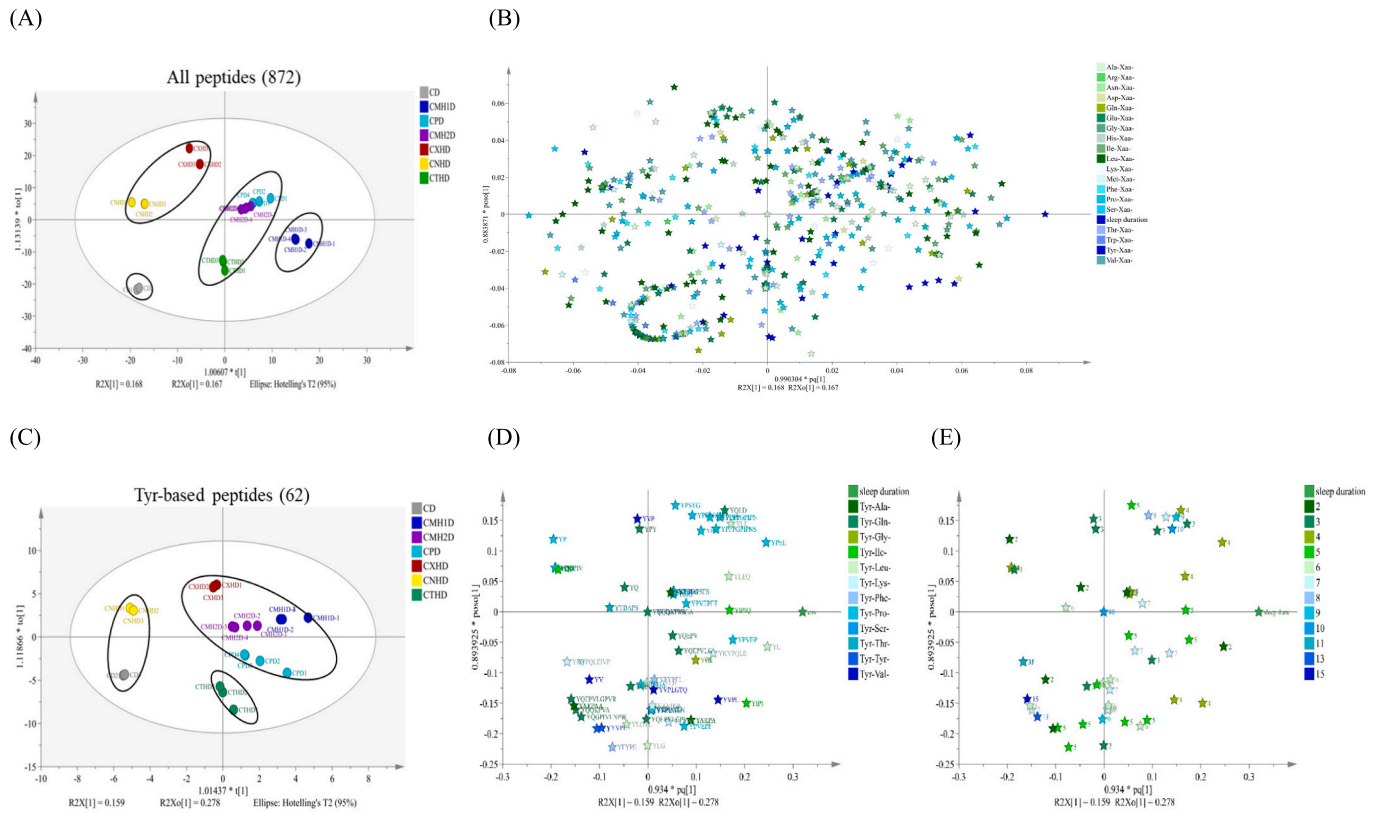

Fig. 3. Score plot (A), loading plot of different type (B) of OPLS model of all peptides (872) identified from CD and CHDs. (C) Score plot, loading plot of different type (D) and length (E) of OPLS model of 62 Tyr-based peptides identified from CD and CHDs. 3–4 batches of the digests were prepared and analyzed.

relationship.

