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Separation, identification, and anti-melanogenic efficacy validation of tyrosinase inhibitory peptides from pufferfish (*Takifugu obscurus*) hydrolysates

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ABSTRACT: Abnormal melanin overproduction leads to hyperpigmentation disorders, and tyrosinase is the rate-limiting enzyme in melanin biosynthesis. To develop natural and safe tyrosinase inhibitors, this study systematically explored anti-melanogenic peptides from the muscle proteins of *Takifugu obscurus*. Five commercial proteases (pepsin, trypsin, flavourzyme, papain, and alkaline protease) were used for enzymatic hydrolysis, and pepsin was identified as the optimal enzyme. The pepsin-hydrolyzed peptide fraction (300–1000 Da) exhibited a tyrosinase inhibition rate of $87.01\% \pm 2.64\%$ at 10 mg/mL, comparable to arbutin ($96.76\% \pm 1.12\%$) but with significantly lower cytotoxicity (cell viability > 90% at 4.0 mg/mL vs $56.43\% \pm 1.20\%$ for arbutin). Peptide identification via nano-LC-MS/MS and virtual screening using molecular docking with mushroom tyrosinase led to the selection of five candidate peptides (Pep1–Pep5). Notably, Pep2 (LRVAPEEHPTL) showed the highest binding affinity and significantly outperformed arbutin in *in vitro* tyrosinase inhibition at certain concentrations. In α -MSH-stimulated B16-F10 melanoma cells, both crude pepsin hydrolysates and synthetic Pep2 effectively reduced melanin content and cellular tyrosinase activity without obvious cytotoxicity. Furthermore, an *in vivo* zebrafish model confirmed that the pepsin-hydrolyzed peptides effectively inhibited melanin accumulation while maintaining excellent biosafety (near 100% survival), overcoming the severe toxicity limitations of arbutin. Mechanistic investigation using real-time quantitative reverse transcription PCR and global RNA-seq transcriptomic analysis revealed that Pep2 downregulated key genes involved in melanogenesis, including *Mitf*, *Tyr*, *Tyrp1*, *Dct*, *Mclr*, and *Mapk1*. These findings demonstrate that *T. obscurus* peptides, particularly LRVAPEEHPTL, are promising natural tyrosinase inhibitors with a multi-level regulatory network. Alongside direct enzyme binding and classical transcriptional suppression, transcriptomic profiling suggests their potential to modulate the cAMP-PDE axis, further blunting the upstream melanogenic signal. These synergistic properties offer great potential for applications in functional foods and cosmetic formulations.

Keywords: *Takifugu obscurus*; Tyrosinase inhibitory; Anti-melanogenesis; Molecular docking; Bioactive peptides

1. Introduction

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Melanin, a pivotal pigment in skin pigmentation, plays critical roles in protecting skin cells from ultraviolet radiation-induced damage [1]. However, aberrant melanin overproduction and deposition can lead to hyperpigmentation disorders such as melasma, solar lentigines, freckles, age spots, post-inflammatory melanoderma, pigmented acne scars, and even malignant melanoma, posing significant threats to skin health and aesthetic needs (Pillaiyar et al., 2018; Zhang et al., 2017). In addition, research reports indicate that many melanogenic diseases are associated with neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, and Huntington's disease [4–6]. Tyrosinase, a copper-containing oxidase, is the rate-limiting enzyme in the melanin biosynthetic pathway, catalyzing the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of L-DOPA to *o*-quinone, finally the *o*-quinone is converted to melanin [7]. Thus, targeted inhibition of tyrosinase activity has become the core strategy for developing anti-hyperpigmentation agents.

Traditional synthetic tyrosinase inhibitors, such as arbutin, hydroquinone, and kojic acid, azelaic acid, and electron-rich phenols, have been widely used in cosmetics and pharmaceuticals. However, their application is restricted by inherent drawbacks, including high cytotoxicity, poor stability, and potential side effects such as skin irritation and mutagenicity [8–12]. This has spurred significant interest in identifying novel, natural, and biocompatible alternatives with high efficacy and low toxicity. In recent years, natural bioactive peptides derived from food proteins have garnered increasing attention as alternative tyrosinase inhibitors, attributed to their advantages of low toxicity, high biocompatibility, easy absorption, and diverse biological activities [1,13,14]. Bioactive peptides and protein hydrolysates derived from natural food sources have gained significant attention in the nutricosmetic industry, owing to their diverse health-promoting properties, including skin protection, anti-photoaging, anti-inflammatory, and systemic antioxidant effects [15–17]. To date, tyrosinase inhibitory peptides have been successfully isolated from various protein sources, including rice-bran albumin [18,19], walnut meal [1], crocodile serum [20], fish scale [21], and nacre powders [22], verifying the feasibility of screening natural anti-melanogenic peptides from edible resources.

To meet the demand for safe and natural alternatives, aquatic organisms represent a particularly rich and underexplored source of bioactive peptides. Pufferfish (*Takifugu* genus) is a valuable aquatic product with high nutritional value, rich in high-quality proteins, essential amino acids, and bioactive substances (Kaleshkumar et al., 2022; Yang et al., 2025). Among them, *Takifugu obscurus* (dark-striped pufferfish) is a commercially important cultured species in China that has been legally approved by Chinese authorities for aquaculture, processing, and consumption, as one of the two pufferfish species permitted for conditional processing and operation with standardized breeding techniques and strict processing criteria to ensure food safety. Consequently, *T. obscurus* boasts abundant muscle protein resources that remain underutilized, especially in the development of functional peptides (Yang et al., 2025). Although significant attention has been paid to the toxicology of certain pufferfish species [14], the muscle tissues of legally cultured *T. obscurus* are nontoxic after standardized processing and constitute an excellent and safe protein resource.

Previous studies on *T. obscurus* have mainly focused on umami peptides and angiotensin-converting enzyme (ACE)-inhibitory peptides (Su et al., 2023; Zhang et al., 2022). However, the potential of *T. obscurus* protein hydrolysates as a source of anti-melanogenic peptides remains largely unexplored, with systematic investigation into their tyrosinase inhibition and mechanisms of action in cellular melanogenesis models being relatively scarce.

Given the above research gaps, the present study aimed to comprehensively explore the tyrosinase inhibitory and anti-melanogenic potential of bioactive peptides derived from *T. obscurus* muscle proteins. The specific objectives were: (1) to optimize the enzymatic hydrolysis conditions of *T. obscurus* muscle proteins using five commercial proteases (pepsin, trypsin, flavourzyme, papain, and alkaline protease) and screen the hydrolysate with the highest tyrosinase inhibitory activity; (2) to isolate, purify, and identify key active peptides from the optimal hydrolysate via ultrafiltration, dialysis, nano-liquid chromatography–tandem mass spectrometry (nano-LC–MS/MS), and virtual screening (molecular docking); (3) to chemically synthesize the top candidate peptides and validate their tyrosinase inhibitory activity *in vitro*, as well as their anti-melanogenic efficacy and safety in α -MSH-induced B16-F10 melanoma cells and an *in vivo* zebrafish model; (4) to clarify the molecular mechanism underlying the anti-melanogenic effect by analyzing the mRNA expression levels of key melanogenesis-related genes (e.g., *Mitf*, *Tyr*, *Tyrp1*, *Dct*, *Mclr*, *Mapk1*), complemented by global RNA-seq transcriptomic profiling to uncover novel regulatory networks; and (5) to evaluate the safety of the active peptides by assessing the viability of B16-F10 cells. This study provides a theoretical basis for the high-value utilization of *T. obscurus* protein resources and the development of natural, safe anti-hyperpigmentation agents for food, cosmetic, and pharmaceutical applications.

2. Materials and methods

2.1. Materials and reagents

Pufferfish (*T. obscurus*) were supplied by Beijing Pufferfish Family Technology Industry Co., Ltd. Pepsin (3000 U/mg, Cat# P6322) was purchased from Macklin (Shanghai, China). Trypsin (250 N.F.U/mg, Cat# T8150), flavourzyme (30,000 U/g, Cat# F8270), papain (800,000 U/g, Cat# G8430), and alkaline protease (200,000 U/g, Cat# B8360) were obtained from Solarbio (Beijing, China). Tyrosinase (500 U/mg, Cat# T828256) and Arbutin (Cat# A828191) were acquired from Macklin. L-Tyrosine (Cat# ST8360) was sourced from Solarbio. α -Melanocyte-stimulating hormone (α -MSH) was purchased from MCE (Cat# HY-P0252, NJ, USA). The RNA extraction kit (Cat# RC112), reverse transcription kit (Cat# R433), and quantitative PCR kit (Cat# Q712) for real-time quantitative reverse transcription PCR (RT-qPCR) were procured from Vazyme (Nanjing, China). All other chemicals and reagents were of analytical grade.

2.2. Preparation of pufferfish peptides

Pufferfish meat was homogenized using a tissue homogenizer. The homogenate was mixed with deionized water at a ratio of 1:4 (w/v). The selection of enzymatic hydrolysis parameters, including this

liquid-to-solid ratio, as well as the enzyme dosage, pH, temperature, and time for each protease, was determined based on preliminary experiments and the recommended optimal reaction conditions for each enzyme to ensure sufficient protein hydrolysis. Specifically, the pH of the reaction mixtures for pepsin, trypsin, flavourzyme, papain, and alkaline protease was adjusted to 3, 8, 7, 7, and 10, respectively. The enzyme activity in each reaction system was maintained at 1500 U/g of pufferfish meat. The hydrolysis with pepsin and trypsin was conducted at 37 °C for 12 h with shaking, whereas flavourzyme, papain, and alkaline protease hydrolyses were performed at 50 °C for 4 h with shaking. After hydrolysis, the mixtures were centrifuged, and the supernatants were collected. The supernatants were then subjected to ultrafiltration using Microsep™ Advance Centrifugal Devices with a 1000 Da molecular weight cut-off (MWCO) (Pall Life Sciences, Cat# MAP001C36) by centrifugation for 90 min. The fractions with molecular weight < 1000 Da were collected. Subsequently, these fractions were dialyzed using a 300 Da MWCO dialysis membrane to remove free amino acids thoroughly. The resulting peptide mixture (300–1000 Da) was lyophilized.

2.3. Determination of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was determined by measuring the formation of dopachrome at 475 nm, according to a previously reported method with slight modifications [27]. The total reaction volume was 200 µL. Briefly, sample solutions at different concentrations were prepared in a potassium phosphate buffer (50 mM, pH 6.8). A volume of 40 µL of the sample solution and 100 µL of potassium phosphate buffer (50 mM, pH 6.8) were mixed with 40 µL of tyrosinase (500 U/mL in phosphate buffer) in a 96-well plate and incubated at 37 °C for 10 min. Then, 20 µL of the substrate (L-tyrosine) was added to each well to yield a final concentration of 0.8 mM, followed by incubation at 37 °C for another 10 min. The absorbance was measured at 475 nm using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Arbutin was used as a positive control. The inhibition rate of tyrosinase was calculated using the following formula:

$$\text{Inhibition}(\%) = \left(1 - \frac{OD_{\text{sample}} - OD_{\text{sample_blank}}}{OD_{\text{control}} - OD_{\text{control_blank}}}\right) \times 100\%$$

Where: OD_{sample} is the absorbance of the test well (containing sample, tyrosinase, L-tyrosine, and buffer); $OD_{\text{sample_blank}}$ is the absorbance of the sample blank well (containing sample, L-tyrosine, and buffer, but without tyrosinase, used to correct for the sample's background color); OD_{control} is the absorbance of the control well (containing tyrosinase, L-tyrosine, and buffer, but without sample); $OD_{\text{control_blank}}$ is the absorbance of the control blank well (containing only L-tyrosine and buffer, used as the true baseline).

2.4. Peptide identification

The amino acid sequences and molecular weights of the peptide fraction exhibiting the highest tyrosinase inhibitory activity were analyzed using nano-LC–MS/MS on an Ultimate 3000 system (Thermo Fisher Scientific, USA) coupled online to an Orbitrap Elite™ hybrid ion trap–Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) equipped with a nano-electrospray ionization (ESI) source. The sample was loaded onto a 75 µm × 150 mm Acclaim PepMap C18 analytical column packed with C18 AQ beads (3 µm, 100 Å) for peptide separation. Mobile phase A consisted of 0.1% (v/v) formic acid in water, and mobile

phase B was 0.1% (v/v) formic acid in acetonitrile. A flow rate of 300 nL/min was used with a linear gradient of mobile phase B from 6% to 95% (v/v) over 78 min. The column temperature was maintained at 30 °C. Both MS and MS/MS spectra were acquired in data-dependent mode with collision-induced dissociation energy set at 35%. The parameters were as follows: (1) MS: scan range (m/z) = 100–1500; resolution = 70,000; AGC target = 3e6; maximum injection time = 40 ms; dynamic exclusion time = 20 s; (2) MS/MS: resolution = 17,500; AGC target = 1e5; maximum injection time = 60 ms; top N = 20; normalized collision energy = 27; scan range (m/z) = 100–1500. Only peptides with high confidence were selected for downstream protein identification analysis.

2.5. Virtual screening and molecular docking

The X-ray crystal structure of mushroom tyrosinase (PDB ID: 2Y9X) was obtained from the RCSB Protein Data Bank (<http://www.rcsb.org>, accessed on 2025.09.05). The protein structure was prepared using Autodock Tools-1.5.6 Discovery Studio 2.5 software (Accelrys Software Inc., San Diego, CA, USA) for rigid molecular docking [28,29]: water molecules were removed, hydrogen atoms were added based on the physiological pH (7.4), and the native binuclear copper ions (Cu^{2+}) in the active site were retained with their original coordination bonds to avoid disrupting the active cavity structure.

Three-dimensional structures of the identified peptides were constructed using ChemDraw20.0 [30], followed by energy minimization using the MMFF94 force field (convergence criterion: RMS gradient = 0.01 kcal/mol·Å) to eliminate structural redundancy.

Subsequently, molecular docking of the tyrosinase inhibitory peptides was performed using AutoDock Vina [31,32]. A grid box (40 Å × 40 Å × 40 Å, spacing = 0.375 Å) was centered at the coordinates ($x = 11.2$ Å, $y = 28.5$ Å, $z = 35.7$ Å) to fully encompass the active site. For each peptide, the docking model with the lowest binding free energy was visualized with PyMOL 2.5 (Schrödinger, USA).

2.6. Peptide synthesis

To verify the tyrosinase inhibitory activity of the screened key peptides, peptide synthesis and purification were commissioned to GL Biochem (Shanghai) Co., Ltd. The peptides were synthesized using the standard Fmoc solid-phase method with an automated peptide synthesizer (Applied Biosystems, Warrington, UK).

After synthesis, the crude peptides were purified using reverse-phase high-performance liquid chromatography (RP-HPLC) on a C18 analytical column (4.6 mm × 250 mm, 5 μm, 100 Å). The mobile phase consisted of solvent A (0.1% v/v trifluoroacetic acid in water) and solvent B (0.1% v/v trifluoroacetic acid in acetonitrile), with a linear gradient elution program: 5%–95% solvent B over 30 min at a flow rate of 1.0 mL/min. The eluate was monitored at a detection wavelength of 220 nm.

The purity of the purified peptides was determined to be ≥98% using RP-HPLC, and their molecular weights were confirmed to match the theoretical values using ESI mass spectrometry.

2.7. Cell culture and induction of the melanogenesis model

B16-F10 mouse melanoma cells, obtained from Wuhan Pricella Biotechnology Co., Ltd, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. The medium was changed every 2 days, and cells were passaged when they reached over 90% confluence.

To evaluate the anti-melanogenic effects of the peptides, B16-F10 cells were seeded in 6-well plates. After cell attachment, the culture medium was replaced with fresh DMEM (supplemented with 10% FBS and 1% penicillin/streptomycin) containing both 1 μM α-MSH and varying concentrations of samples (pepsin-hydrolyzed peptides at 0.25–4.0 mg/mL, or synthetic peptides Pep2 and Pep5 at 0.125–1.0 mg/mL). The concentration of 1 μM α-MSH was selected to induce the melanogenesis model based on previously established protocols [33]. Arbutin served as the positive control. The cells were co-incubated for 24 h before the subsequent determination of melanin content and intracellular tyrosinase activity.

2.8. Measurement of melanin content in B16-F10 cells and in vivo zebrafish model

For *in vitro* evaluation, cells were seeded into 6-well plates at a density of 1×10^8 cells/L (2 mL per well) and allowed to adhere for 12 h. The medium was then replaced with DMEM complete medium containing 1 μM α-MSH (to induce melanogenesis) co-administered with different concentrations of pepsin-hydrolyzed peptides, arbutin, or synthetic peptides. After 24 h of incubation, cells were harvested. To ensure accurate quantification independent of cell proliferation or cytotoxicity, the total protein concentration of the cells was determined using a BCA protein assay kit. Subsequently, one milliliter of 1 M NaOH containing 10% DMSO was added to lyse the cells, followed by incubation in an 80 °C water bath for 30 min. Then, 200 μL of the solution was transferred to a 96-well plate, and the absorbance was measured at 405 nm. The final relative melanin content was normalized to the total protein amount to eliminate the confounding effect of variations in cell numbers.

For *in vivo* evaluation, healthy wild-type AB strain zebrafish embryos (2–4 h post-fertilization) were maintained in E3 medium at 28.5 °C under a strict 14 h light/10 h dark cycle. After hatching and a 24 h dark adaptation period to eliminate endogenous melatonin interference, the larvae (10 per well) were transferred to 6-well plates containing 2 mL of E3 medium supplemented with pepsin-hydrolyzed peptides (50 and 100 μg/mL) or the positive control arbutin (100 μg/mL). Half of the culture medium was replaced daily with fresh corresponding solutions. The survival rates and phenotypes of the zebrafish were monitored and photographed at designated time points. To quantify *in vivo* melanin content, the larvae were collected, washed twice with pre-chilled PBS, and completely lysed in 1 mL of 1 M NaOH containing 10% DMSO at 80 °C for 30 min. After centrifugation at 12,000 rpm for 5 min, the absorbance of the supernatant was measured at 405 nm.

2.9. Determination of cellular tyrosinase activity

After co-treatment for 24 h as described in Section 2.8, the culture supernatant was discarded, and the cells were washed twice with PBS. Cells were lysed using 1% Triton X-100. The cell lysates were scraped off and transferred to 1.5 mL microcentrifuge tubes, followed by centrifugation at $12,000 \times g$ for 10 min at

4 °C. The total protein concentration of the supernatant was measured using a BCA protein assay kit. A volume of 100 µL of the supernatant was added to each well of a 96-well plate, followed by the addition of 100 µL of 2 mmol/L L-DOPA. The mixture was incubated at 37 °C for 1 h, and the absorbance was measured at 475 nm. The cellular tyrosinase activity was normalized to the total protein content and expressed as units per milligram of protein (U/mg prot).

2.10. RT-qPCR analysis of mRNA expression levels of genes related to the melanin signaling pathway in B16-F10 cells

After treatment for 24 h as described in Section 2.8, cells were collected, and total RNA was extracted using an RNA extraction kit according to the manufacturer's instructions. The quality of the RNA was assessed by measuring the absorbance at 260 nm and 280 nm.

Using the extracted RNA as a template, cDNA was synthesized following the instructions of the reverse transcription kit. RT-qPCR was performed using the ChamQ Universal SYBR qPCR Master Mix kit according to the manufacturer's protocol. The total reaction volume was 20 µL, containing 10 µL of SYBR qPCR Master Mix, 0.4 µL of each primer (10 µmol/L), 1 µg of cDNA, and RNase-free water added to a final volume of 20 µL. Gene sequences were retrieved from NCBI, and primer information was obtained from Primer Bank. The PCR conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, then 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s for melt curve analysis. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Table 1. Primers for RT-qPCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
<i>Mitf</i>	CCAACAGCCCTATGGCTATGC	CTGGGCACTCACTCTCTGC
<i>Tyr</i>	CAAAGGGGTGGATGACCGTG	AACTTACAGTTTCCGCAGTTGA
<i>Tyrp1</i>	CCCCTAGCCTATATCTCCCTTTT	TACCATCGTGGGGATAATGGC
<i>Dct</i>	TTCCCCGAGTCTGCATGAC	TGCATGTCCGGTTGAAGAATTT
<i>Mclr</i>	GTGCTGGTTGTGATAGCCATC	TGCTGACACTTACCATCAGGT
<i>Mapk</i>	GGTTGTTCCCAAATGCTGACT	CAACTTCAATCCTCTTGTGAGGG

2.11. RNA sequencing and transcriptomic analysis

Total RNA from B16-F10 cells was extracted using TRIzol reagent (Thermo Fisher, USA) and evaluated for quality. Strand-specific cDNA libraries were sequenced on an Illumina Novaseq™ X Plus platform by LC Bio Technology CO., Ltd. (Hangzhou, China). After quality control via FastQC (v0.10.1), clean reads were mapped to the *Mus musculus* reference genome (Ensembl v112) using HISAT2 (v2.2.1), and expression levels were quantified as FPKM using StringTie (v2.1.6). Differentially expressed genes (DEGs) were identified using the DESeq2 R package (v1.22.2), with significance thresholds set at $|\log_2(\text{Fold Change})| \geq 1$ and $q\text{-value} < 0.05$. Finally, R software (v3.6) was used to generate volcano plots and hierarchical clustering heatmaps, and to perform Gene Ontology (GO) enrichment analysis.