3.2. Multivariate statistical analysis

3.2.1. Structural features of potential sleep-enhancing peptides in different casein hydrolysates

The activity of diverse CHs varied significantly based on their released peptide composition and the concentrations of potent sleep-enhancing peptides. To investigate which type of peptides were primarily responsible for the sleep-enhancing effects of CHs produced by different enzymes, OPLS and Pearson's correlation analysis were firstly used to elucidate the vital structural features of potent sleep-enhancing peptides. As shown in Fig. 2A, a total of 1283 peptides were identified within the six CHs, each demonstrating varying sleep-enhancing effects. These peptides were classified into four regions based on their distinct sleep-enhancing activities using a well-fitted OPLS model, which exhibited strong variance and predictive power ($R^2Ycum = 0.992$, $Q^2cum = 0.987$, validation intercepts of $R^2 = (0.0, 0.281)$, $Q^2 = (0.0, -0.797)$; $p = 7.07465e\text{-}014$). The distinct separation pattern observed in the OPLS model corresponds with the sleep duration results, where CMH1 demonstrated the strongest activity, followed by CP, CMH2, CTH, CXH, and CNH. The loading plot reveals that casein peptides contribute to the sample separation, categorized by peptide type, with most peptides being Tyr-based (represented by blue dots) and Gln-based (represented by pink diamonds) as shown in Fig. 2B. Tyr is a precursor to the key neurotransmitter dopamine, which is implicated in the co-occurrence of insomnia and depression (Finan & Smith, 2013; Kim et al., 2019). Tyr-based peptides contributed more to the sleep-enhancing activity of CHs than other types of peptides as indicated in our previous results. Hence, 122 Tyr-based peptides from six CHs were used to establish a novel OPLS model to further investigate the correlation between detailed structural features of potential Tyr-based peptides and the sleep duration. Notably, the score plot of Tyr-based peptides ($R^2Y_{cum} = 0.963$, $Q^2_{cum} = 0.944$, validation intercepts of $R^2 = (0.0, 0.256)$, $Q^2 = (0.0, -0.684)$; $p = 3.16901e\text{-}009$) exhibited separate distributions in three regions according to their different sleep-enhancing activities (Fig. 2C). As for Tyr-based peptides, the amino acid residues at position 2 of N-terminus and the specific peptide length may cause significant differences in their sleep-enhancing activity. Obvious discrepancies in the distribution of YQ- (dark blue), YL/I- (sky blue and light purple), YK- (light blue) and YP- type (pink) peptides were observed (numbers of 13, 11, 3, and 8, respectively with VIP value >1.0 in Table S4), as well as their distributed number increased as the activity increased in Fig. 2D. However, the release of YK-type peptides was limited (only 7, as shown in Table S3), while YQ-, YL/I-, and YP-type peptides were more prevalent among the 122 Tyr-based peptides (with 27, 21, and 33 respectively, as detailed in Table S3). This indicates that YQ-, YL/I-, and YP-type peptides contribute significantly to sleep duration. It suggests that the majority of YP/Q/I/L-type peptides might play a greater role in sleep-enhancing activity in CHs. Additionally, the distribution of most peptides with 2–10 amino acids distributed in high activity area, although few peptides with >10 amino acids were observed (Fig. 2E and Fig. S2A).

The Pearson's correlation analysis was used to further explore the potential structural features on peptide length and other properties of sleep-enhancing peptides. As shown in Fig. 2E, a significant increase from 0.14 to 0.80 in correlation was observed when the length (Len) range expanded from Len ≤ 3 to Len ≤ 6, with the highest accumulated correlation noted at Len ≤ 10 (PCC = 0.90). Mizushige et al. (2013) also determined that tripeptide YLG and dipeptide YL displayed the weaker anxiolytic effects at an equal 3.5 μmol/kg dose compared to decapeptide YLGYLEQLLR in elevated plus-maze test. However, the PCC of Sum (Len ≥ 10) was significantly decreased into only 0.31. Furthermore, a gradual increase in correlation, from 0.84 to 0.91, was observed as the length range expanded from Len 4–5 to Len 4–10. Lecouvey et al. (1997) proved that the distance between the aromatic rings of Tyr within four