2.12. Quantification and statistical analysis

Quantification and statistical analyses were performed using GraphPad Prism 8. Unpaired *t* tests were used for two-group comparisons, and analysis of variance (one-way or two-way) was used for multigroup comparisons. Tukey's test was used for post hoc multiple comparisons. The results are presented as mean $\pm$ SEM, with the biological replicates (*n*) explicitly defined. *P*-values were as follows: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

3. Results and discussion

3.1. Preparation of pufferfish peptides and their inhibitory effects on tyrosinase activity

Enzymatic hydrolysis is a critical step for releasing bioactive peptides from parent proteins, and the type of protease, hydrolysis conditions (pH, temperature, time), and enzyme-to-substrate ratio directly affect the yield and activity of target peptides [34]. In this study, pufferfish proteins were hydrolyzed separately using pepsin, trypsin, flavourzyme, papain, and alkaline protease, followed by ultrafiltration and dialysis to obtain peptide mixtures with molecular weights ranging from 300 to 1000 Da (Fig. 1A). The tyrosinase inhibitory activities of the five peptide preparations were evaluated using arbutin, a known potent tyrosinase inhibitor (with 96.76% $\pm$ 1.12% of tyrosinase inhibitory activity at 10 mg/mL), as a positive control (Fig. 1B). Pepsin, which exhibits optimal activity under acidic conditions (pH 3.0, 37 °C), produced hydrolysates with the strongest tyrosinase inhibitory activity (87.01% $\pm$ 2.64% at 10 mg/mL) (Fig. 1C), significantly higher than trypsin (33.75% $\pm$ 1.70%) (Fig. 1D), flavourzyme (19.75% $\pm$ 1.50%) (Fig. 1E), papain (31.03% $\pm$ 1.11%) (Fig. 1F), and alkaline protease (31.79%) (Fig. 1G). This result is consistent with the finding of Zu et al. [35], who reported that pepsin hydrolyzes scale collagen peptides with superior tyrosinase inhibitory activity than neutral and alkaline proteases. The high activity of pepsin-hydrolyzed peptides may be attributed to two factors: first, pepsin preferentially cleaves peptide bonds adjacent to hydrophobic amino acids (e.g., Phe, Tyr, Leu), which are critical for forming stable interactions with the hydrophobic active site of tyrosinase [36]; second, the 12 h hydrolysis duration at 37 °C ensured sufficient protein degradation, leading to the enrichment of small-molecular-weight peptides (300–1000 Da) after ultrafiltration and dialysis. Previous studies have confirmed that peptides with molecular weights < 3 kDa, especially < 1000 Da, exhibit higher bioactivity due to their better solubility, membrane permeability, and ability to bind to enzyme active centers (Hao et al., 2024; Yang et al., 2023). In summary, five different pufferfish peptide preparations were obtained using various proteases and hydrolysis conditions, and the pepsin-hydrolyzed peptides, which showed the strongest tyrosinase inhibition, were selected for subsequent experiments.

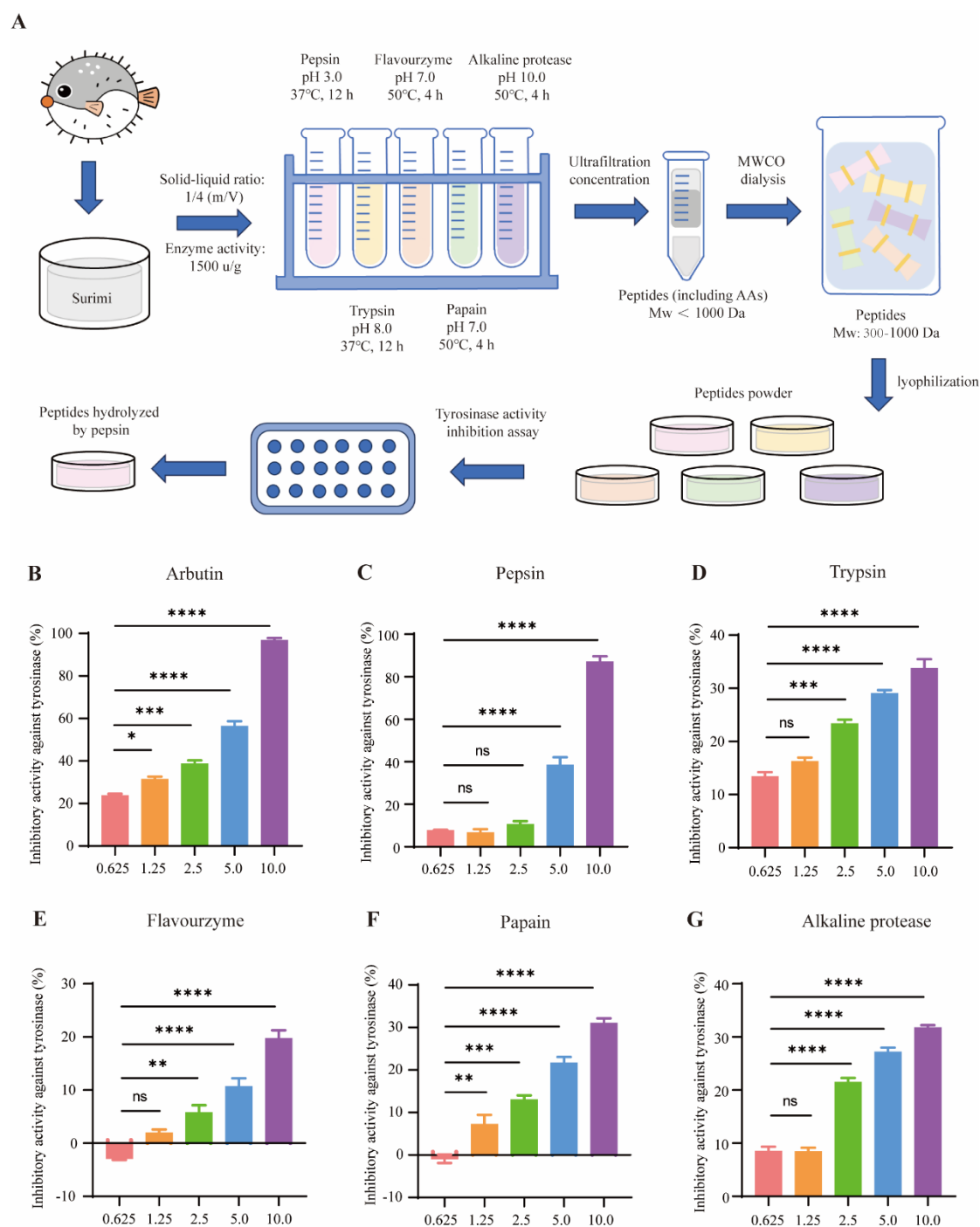


Figure 1. Preparation of pufferfish peptides and their inhibitory effects on tyrosinase activity. **A.** Schematic diagram of the enzymatic preparation of five pufferfish peptide mixtures and the screening of the most potent tyrosinase inhibitor *in vitro*. Tyrosinase inhibitory activity of the positive control arbutin (**B**), pepsin-hydrolyzed peptides (**C**), trypsin-hydrolyzed peptides (**D**), flavourzyme-hydrolyzed peptides (**E**), papain-hydrolyzed peptides (**F**), and alkaline protease-hydrolyzed peptides (**G**) at different concentrations. $n = 3$. Data are presented as mean $\pm$ SEM. A one-way ANOVA test was used: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; “ns” indicates no significant difference; “n” represents the number of biological replicates.

3.2. Inhibitory effects of pepsin-hydrolyzed peptides on α -MSH-induced melanogenesis in B16-F10 cells

After confirming that pepsin-hydrolyzed peptides exhibited the strongest tyrosinase inhibitory activity *in vitro* (Section 3.1), we further validated their anti-melanogenic potential and biocompatibility in α -MSH-induced B16-F10 melanoma cells, using arbutin as a positive control for comparative analysis. Pepsin-hydrolyzed peptides showed no significant effect on the viability of B16-F10 cells at concentrations

ranging from 0.25 to 4.0 mg/mL (Fig. 2A). By contrast, arbutin exhibited concentration-dependent cytotoxicity within the same concentration range, reducing cell viability to $56.43\% \pm 1.20\%$ at 4.0 mg/mL (Fig. 2B), indicating significantly higher cytotoxicity than the pepsin-hydrolyzed peptides. α -MSH-induced B16-F10 cells are a well-established model for evaluating anti-melanogenic agents, as α -MSH activates the MC1R signaling pathway, leading to upregulation of *Mitf* and subsequent induction of melanin synthesis [39]. α -MSH stimulation induced noticeable melanin deposition in B16-F10 cells, whereas both pepsin-hydrolyzed peptides and arbutin reduced melanin accumulation (Fig. 2C), with a clearly visible lightening effect at concentrations of 1.0–4.0 mg/mL. Further quantification revealed that pepsin-hydrolyzed peptides significantly decreased intracellular melanin content at concentrations of 0.25–4.0 mg/mL compared with the model group (Fig. 2D), whereas arbutin showed similar effects at 0.5–4.0 mg/mL (Fig. 2E). In addition, pepsin-hydrolyzed peptides significantly suppressed intracellular tyrosinase activity at 1.0–4.0 mg/mL (Fig. 2F), whereas arbutin exhibited stronger inhibition at 0.5–4.0 mg/mL (Fig. 2G).

To further validate the anti-melanogenic efficacy and biosafety of the peptides in a complex physiological environment, an *in vivo* zebrafish model was employed. As shown in the microscopic images (Fig. 2H), treatment with pepsin-hydrolyzed peptides (50 and 100 μ g/mL) visibly reduced melanin deposition in zebrafish in a dose-dependent manner. This visual observation was corroborated by the quantitative analysis of total melanin content (Fig. 2J), which demonstrated that both the positive control arbutin (100 μ g/mL) and the peptide treatments significantly inhibited *in vivo* melanogenesis. Crucially, the evaluation of *in vivo* toxicity revealed an obvious contrast between the treatments (Fig. 2I). Arbutin intervention resulted in relative severe toxicity, plummeting the zebrafish survival rate to approximately 70%. In contrast, all peptide-treated groups maintained survival rates nearing 100%, indicating minimal *in vivo* toxicity. These animal experiments establish that while arbutin exhibits strong depigmenting effects, its practical application is hindered by significant systemic toxicity. Conversely, the *T. obscurus* derived peptides exhibit a favorable balance of *in vivo* anti-melanogenic efficacy and biosafety, highlighting their potential as natural and safe tyrosinase inhibitors.