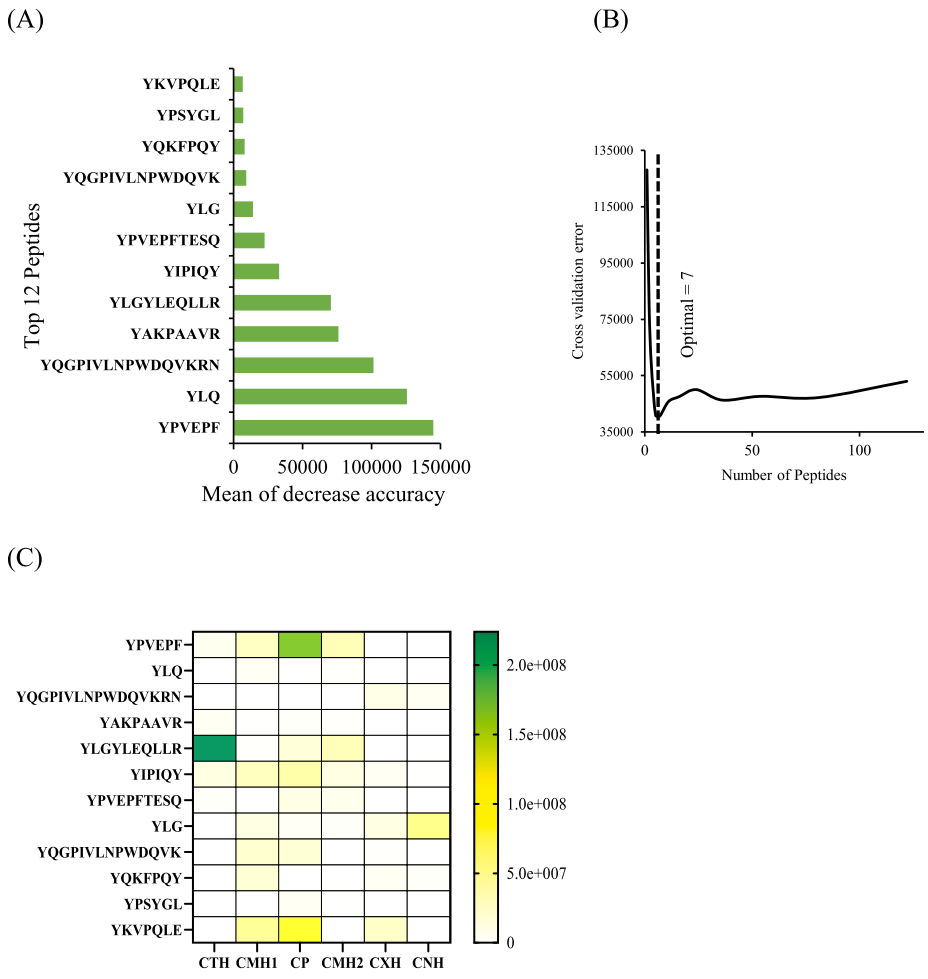

Fig. 4. Regression random forest analysis was performed on 122 Tyr-based type casein peptides. Random Forest analysis diagram (A-B), the peak area of the selected top 12 peptides by random forest model in six CHs (C).

amino acids of the N-terminus of peptide YLGYLEQLLR overlapped with the aromatic groups of benzodiazepines in the $GABA_AR$ binding pocket eliciting anxiolytic activity. These findings indicated that Tyr-based peptides with 4–10 amino acids might contribute significantly to the sleep-enhancing activity of CHs. Moreover, the activation of the $5\text{-}HT_{1A}$ receptor with sleep-enhancing effects meditated by YPVEPF might be influenced by amino acids in the second position from the C-terminus (Qian, Zheng, et al., 2024). Fig. 2G further shows that Glu in the second position of the C-terminus of Tyr-based peptides displayed the highest PCC of 0.75, followed by that of Ala, Gln, Val, Pro, and Gly (numbers of 3, 11, 13, 1, 17) residue (PCC > 0.5). Furuse et al. found that Arg, Lys, Pro, Trp, and Ala showed sedative and hypnotic effects by a chick model and that Gln, Tyr, Ile/Leu, and Phe positively affected depressive-like behavior in rats (Furuse, 2015; Ihara et al., 2023). However, the occurrences of Tyr-based peptides with Ala and Gly in the second position of the C-terminus were notably scarce as indicated in Table S4, showing that high PCC values of the two peptide types were not representative because of the narrow sample capacity. To summarize, YP-, YI/L- and YQ-type peptides with 4–10 amino acid residues, especially containing Gln/Pro at the second position of the C-terminus, might support improved sleep for CHs.

3.2.2. *Structural features of potential sleep-enhancing peptides in the digests of different casein hydrolysates*

It is well-established that bioactive peptides administered orally must traverse the gastrointestinal digestive system to effectively reach their targets *in vivo* (Li et al., 2020; Yang et al., 2024). After digestion, the numbers of identified total peptides and those peptides with ≥7 amino acids were decreased compared with that of CHs, however, the numbers of these peptides consisting of 2 to 6 amino acids were increased (in Table S3). This might be attributed to the gastrointestinal enzymatic functions, which cleaved precursor peptides to release common shorter peptides. To gain deeper insights into the structural features of sleep-enhancing precursor peptide in CHs, the OPLS model was established among the 872 identified peptides in seven digests of casein hydrolysates (CHDs) and casein (as a reference, CD). As illustrated in the OPLS score plot of all peptides (R^2Y_{cum} = 0.979, Q^2_{cum} = 0.958, with validation intercepts of R^2 = (0.0, 0.264) and Q^2 = (0.0, −0.719); p = 4.21801e-012, in Fig. 3A), CD and CHDs were distributed in four regions according to their varying sleep durations. More peptides with Tyr, Phe, Pro, Ile, Leu and Val at N-terminus were observed in the region with higher sleep-enhancing activity in Fig. 3B. Moreover, abundant peptides with 2–6 amino acids were distributed in the region with higher sleep-enhancing activity in Fig. S2B (supplementary data). Few di−/tripeptides were observed in the region with lower sleep-enhancing activity. As observed in the loading plots by peptide type and length, there is no significant tendency of all peptides in CD and CHDs.

The Tyr-based peptides that remained after digestion were chosen for further analysis by OPLS model. As depicted in Fig. 3C, 62 Tyr-based peptides were categorized into three distinct sleep-enhancing activity regions, which achieved high values with R^2Y_{cum} (0.904) and Q^2_{cum} (0.886), with validation intercepts of R^2 = (0.0, 0.190) and Q^2 = (0.0, −0.663); p = 2.84568e-008. As shown in Fig. 3E and F, YP-, YI/L-, and YQ- with 2–10 amino acids were predominantly distributed in the high

Table 1
Quantification of the potential Tyr-based peptides in casein hydrolysates and their digests.

Content (mg/g)	VIP	RF	CTH	CMH1	CXH	CP	CMH2	CNH	CD	CTHD	CMH1D	CXHD	CPD	CMH2D	CNHD
YP	0.81	107	0	0.16±0.10	0.59±0.08	0	0	0	0	0	0	0.10±0.06	0	0	1.53±0.22
YPVEP	1.52	75	0	6.90±0.69	0	0	0	0	2.54±1.11	3.95±0.36	13.17±4.85	2.66±0.17	1.64±0.29	1.22±0.08	0.35±0.00
YPVEPF	0.93	1	0.41±0.00	1.22±0.01	0	12.04±0.39	0.80±0.06	0	5.22±0.29	4.61±0.28	1.04±0.30	0	5.43±0.37	4.65±0.22	0.09±0.04
YPS	0.93	117	0	0	0	0	0	0	0	0	0	0	0	0	0
YPSYG	0.86	/	0	0.61±0.11	0.33±0.01	0	0	0	0	0	0	0.21±0.001	0	0	0.34±0.01
YPSYGL	0.67	11	0	0±0	0±0	0.04±0.07	0	0	0	0	0	0	0	0	0
YPSYGLN	0.71	32	0	0±0	0±0	0.24±0.08	0	0	0	0	0	0	0	0	0
YPSGAWY	0.63	33	0	0.12±0.01	1.07±0.04	0±0	0	0	0	0	0	0	0	0	0
YPY	1.24	61	0	0	0	0	0	0	0	0	0	0	0	0	0
YPFPGPI	1.11	/	0	0	0	0	0	0	0	0	3.37±1.16	1.85±0.004	0	0	0
YPFPGPIPN	1.06	69	0	6.55±0.51	8.36±0.08	0	0.11±0.07	0	0	0.06±0.08	20.89±2.49	9.53±1.26	0	2.49±0.08	0
YF	1.41	18	0	0	0	0	0	0	0	0	0	0	0	0	0
YFYPE	1.66	14	0	0.82±0.01	0	0	0	0	3.37±0.19	2.59±0.16	0.31±0.09	0	1.02±0.01	0	0
YFYPEL	0.50	/	0	0.26±0.01	0	0	0	0	0	0	0	0	0	0	0
YIPIQY	1.56	6	1.01±0.05	1.70±0.10	0.25±0.03	1.55±0.10	0.27±0.01	0	0	0	0	0	0	0	0
YLQ	1.59	2	0	0	0	0	0	0	0	0	0	0	0	0	0
YQ	1.05	43	0	1.19±0.33	0	0	0	0	0	0	5.69±3.92	0	0	0	0
YQKFPQY	1.37	10	0	1.22±0.02	0.19±0.02	0	0	0	0	0	0	0	0	0	0
YQEPVLGPVR	1.40	26	0	3.74±0.16	0	3.68±0.14	0	0	0	0	0	0	0	0	0
YYQQKPVAL	1.71	27	0	0	0	0	0	0	0	0	0	0	0	0	0
YVPL	1.08	17	0	4.03±0.39	1.44±0.07	4.44±0.32	0	0	3.70±0.15	3.32±0.15	4.54±0.21	1.39±0.09	4.77±0.24	0.17±0.02	2.15±0.07
YYVPL	1.06	/	0	0	0	0.20±0.08	0	0	1.55±0.04	1.82±0.09	0	0	0	0	0.10±0.00
YAKPAAVR	0.77	4	0.36±0.01	0	0	0	0	0	0	0	0	0	0	0	0
YL	1.08	15	0.92±0.32	1.88±0.17	3.71±2.77	1.66±0.35	2.31±0.08	0	0	1.15±0.24	1.54±0.31	1.44±1.32	1.24±0.21	1.65±0.16	0
YLG	1.24	8	0	0.29±0.001	0	0	0	0	0	2.40±0.16	0.30±0.15	0	0	0	0
YLGYLEQ	0.74	58	0	0.070±0.04	0	0.59±0.20	0	0	0	0	0	0	0	0	0
YLGYLEQLLR	0.71	5	11.83±0.30	1.70±0.70	0.51±0.002	2.99±0.98	1.10±0.55	0.51±0.001	1.56±0.83	0.25±0.25	1.02±1.10	0.51±0.003	1.53±1.02	0.26±0.26	0.88±0.52