Overall, the intracellular melanin and tyrosinase inhibitory activity of pepsin-hydrolyzed peptides was slightly lower than that of arbutin, consistent with *in vitro* tyrosinase inhibitory activity (pepsin-hydrolyzed peptides of 87.01% vs arbutin of 96.76%) at 10 mg/mL. Notably, pepsin-hydrolyzed peptides showed no significant cytotoxicity to B16-F10 cells even at 4.0 mg/mL (cell viability > 90%), whereas arbutin reduced cell viability to $56.43\% \pm 1.20\%$ at the same concentration (Fig. 2A, B). This contrast in safety was further mirrored in our *in vivo* findings, where arbutin compromised zebrafish survival (approx. 70%), while the peptides maintained near 100% survival (Fig. 2I). It is important to note that because our melanin and tyrosinase assays were normalized to the total protein content of viable cells, the strong inhibitory effects of high-dose arbutin are genuine rather than an artifact of reduced cell numbers. However, the severe cytotoxicity and systemic toxicity of arbutin suggests that its efficacy comes at the cost of extreme cellular stress and organismal damage. In contrast, the pufferfish peptides exert their targeted anti-melanogenic

mechanisms without compromising cell health or animal survival. This safety advantage is crucial for the practical application of pufferfish peptides, as arbutin-induced cytotoxicity at high concentrations has been a major concern in cosmetic formulations [40]. The low toxicity of pufferfish peptides may be related to their natural origin and amino acid composition, which are compatible with biological systems and less likely to induce oxidative stress or cell membrane damage [41]. Altogether, these results indicate that pepsin-hydrolyzed peptides effectively inhibit melanin accumulation and tyrosinase activity both *in vitro* and *in vivo* without significantly affecting cell viability and organismal safety.

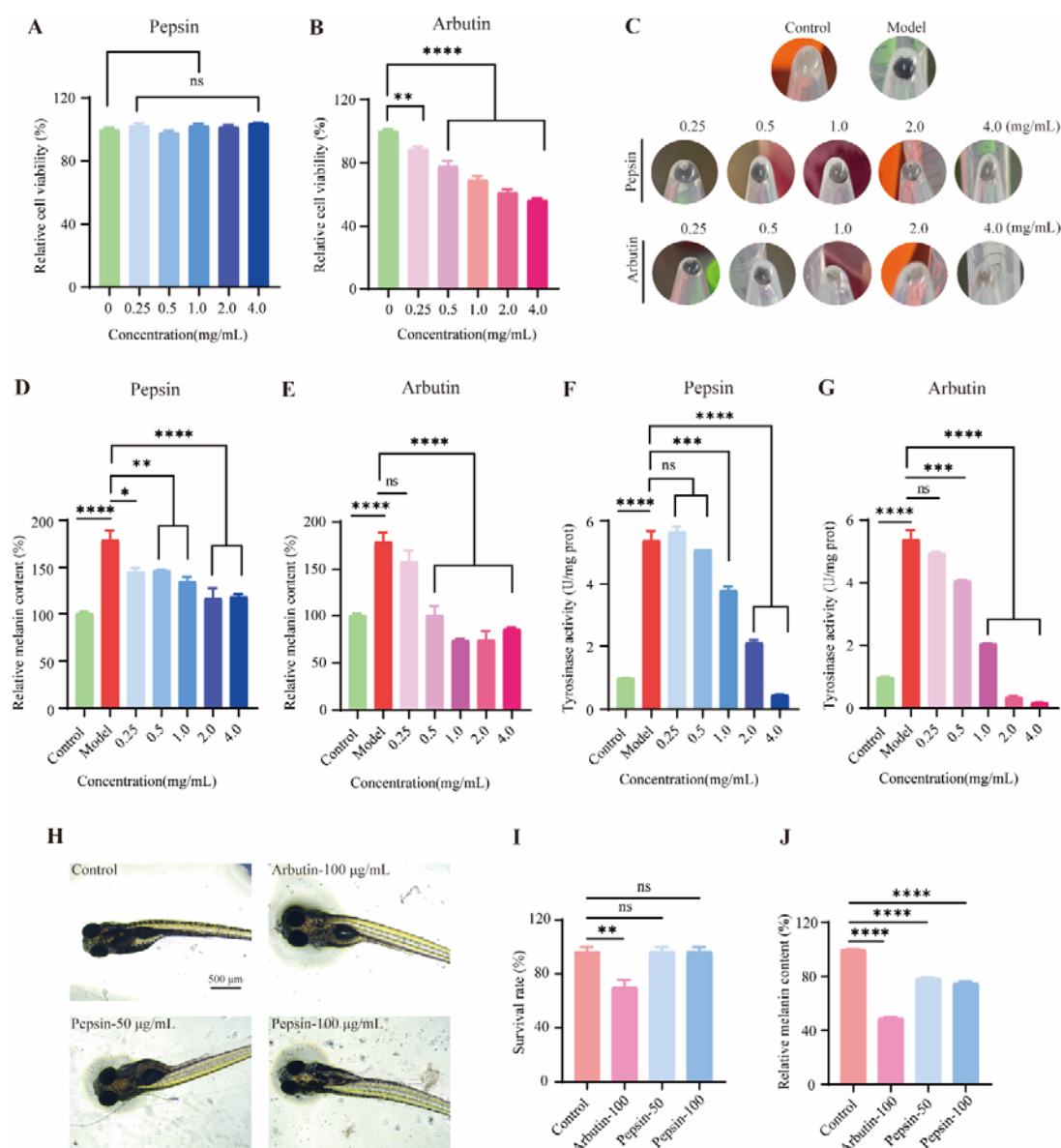


Figure 2. Inhibitory effects of pepsin-hydrolyzed peptides on α -MSH-induced melanogenesis in B16-F10 cells. A, B.

Relative cell viability was assessed by CCK-8 assay after treatment with different concentrations of pepsin-hydrolyzed peptides (A) and the positive control arbutin (B). C. Representative images of α -MSH-induced melanin-producing B16-F10 cells treated with different concentrations of pepsin-hydrolyzed peptides or arbutin, which showed a visible reduction in melanin deposition. D, E. Relative melanin content in cells treated with different concentrations of pepsin-hydrolyzed peptides (D) and arbutin (E). F, G. Intracellular tyrosinase activity in cells treated with different concentrations of pepsin-hydrolyzed peptides (F) and arbutin (G). H. Representative microscopic images (scale bar: 500 μ m) of zebrafish treated with arbutin (100 μ g/mL) or varying concentrations of pepsin-hydrolyzed peptides (50 and 100 μ g/mL). I. Survival rate of zebrafish following the respective treatments. J. Relative *in vivo* melanin content of zebrafish across the treatment groups. n = 6. Data are presented as mean $\pm$ SEM. A one-way ANOVA test was used: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; “ns” indicates no significant difference; “n” represents the number of biological replicates.

3.3. Peptide identification of pepsin-hydrolyzed peptides and molecular docking with tyrosinase

The peptide mixture obtained from pepsin hydrolysis was identified using LC–MS/MS to determine its peptide composition (Fig. 3A). To screen for potential tyrosinase inhibitory peptides, molecular docking analysis was performed. Tyrosinase (PDB: 2Y9X) was used as the docking target, with lower binding energy values indicating stronger and more stable interactions between the ligand and the protein [1]. The top five peptides with favorable binding affinity were ultimately identified (Table 2). Among them, Pep2 exhibited the most stable complex with the best binding affinity (−7.3 kcal/mol), followed by Pep5 with a binding affinity of −7.2 kcal/mol. The structural characteristics of the identified peptides provide insights into their structure–activity relationship. Pep2, a linear peptide with 11 amino acid residues, contains multiple hydrophilic residues (Glu, Asp) and hydrophobic residues (Leu, Val, Phe). The hydrophilic residues facilitate the formation of hydrogen bonds with tyrosinase, while the hydrophobic residues enhance the hydrophobic interactions with the enzyme's active site, synergistically improving binding affinity [42]. Pep5, with nine amino acid residues, is rich in Tyr and Phe residues at the N- and C-termini, which are known to be critical for tyrosinase inhibition. Tyr residues can compete with the substrate L-tyrosine for binding to the active site, whereas Phe residues enhance hydrophobic interactions [43]. In addition, the presence of Pro residues in both Pep2 and Pep5 may contribute to the formation of a stable secondary structure (e.g., β -turns), which is beneficial for maintaining peptide conformation and binding to the enzyme [44].

Table 2. Information on the five peptides exhibiting high binding affinity to tyrosinase.

Serial number	Peptide	Binding sites	Affinity (kcal/mol)
Pep1	YALPHALM	ASN70, GLY68, ASP49, LEU9, ASN70, GLN48	−6.9
Pep2	LRVAPEEHPTL	ASN70, ASN71, ASN70, MET67, GLN48, GLN48	−7.3
Pep3	AGDDAPRAVF	ASP10, GLN48, GLY68, ASN70, LEU9	−6.7
Pep4	DRVGDSAGVAF	ASN70, ASP10, ASP49, GLN48, GLN48, LEU9, ASN70, LEU9	−6.7
Pep5	TYVTPHQTF	ASP10, ASN70, LEU9, ASP10, ASN70, MET67, GLN48, ASP49, MET67, ASN70	−7.2

Furthermore, more precise docking analyses were performed to further clarify the exact binding sites and interactions between these five peptides and tyrosinase. The chemical structural formulas of the ligand peptides and the three-dimensional interaction diagrams with the receptor 2Y9X are shown in Fig. 3B–F. As illustrated, all five peptides could dock deeply and stably into the hydrophobic cavity of the receptor protein and interact with multiple amino acid residues, including ASN70, GLY68, ASP49, LEU9, ASN71, GLN48, MET67, and ASP10. The multiple hydrogen bonds and electrostatic interactions between the peptides and the amino acid residues of tyrosinase suggest that these peptides may possess strong tyrosinase inhibitory potential. Regarding the physiological relevance of these docking results, it is worth noting that although the overall amino acid sequence homology between mushroom tyrosinase (2Y9X) and human tyrosinase is relatively low, their catalytic active centers—specifically the binuclear copper pocket coordinated by six conserved histidine residues—are highly structurally conserved across species[45–48]. Since the active tyrosinase inhibitory peptides identified in our study (e.g., Pep2 and Pep5) primarily interact with this

conserved catalytic domain through hydrogen bonding, hydrophobic interactions, and π - π stacking, the molecular docking models based on 2Y9X possess substantial reference value. These models provide a reliable structural and mechanistic basis for predicting how these peptides might interact with and inhibit human tyrosinase in physiological environments, which is further supported by our *in vitro* cellular efficacy results in murine B16-F10 melanoma cells. Therefore, we proceeded to purify and synthesize these five peptide sequences for further validation of their inhibitory effects on tyrosinase activity.

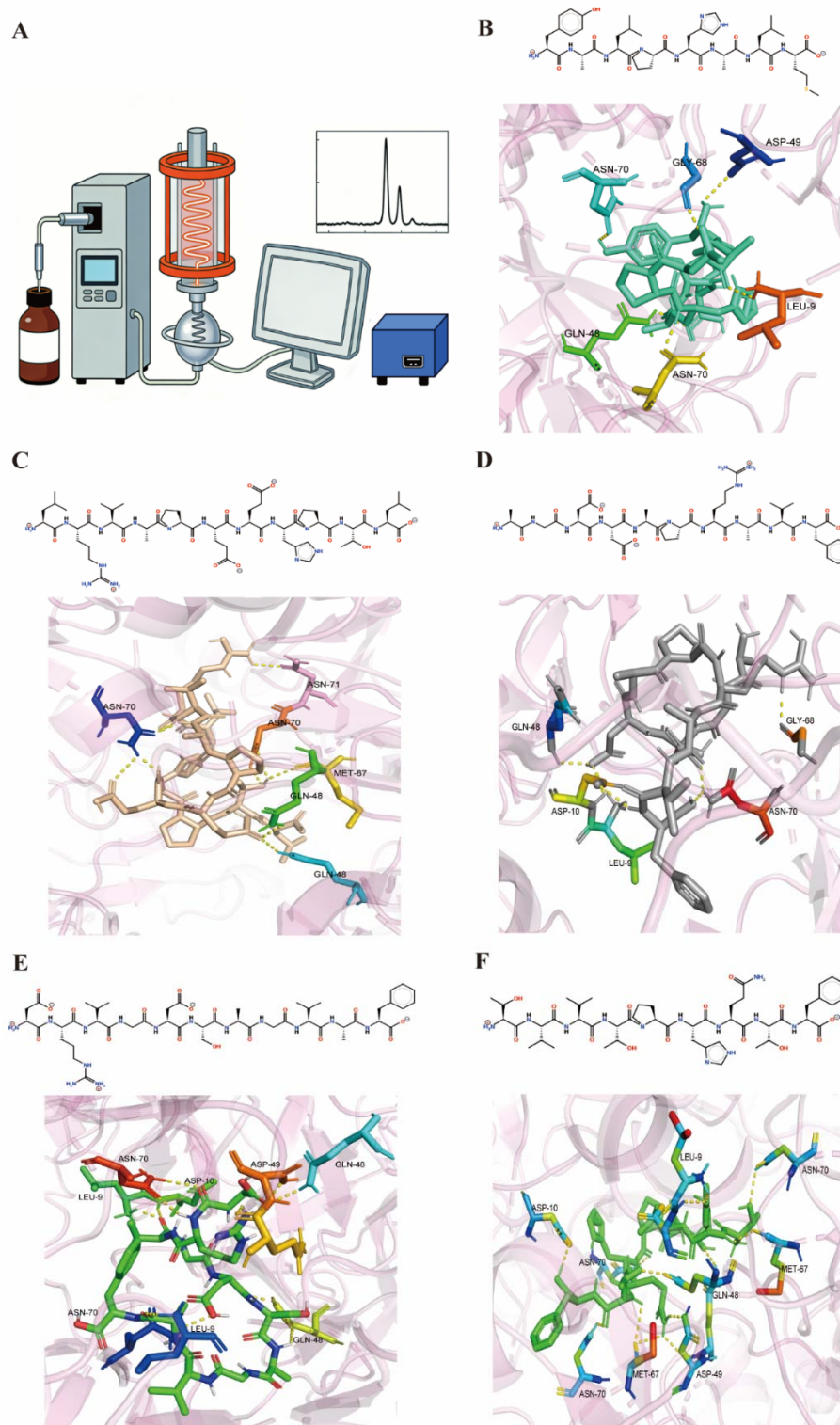


Figure 3. Peptide identification of pepsin-hydrolyzed peptides and molecular docking with tyrosinase. A. Schematic workflow of LC-MS/MS analysis for identifying peptides in the pepsin-hydrolyzed mixture. B-F. Docking poses of tyrosinase (PDB: 2Y9X) with the identified peptides: Pep1 (B), Pep2 (C), Pep3 (D), Pep4 (E), Pep5 (F).

3.4. Inhibitory effects of synthetic peptides on *in vitro* tyrosinase activity

To systematically evaluate the tyrosinase inhibitory potential of the five synthetic peptides (Pep1–Pep5) identified via molecular docking, *in vitro* enzyme activity assays were performed using L-tyrosine as the substrate, with arbutin as a positive control. The inhibitory rates of all samples at concentrations of 0.625 mg/mL to 5.0 mg/mL were quantified, and the results are presented in Fig. 4A–F.

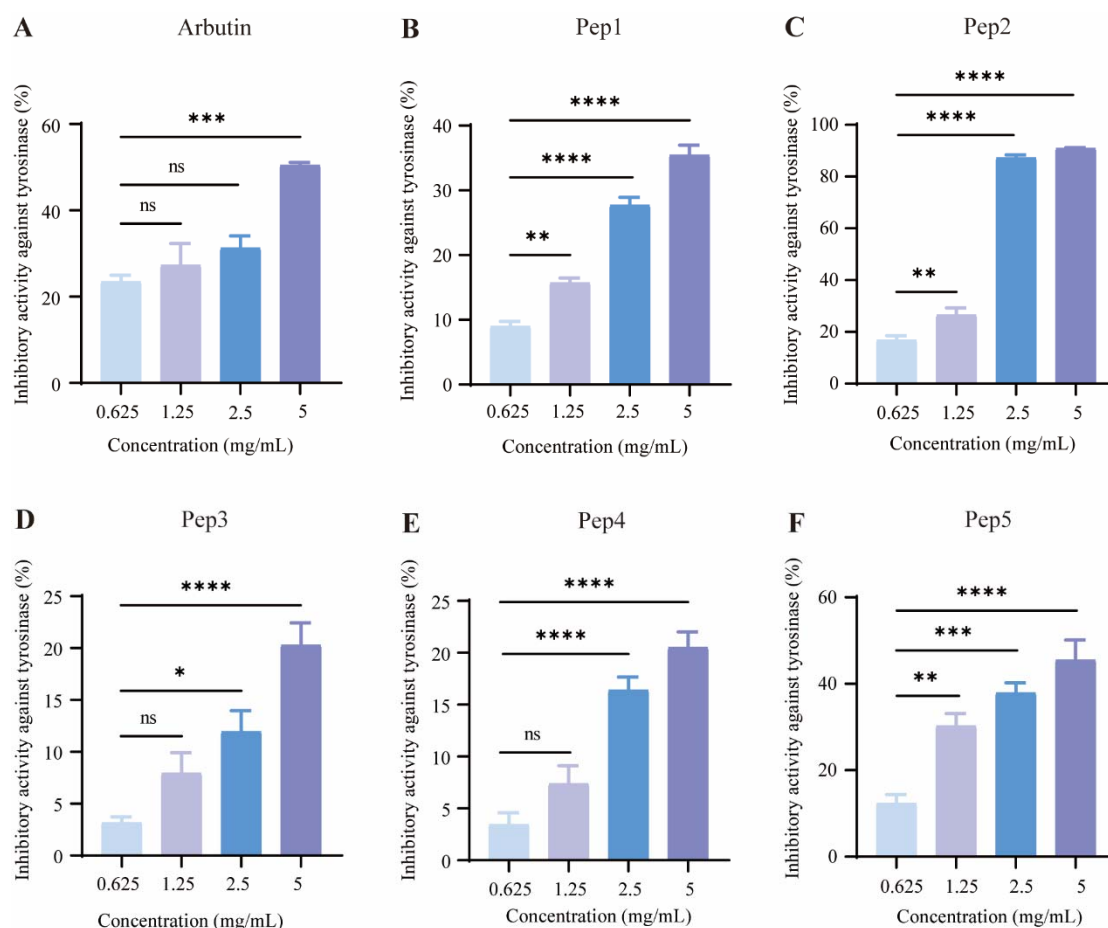


Figure 4. Inhibitory effects of synthetic peptides on tyrosinase activity *in vitro*. A–F. Tyrosinase inhibitory activity was evaluated for the positive control arbutin (A) and five synthetic peptides: Pep1 (B), Pep2 (C), Pep3 (D), Pep4 (E), Pep5 (F) at different concentrations *in vitro*. $n = 6$. Data are presented as mean $\pm$ SEM. A one-way ANOVA test was used: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; “ns” indicates no significant difference; “n” represents the number of biological replicates.

Among all tested peptides, Pep2 (LRVAPEEHPTL) exhibited the most potent tyrosinase inhibitory activity, significantly surpassing arbutin at concentrations of 2.5 and 5.0 mg/mL (Fig. 4A and 4C). Specifically, at 2.5 mg/mL, Pep2 achieved an inhibition rate of $87.31\% \pm 1.05\%$, which was substantially higher than that of arbutin ($31.26\% \pm 2.81\%$, Fig. 4A); at 5.0 mg/mL, Pep2’s inhibitory rate further increased to $90.97\% \pm 0.20\%$, outperforming arbutin’s $50.45\% \pm 0.65\%$. To further highlight the activity advantages of Pep2, its inhibitory efficacy was quantitatively compared with other reported aquatic-derived tyrosinase inhibitory peptides. Based on non-linear regression analysis, the IC_{50} value of Pep2 was determined to be 1.573 mg/mL. This activity is notably superior to several well-documented peptides derived from fish by-products. For instance, previously reported peptides from *Pinctada martensii* and grass carp scale gelatin exhibited higher IC_{50} values ranging from 2.55 to 3.87 mg/mL [49,50]. Furthermore, at the

identical concentration of 5.0 mg/mL, Pep2 achieved a highly potent inhibition rate of 90.97%, which significantly outperformed peptides derived from the skins of bigeye tuna (*Thunnus obesus*) (59.90%–63.10%) [51] and tilapia (*Oreochromis niloticus*) (59.73%) [21]. These comparisons clearly demonstrate that Pep2 possesses highly competitive anti-tyrosinase efficacy, highlighting the immense potential of *Takifugu obscurus* muscle proteins as an excellent source for natural anti-melanogenic agents. This remarkable activity of Pep2 is consistent with its molecular docking results (Table 1), where it displayed the lowest binding free energy (−7.3 kcal/mol) among the five peptides. The potent activity of Pep2 is highly dependent on its specific amino acid profile. Pep2 is notably abundant in hydrophobic amino acids (Leu, Val, Ala, Pro), which facilitate deep penetration and stable embedding into the hydrophobic cavity of the tyrosinase active center. Furthermore, the Pro residues promote the formation of a stable turn structure, optimizing the spatial matching between the peptide and the enzyme's active pocket. This structure-activity relationship aligns perfectly with previous findings that hydrophobic amino acids form the critical structural basis for potent tyrosinase inhibitory peptides [52,53]. The strong binding affinity is attributed to its multiple hydrogen bonds with key residues (e.g., ASN70, ASN71, MET67) and hydrophobic interactions with the tyrosinase active site, which effectively block substrate access and stabilize the enzyme–ligand complex [54–56]. Furthermore, to clarify the sequence novelty of the highly active Pep2 (LRVAPEEHPTL), a comprehensive sequence search was conducted against the BIOPEP-UWM database and existing literature. The sequence LRVAPEEHPTL was previously identified from chicken breast myofibrillar protein hydrolysate and reported exclusively as an antioxidant peptide [57]. However, to the best of our knowledge, no previous studies have reported its anti-tyrosinase or anti-melanogenic activity. Therefore, this study represents the first discovery of LRVAPEEHPTL as a potent natural tyrosinase inhibitor, which significantly expands the known bioactive profile of this sequence and highlights the value of *Takifugu obscurus* muscle proteins for mining multi-functional peptides.