activity region in these digests. Furthermore, the top ten peptides among those Tyr-based peptides with VIP value >1.0 predominantly included L/I/P/Q at position 2 of N-terminus (80%), indicating the contributions of YI/L-, YP-, and YQ-type peptides in the hydrolysates and digests in Table S4 and S5. Interestingly, the precursors Tyr-based peptides comprising 4–10 amino acid residues from CHs might be digested into potential peptides with 2–6 amino acids residues. Especially, tetrapeptides contributed more to higher sleep-enhancing activity in Fig. 3F, showcasing structural similarities with the benzodiazepine agonist diazepam. Cakir-Kiefer et al. (2011) also demonstrated that the digestive homologous fragments of anxiolytic peptides YLGYLEQLLR exhibited potential anxiolytic activity including YLGYL and YLGYLEQ. Short peptides are easier to exert biological activity and transport into physicochemical barriers *in vivo* (Xu et al., 2024). However, the dipeptide YP exhibited a lower activity region compared to YPEL in Fig. 3E, indicating the importance of proper peptide length influenced on the sleep-enhancing activity of Tyr-based peptides. Combining the results before and after digestion, YL/I/Q/P-type peptides with 4–10 amino acids contributed more to the sleep-enhancing activity of CHs as vital precursor peptides compared with other types of peptides.

3.3. Screening of sleep-enhancing Tyr-based peptides from casein hydrolysates using random forest model

Random forest is a powerful ensemble algorithm designed to address optimization problems with discrete decision variables. It consists of multiple decision trees for both classification and regression, creating a robust and effective structure (Du et al., 2024). Therefore, a random forest model was developed using 122 Tyr-based peptides across six CHs to facilitate the rapid screening of potent sleep-enhancing peptides from casein (Table S6). As shown in Fig. 4A, the random forest model achieved an accuracy of 97.85% in predicting indicators for the 122 Tyr-based peptides, validating the model's reliability. The cross-validation error curve stabilized with the inclusion of the 7 most relevant peptides (Fig. 4B), identifying YPVEPF, YLQ, YAKPAAVR, YLGYLEQLLR, YIPIQY, YPVEPFTESQ, YLG, and YQKFPQY as the dominant peptides (Len $\leq$ 10). Notably, YLQ, YIPIQY, and YQKFPQY exhibited favorable VIP scores of 1.5939, 1.5580, and 1.3679, respectively, in Table S6. Based on these findings, YP-, YL/I-, and YQ-type peptides with 4 to 10 amino acid residues, particularly those with Q/P in the second position at the C-terminus, emerged as significant among the 122 peptides. Consequently, YIPIQY and YQKFPQY were highlighted as strong potential sleep-enhancing peptides. Interestingly, the top-ranking peptides in the random forest model included the previously identified strong sleep-enhancing peptides YPVEPF and YLGYLEQLLR, further supporting the model's accuracy and suitability for predicting and screening potential sleep-enhancing peptides.