Pep5 (TYVTPHQTF) showed the second-highest inhibitory activity among the peptides. At 2.5 mg/mL, its inhibition rate was $37.94\% \pm 2.27\%$, slightly higher than arbutin's $31.26\% \pm 2.81\%$; at 5.0 mg/mL, it reached $45.54\% \pm 4.57\%$, approaching arbutin's $50.45\% \pm 0.65\%$ (Fig. 4F). Structurally, Pep5 is characterized by a high content of aromatic (Tyr, Phe) and hydrophobic (Val, Pro) amino acids. The aromatic rings of Tyr and Phe can form robust π - π interactions with the binuclear Cu^{2+} in the tyrosinase active center, effectively disrupting the enzyme's catalytic structural integrity. Notably, the C-terminal Phe facilitates entry into the active pocket [58], while the Tyr residue functions as a substrate analogue, competitively occupying the active site to block the enzymatic oxidation of L-tyrosine/L-DOPA. These observations are fully corroborated by literature confirming that aromatic amino acids (especially at the sequence termini) serve as core active residues for tyrosinase inhibition [19,58]. This moderate activity aligns with its relatively low binding free energy (−7.2 kcal/mol), as Pep5 forms multiple hydrogen bonds with residues such as ASP10, ASN70, and MET67 in the tyrosinase active cavity (Section 3.3), facilitating stable enzyme binding [43].

By contrast, the three other peptides exhibited weaker tyrosinase inhibitory activities. Pep1 (YALPHALM), Pep3 (AGDDAPRAVF), and Pep4 (DRVGDSAGVAF) showed inhibition rates of $35.46\% \pm 1.51\%$ (Fig. 4B), $20.29\% \pm 2.12\%$ (Fig. 4D), and $20.52\% \pm 1.48\%$ (Fig. 4E) at 5.0 mg/mL. Although these peptides possess specific functional residues (e.g., N-terminal Tyr in Pep1, and C-terminal Phe in Pep3 and Pep4), their overall efficacy is comparatively low due to structural compromises. Firstly, unfavorable electrostatic interference occurs in Pep3 and Pep4, which contain a higher proportion of charged or highly polar residues (such as Asp and Arg). These residues introduce electrostatic repulsions or hydration shells that hinder the peptides from firmly anchoring into the highly hydrophobic core of the tyrosinase active site [52,53]. Secondly, suboptimal spatial positioning limits their activity: while Pep1 contains an N-terminal Tyr, structural studies suggest that C-terminal positioning of aromatic residues often yields superior substrate analogue effects and stronger coordination with copper ions [19,58]. Consequently, these suboptimal residue arrangements prevent the formation of optimally stable intermolecular interactions, leading to higher binding free energies compared with Pep2 and Pep5.

Based on these *in vitro* screening results, Pep2 (the most active) and Pep5 (the second-most active) were selected for further cellular experiments to validate their anti-melanogenic efficacy in a more physiological context, laying the foundation for subsequent mechanism exploration.

3.5. Inhibitory effects of synthetic peptides on α -MSH-induced melanogenesis in B16-F10 cells

After confirming the potent *in vitro* tyrosinase inhibitory activity of synthetic peptides (especially Pep2 and Pep5) in Section 3.4, we further evaluated their anti-melanogenic efficacy in α -MSH-induced B16-F10 melanoma cells, with arbutin as a positive control, to validate their functional performance in a physiological cellular context. Prior to efficacy evaluation, the cytotoxicity of the synthetic peptides was assessed to ensure that any observed reduction in melanin was not caused by cell death. As shown in Fig. 5A and 5B, treatments with Pep2 and Pep5 at concentrations ranging from 0.125 to 1.0 mg/mL did not exert any significant toxic effects on B16-F10 cells, with relative cell viabilities consistently maintained above 95%. This confirms the high biosafety of Pep2 and Pep5 within their effective dosage ranges.

Compared with the α -MSH-induced model group, arbutin, Pep2, and Pep5 all visibly reduced intracellular melanin deposition (Fig. 5C), confirming their ability to interfere with melanin synthesis in B16-F10 cells. Quantitative analysis revealed that all three treatments decreased intracellular melanin content to varying degrees, with arbutin being the most effective, followed by Pep2, and then Pep5 (Fig. 5D–F). At concentrations of 0.125–1.0 mg/mL, both arbutin and Pep2 significantly inhibited melanin synthesis, whereas Pep5 showed significant effects only at 0.25–1.0 mg/mL. This concentration-dependent difference in activity between Pep2 and Pep5 aligns with their structural characteristics: Pep2 (11 amino acid residues) contains a balanced ratio of hydrophilic (Glu, Asp) and hydrophobic (Leu, Val, Phe) residues, which not only enhances binding to tyrosinase but also improves cell membrane permeability [59,60]. By contrast, Pep5 (nine amino acid residues) has fewer hydrophilic residues, potentially limiting its solubility and cellular uptake efficiency at lower concentrations [61]. In addition, the lower activity threshold of Pep2

(0.125 mg/mL) than Pep5 (0.25 mg/mL) underscores its superior bioavailability, a critical advantage for practical application in functional products.

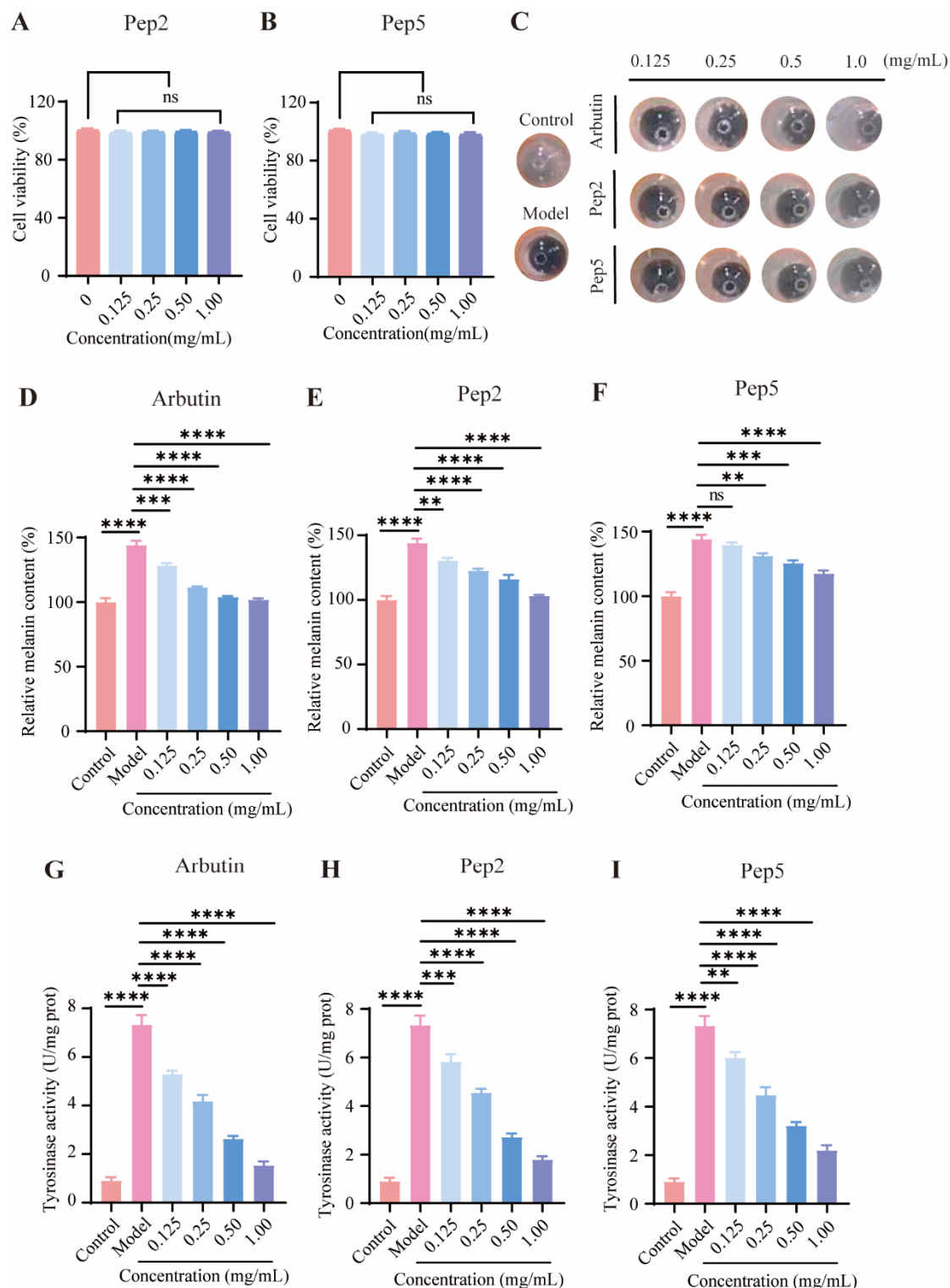


Figure 5. Inhibitory effects of synthetic peptides on α -MSH-induced melanogenesis in B16-F10 cells. A, B. Relative cell viability was assessed by CCK-8 assay after treatment with different concentrations of Pep2 (A) and Pep5 (B). C. Representative images of α -MSH-induced melanin-producing B16-F10 cells treated with different concentrations of arbutin, Pep2, or Pep5, showing a visible reduction in melanin deposition. D-F. Effects of different concentrations of arbutin (D), Pep2 (E), and Pep5 (F) on relative melanin content in cells. G-I. Effects of different concentrations of arbutin (G), Pep2 (H), and Pep5 (I) on intracellular tyrosinase activity. $n = 6$. Data are presented as mean $\pm$ SEM. A one-way ANOVA test was used: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; “ns” indicates no significant difference; “n” represents the number of biological replicates.