In addition, the distribution of potential Tyr-based peptides in CHs with different sleep-enhancing activities was further investigated in Fig. 4C. It was evident that most of these top 7 potential sleep-enhancing peptides were YP- (28.57%), YI/L- (42.86%) and YQ- (14.29%), which was consistent with the feature of sleep-enhancing peptides from chemometric analysis. Notably, the abundant contents of well-documented sleep-enhancing peptides YLGYLEQLLR and YPVEPF were observed in CTH and CP, respectively. They might constitute vital active ingredients in CTH and CP to exert sleep-enhancing effects. It was clear that the total content of the potential Tyr-based peptides in CMH1 was the highest among these six CHs, with CMH1 exhibiting the strongest activity among them. Specifically, YPVEPF, YIPIQY, YQKFPQY and YKVPQLE showed a considerable relative peak area in CMH1, suggesting their vital contributions to the higher activity. However, the peak area of these potential peptides in CXH and CNH with low activity displayed limited values. Therefore, the peak area of potential sleep-enhancing peptides reflected their contents to some degree. It would help to elucidate the intrinsic patterns of activity, but it is not sufficiently accurate. The contents of the featured potential sleep-enhancing peptides in CHs and their digests were necessary to be further quantified by UPLC-MS/MS.

3.4. Distribution of potential sleep-enhancing peptides in casein hydrolysates and their digests

Limited studies have focused on the identification and quantification

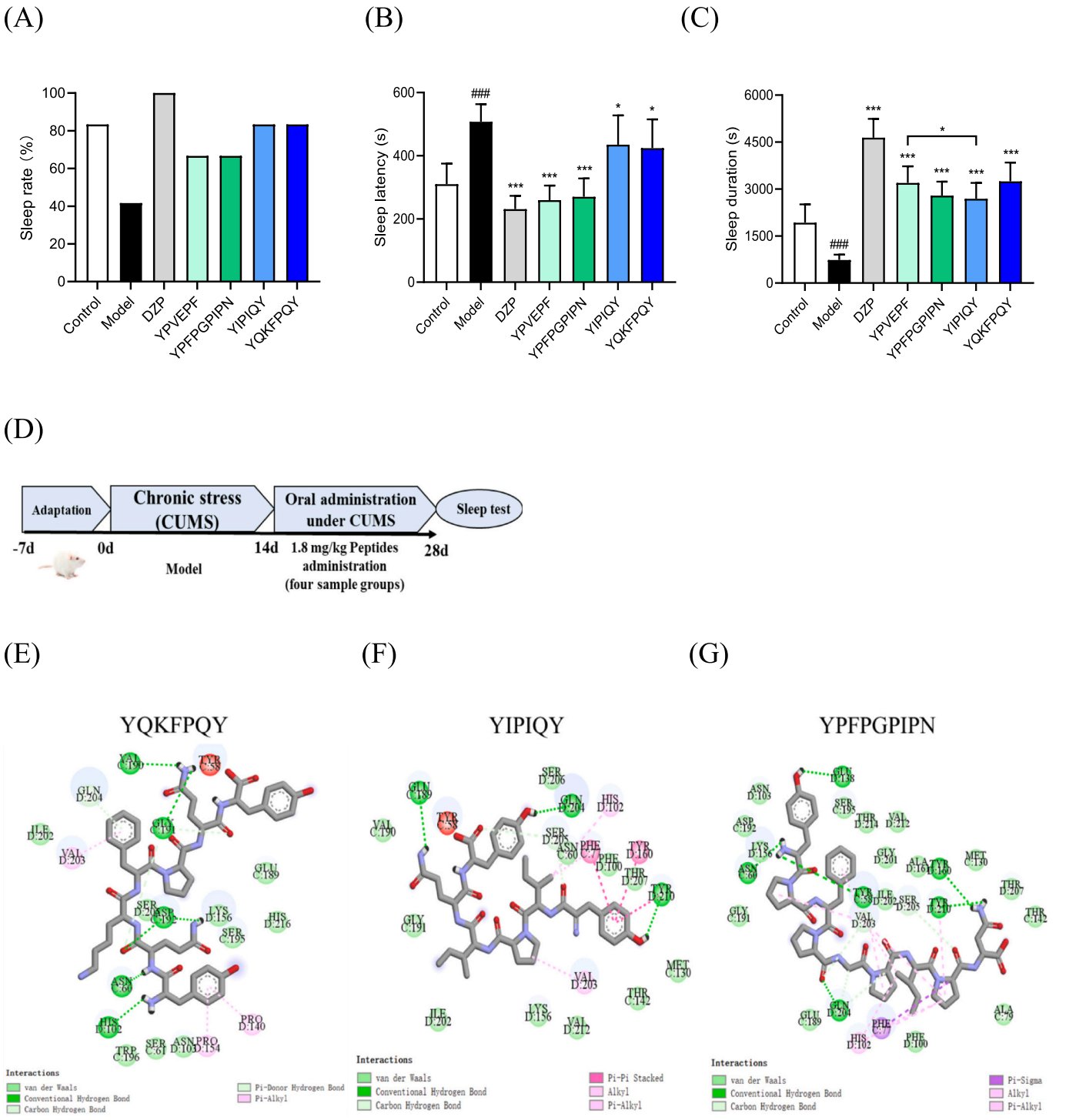

Fig. 5. Effects of the four selected potential peptides (YPVEPF, YPFPGPIPN, YIPIQY and YQKFPQY) by pentobarbital-induced sleeping test on stressed mice ($n = 12$). Each bar represents the mean ± SD of sleep rate (A), sleep latency (B), sleep duration (C), and detail design (D) during the sleeping behavior test. *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* model group. #p < 0.05, ##p < 0.01, and ###p < 0.001 *versus* control group. Abbreviations: DZP-diazepam (1 mg/kg, i.p.). Molecular docking analysis of the three novel potential peptides, (E) YQKFPQY, (F) YIPIQY, and (G) YPFPGPIPN.