Similarly, all three treatments reduced intracellular tyrosinase activity, with the same order of efficacy: arbutin was the most potent, followed by Pep2, and then Pep5 (Fig. 5G–I). At concentrations of 0.125–1.0 mg/mL, all three significantly suppressed tyrosinase activity, consistent with the melanin content results. The slightly weaker cellular activity of Pep2 compared with arbutin may be attributed to differences in their modes of action: arbutin acts as a competitive inhibitor that directly binds to the tyrosinase active site with high specificity [62], whereas Pep2 may exert inhibitory effects through both direct enzyme binding and indirect regulation of melanogenesis-related genes (as discussed in Section 3.5). Notably, however, Pep2 retains a critical safety advantage over arbutin: as demonstrated in Section 3.2, arbutin exhibits significant cytotoxicity at concentrations ≥ 0.25 mg/mL, whereas Pep2 shows no adverse effects on B16-F10 cell viability within the tested concentration range up to 1.0 mg/mL (Fig. 5A). This balance of efficacy and safety is particularly valuable, as cytotoxicity has long been a major limitation for arbutin in high-concentration cosmetic formulations [40].

In summary, among the five synthetic peptides tested, Pep2 demonstrated the best tyrosinase inhibitory activity and melanin synthesis suppression at the cellular level. Its superior binding affinity to tyrosinase, favorable structural characteristics for cellular uptake, and low cytotoxicity make it a promising candidate for the development of natural anti-hyperpigmentation agents. These results lay the foundation for further exploration of the molecular mechanisms underlying Pep2's anti-melanogenic effect and its potential application in food, cosmetic, and pharmaceutical products.

3.6. Effects of synthetic peptides on the expression of melanogenesis-related genes in B16-F10 cells

Melanogenesis involves multiple signaling pathways, with the melanocortin-1 receptor (*Mclr*) serving as a key receptor in α -MSH-triggered melanin synthesis. Upon activation, *Mclr* upregulates microphthalmia-associated transcription factor (*Mitf*), which in turn promotes the expression of genes such as tyrosinase (*Tyr*), tyrosinase-related protein-1 (*Tyrp1*), and dopachrome tautomerase (*Tyrp2/Dct*) [63,64]. To investigate the molecular mechanism underlying Pep2-mediated tyrosinase inhibition, RT-PCR was used to measure mRNA expression levels of *Mitf*, *Tyr*, *Tyrp1*, and *Dct* in α -MSH-stimulated B16-F10 cells. α -MSH significantly upregulated the expression of these genes (Fig. 6A–D), whereas arbutin, Pep2, and Pep5 suppressed their expression to varying degrees, with arbutin showing the strongest inhibition, followed by Pep2, and then Pep5 (Fig. 6A–D). In addition, all three treatments inhibited the overexpression of the upstream signaling molecule *Mclr*, suggesting that Pep2 and Pep5 may inhibit pigmentation by downregulating the transcription of key melanogenesis-related genes and modulating upstream signaling pathways to some extent.

Furthermore, Pep2 and Pep5 significantly downregulated the expression of *Mapk1* (encoding ERK1), a key gene in the ERK–MAPK signaling pathway (Fig. 6F). The ERK–MAPK pathway is involved in the post-transcriptional regulation of *Mitf*: activation of ERK1 phosphorylates *Mitf*, leading to its ubiquitination and degradation [65,66]. Thus, the downregulation of *Mapk1* by Pep2 and Pep5 is expected to reduce ERK1-mediated *Mitf* degradation, exerting a potential “*Mitf*-stabilizing” effect. However, this effect was

overridden by the stronger upstream inhibitory signal from *Mclr* downregulation: as α -MSH binding to MC1R was blocked, the transcriptional activation of *Mitf* was substantially suppressed, leading to an overall decrease in *Mitf* expression (Fig. 6A and 6E). These results indicate that the anti-melanogenic effect of Pep2 and Pep5 is primarily mediated by the downregulation of the MC1R–Mitf–Tyr axis (transcriptional level), with the ERK–MAPK pathway (post-transcriptional level) serving as a synergistic regulatory branch to fine-tune Mitf stability. This multilevel regulatory mode is consistent with the mechanism of action of other natural tyrosinase inhibitory peptides [20,67], highlighting the complexity and comprehensiveness of the peptide-mediated anti-melanogenic effect. However, as the current findings regarding the MAPK pathway are predominantly based on transcriptional profiling (*Mapk1* mRNA expression) and supported by transcriptomic trends, further experimental evidence at the protein translation and post-translational modification levels is required.

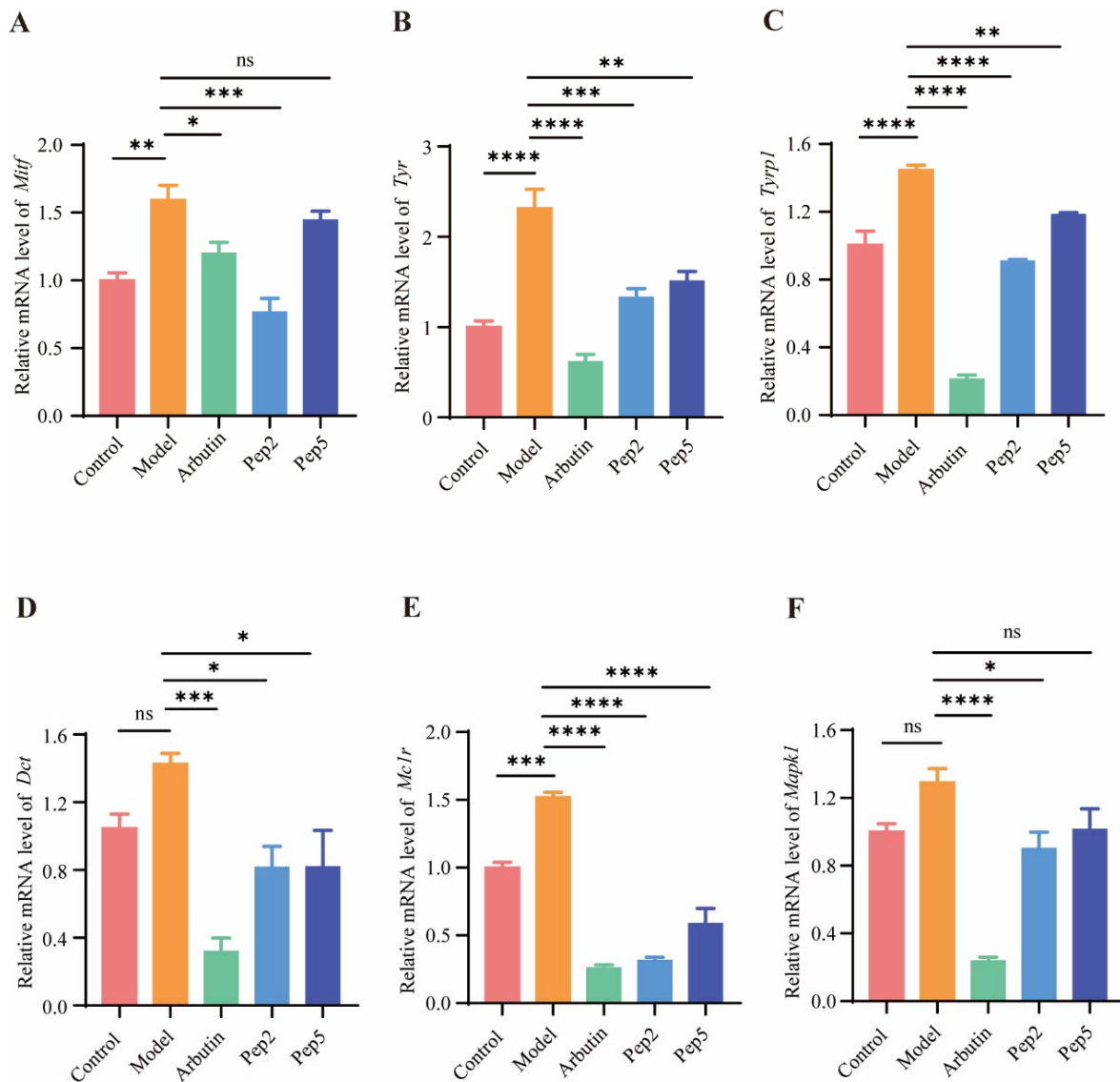


Figure 6. Effects of synthetic peptides on the expression of melanogenesis-related genes in B16-F10 cells. A-F. Changes in mRNA expression levels of *Mitf* (A), *Tyr* (B), *Tyrp1* (C), *Dct* (D), *Mclr* (E), and *Mapk1* (F) in α -MSH-induced melanin-producing B16-F10 cells, following treatment with arbutin, Pep2, or Pep5. $n = 3$. Data are presented as mean $\pm$ SEM. A one-way ANOVA test was used: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; “ns” indicates no significant difference; “n” represents the number of biological replicates.

3.7. Transcriptomic analysis reveals global regulatory networks and novel targets of Pep2

To address the complexity of melanogenesis and explore potential novel targets beyond the classical MC1R-MITF-TYR axis, global RNA-seq transcriptomic analysis was performed. Differential expression analysis was first conducted to identify the specific genes regulated by Pep2 treatment. The volcano plot (Fig. 7A) highlights the significantly up- and down-regulated genes in the Pep2 group compared to the Model group, providing a direct view of the primary genomic targets of Pep2. Subsequently, multi-group functional enrichment and clustering analyses were utilized to further elucidate how these targets contribute to the global transcriptomic shift and effectively reverse the abnormalities induced by α -MSH (Fig. 7B and 7C).