of a range of bioactive peptides derived from casein, which might be attributed to the complexity of components and limitations in the detection or quantification (LOD or LOQ, respectively) capabilities of analytical equipment (Cruz-Huerta et al., 2015; Nguyen et al., 2019). Only some specific peptides such as beta-casomorphin peptides and angiotensin I-converting enzyme inhibiting tripeptides were systematically studied on their quantification and identification in dairy products (Moosmang et al., 2019; Nguyen et al., 2019). However, given that most food-derived peptides exhibit functions with a dose-activity relationship, it is essential to quantify the potential Tyr-based peptides to better understand their sleep-enhancing effects of CHs and to facilitate the screening of bioactive peptides. *A potential Tyr-based peptide library* was established including 27 peptides based on structural features, high VIP score and RF rank in Table 1, which further narrowed the range of potential sleep-enhancing peptides in CHs. Thirteen samples of CHs and their digests were analyzed in triplicate to identify potential Tyr-based

peptides by UPLC-MS/MS using external standard quantitative method. CMH1 and its digest with strong sleep-enhancing effects showed the highest sum contents of YP-type peptides among all hydrolysates and digests, even in the overall contents of Tyr-based peptide library, followed by CP. Compared with other hydrolysates, CMH1 and CP with higher activity had a better ability to release these potential sleep-enhancing peptides. The properties of the identified 53 Tyr-based peptides with 4–10 amino acid residues in CHs were shown in Table S7. Among these, YP-, YI/L-, and YQ-type peptides exhibited high VIP scores, outstanding RF ranks, and substantial peak areas (with 15, 15, and 8 peptides, respectively). This indicates that these featured peptides possessing both essential structural features and abundant content contributed more to the effective sleep-enhancing hydrolysates. Moreover, YLGYLEQLLR, YPVEPF, YPVEP, and YPFPGPIPN was detected with the highest contents of 11.83 ± 0.30, 12.04 ± 0.39, 13.17 ± 4.85, and 20.89 ± 2.49 mg/g in CTH, CP, CMH1D, and CMH1D, respectively. The potential peptide YPFPGPIPN possessing appropriate structures and abundant content, might serve as a strong sleep-enhancing candidate. Therefore, four potential Tyr-based peptides, YPVEPF (previously reported, as a reference), YPFPGPIPN, YIPIQY, and YQKFPQY, were chosen to validate our analysis, considering their structural features, contents, RF rank and VIP scores.

3.5. Validating the sleep-enhancing effects and explaining the potential molecular mechanisms of the screened potential casein peptides

As shown in Fig. 5, four selected Tyr-based peptides exhibited various sleep-enhancing effects in pentobarbital-induced sleep behavior test. The pre-treatment of YIPIQY and YQKFPQY (83.33%) increased sleep rates compared to other peptides and the model group (41.67%), followed by YPVEPF (66.67%), and YPFPGPIPN (66.67%). The sleep latency was all significantly shortened in four peptide treatments compared to the model group (Fig. 5B). Furthermore, the pre-treatments with four Tyr-based peptides significantly prolonged the sleep duration compared to the model group. The increasing rates of sleep duration compared to model group ranked from high to low in the order YQKFPQY > YPVEPF > YPFPGPIPN > YIPIQY (Fig. 5C). Interestingly, there was a common feature that a symmetrical amino acid (Gln or Pro) at the second position of both N and C terminus was observed among the top three sleep-enhancing peptide. In our previous study, we suggested that the symmetrical structure of YPVEPF facilitates simultaneous activate the $GABA_A$ receptors and 5-HT_{1A} receptor resulting in strong sleep-enhancing effects (Qian, Zheng, et al., 2024). Lv et al. (2021) similarly suggested that the repeated amino acid structure in plastron of *Mauremys mutica* peptides might constitute the potential primary structure of sleep-promoting peptides. The intriguing symmetry in YP/Q-type peptides appears to be a vital structure for sleep-enhancing peptides in exerting potent function. Moreover, molecular docking results indicated that the presence of Tyr and Gln at the first and second positions in the N-terminus of YQKFPQY played crucial roles in forming vital π-Alkyl and hydrogen bonds, respectively, with His-102 and Asn-60 of $GABA_AR$ (Fig. 5, 3D illustrations showed in Fig. S3). As described in our previous study, the sleep-enhancing peptide YPVEPF exhibited strong interactions with the Phe-77, Ser-205, Ser-206, Tyr-58, His-102, Val-203, and Gln-204 residues of $GABA_AR$ (Qian, Zheng, et al., 2024). In YIPIQY, the residues at the first and second positions from the N-terminus, Tyr and Ile, remained consistent due to strong hydrogen-bond interactions with Tyr-210, Tyr-160, and Phe-77, and π-π/Alkyl interactions with His-102 and Ser-205. The presence of YP- at the N-terminus in YPFPGPIPN formed vital hydrogen bonds with Asn-60 and π-Alkyl interactions with Val-203. This could explain the activity of peptides with YQ/I/P- as the N-terminal residues to act as $GABA_AR$ ligands to exert sleep-enhancing effects. Interesting, the residues at the first and second positions from the C-terminus of YIPIQY formed strong interactions with the vital residues Gln-204 and Ser-205, as well as YPFPGPIPN activated the residues Phe-77, His-102, Tyr-58 and Tyr-210 of $GABA_AR$. The structure of the three strong sleep-enhancing peptides was consistent with our above hypothesis. The identification of these structural features supports future studies aimed at developing novel, potent sleep-enhancing peptides.

4. Conclusion

This study firstly revealed the important structural features of potent sleep-enhancing peptides from casein hydrolysates by peptidomics and chemometrics. A potential Tyr-based peptide library with essential structural features was established, and the peptides contents were quantified. Our results indicated that variations in the sleep-enhancing effects of different CHs attributed to significant differences in the release of these potent Tyr-based peptides with some specific structures that included YP/Q/L/I- at N terminus, 4–10 amino acids residues, and Q/P at the second position of C terminus supporting potential activation of the $GABA_AR$. Three novel strong sleep-enhancing peptides YQKFPQY, YPFPGPIPN, and YIPIQY were investigated by the integrated assessments of chemometrics, random forest results and contents, as well as their activity and molecular mechanisms were explored. These findings regarding the potential structural features of sleep-enhancing peptides derived from casein help to establish a theoretical basis for screening other potent protein sources. Further investigations are required to explore the detailed structure-activity relationship and the bioavailability discrepancies of YP-, YI/L-, and YQ-type sleep-enhancing peptides.

CRediT authorship contribution statement

Jingjing Qian: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fengjie Yu:** Methodology, Data curation. **Leggy A. Arnold:** Methodology, Visualization, Writing – review & editing. **Arjun Saha:** Methodology, Software, Visualization, Writing – review & editing. **Lin Zheng:** Writing – review & editing, Methodology, Conceptualization. **Mouming Zhao:** Writing – review & editing, Conceptualization.

Declaration of competing interest

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

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140838.

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