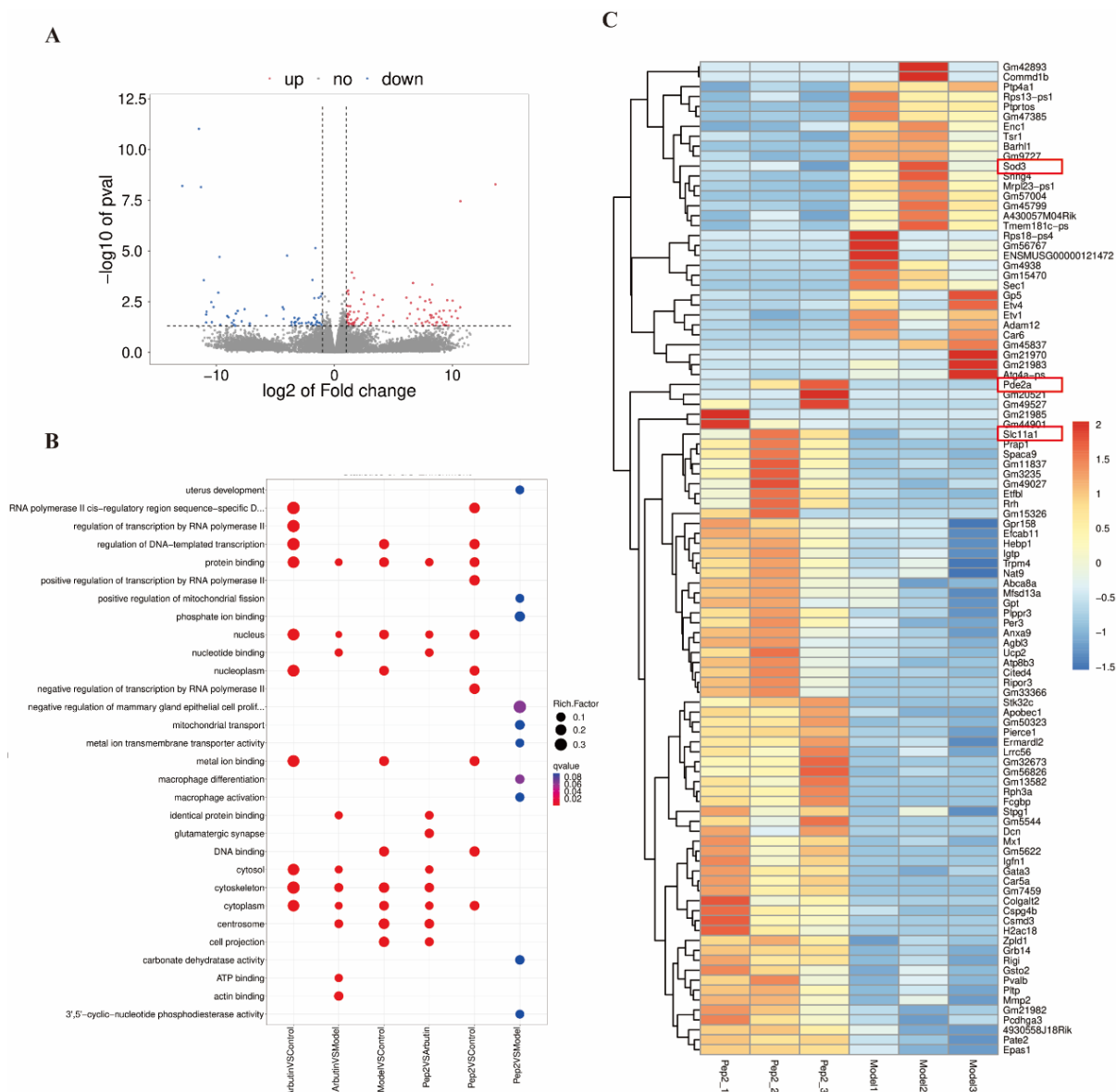


Figure 7. Transcriptomic analysis reveals the global regulatory network and potential novel targets of Pep2 in B16-F10 cells. **A.** Volcano plot of differentially expressed genes (DEGs) between the Pep2 and Model groups. Red and blue dots represent significantly upregulated and downregulated genes, respectively ($|\log_2(\text{Fold Change})| \geq 1$ and $P < 0.05$). **B.** Multi-group GO enrichment scatter plot comparing the functional distribution shifts across different groups. Circle size reflects the number of DEGs, and the color gradient represents the significance level (q -value). **C.** Hierarchical clustering heatmap of the top 100 DEGs between the Pep2 and Model groups. Red and blue indicate high and low expression levels, respectively, with key novel targets (*Pde2a*, *Sod3*, and *Slc11a1*) highlighted. $n = 3$; “n” represents the number of biological replicates.

To further interpret the biological significance of these DEGs, a multi-group Gene Ontology (GO) enrichment analysis was conducted to compare the functional shifts among the Control, Model, Arbutin, and Pep2 groups (Fig. 7B). Consistent with the RT-PCR results, both Arbutin and Pep2 effectively reversed the α -MSH-induced transcriptomic abnormalities, particularly enriching pathways related to the nucleus and transcriptional regulation. Intriguingly, Pep2 exhibited a unique multi-target regulatory profile compared to arbutin. Specifically, Pep2 exclusively enriched pathways associated with 3',5'-cyclic-nucleotide phosphodiesterase (PDE) activity, mitochondrial dynamics (e.g., mitochondrial fission and transport), and metal ion transmembrane transporter activity.

To pinpoint the specific gene targets driving these functional pathways, a hierarchical clustering heatmap of the top 100 DEGs between the Pep2 and Model groups was generated (Fig. 7C). The heatmap revealed a profound transcriptomic reprogramming, where Pep2 distinctively reversed the abnormal gene expression patterns induced by α -MSH. Notably, the expression of *Pde2a* (Phosphodiesterase 2A) was significantly upregulated by Pep2. Given that PDEs are the primary enzymes responsible for the degradation of intracellular cAMP [68,69]—the crucial upstream secondary messenger in α -MSH/MC1R signaling [70]—this finding suggests a novel intervention mechanism: Pep2 may intercept the melanogenic cascade at the post-receptor stage by accelerating cAMP clearance, thereby dampening MITF transcription.

Furthermore, the remarkable transcriptomic regulation of antioxidant enzyme genes (such as the normalization of the compensatory overexpressed *Sod3*) and metal ion transporters (such as *Slc11a1*) indicates a potential for comprehensive cellular rescue. This suggests that Pep2 might help restore cellular redox homeostasis to alleviate α -MSH-induced oxidative stress and could potentially disrupt the essential metal ion (e.g., copper) availability required for the maturation and catalytic activity of tyrosinase within melanosomes [71,72]. Taken together, these transcriptomic insights provide a holistic understanding of Pep2's putative multi-target mechanism, proposing synergistic actions primarily through cAMP-PDE axis modulation and classical gene suppression. However, as these inferences are primarily based on mRNA-level observations, further functional validations—such as quantifying intracellular cAMP levels and PDE protein expression—are warranted in future studies to definitively confirm these proposed regulatory axes.

4. Conclusion

This study systematically explored the anti-melanogenic potential of bioactive peptides from *T. obscurus* muscle proteins to develop natural, safe tyrosinase inhibitors. The key findings are summarized as follows:

Pepsin was identified as the optimal protease for generating tyrosinase inhibitory peptides, with its hydrolyzed fraction exhibiting a tyrosinase inhibition rate of $87.01\% \pm 2.64\%$ at 10 mg/mL—comparable to arbutin but with significantly lower cytotoxicity. Nano-LC–MS/MS and molecular docking identified five high-affinity peptides, among which Pep2 (LRVAPEEHPTL) showed the strongest *in vitro* tyrosinase inhibitory activity and anti-melanogenic effects in α -MSH-induced B16-F10 cells. Importantly, *in vivo*

zebrafish experiments further confirmed that the peptides could effectively reduce melanin accumulation while maintaining excellent biosafety, overcoming the toxicity limitations observed with arbutin.

Mechanistically, alongside direct tyrosinase inhibition, transcriptomic and PCR analyses revealed that Pep2 suppressed melanogenesis through a potential multi-target network. Pep2 downregulated the classical MC1R-Mitf-Tyr signaling axis and modulated the MAPK pathway. Furthermore, transcriptomic data suggested its intervention at novel targets, pointing towards the acceleration of cAMP clearance via the cAMP-PDE axis and the restoration of cellular redox balance. This study realizes the high-value utilization of underutilized *T. obscurus* protein resources and establishes a robust workflow for screening aquatic organisms-derived bioactive peptides (Fig. 8).

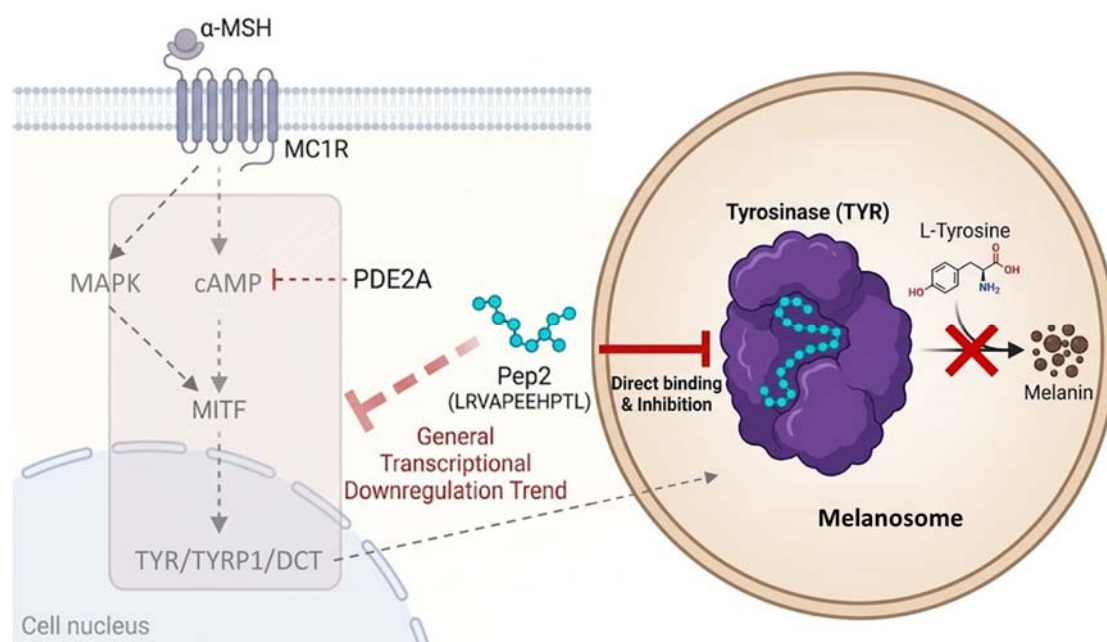


Figure 8. Schematic diagram of the proposed anti-melanogenic mechanisms of bioactive peptides derived from *T. obscurus*. The illustration depicts a multi-target network through which the derived peptides (e.g., Pep2) suppress melanogenesis. The solid line highlights the primary confirmed mechanism: direct binding and inhibition of tyrosinase (TYR) catalytic activity within the melanosome, which completely blocks the conversion of L-tyrosine to melanin. Dashed lines represent classical signaling pathways and transcriptional regulation. α -MSH drives melanogenesis via the MC1R-mediated cAMP and MAPK pathways, leading to MITF activation and the subsequent expression of downstream melanogenic enzymes (TYR/TYRP1/DCT). The broad dashed line indicates a general transcriptional downregulation trend exerted by Pep2 on this classical cascade. Furthermore, transcriptomic insights suggest the involvement of the cAMP-PDE axis, where PDE2A accelerates cAMP degradation to further blunt the upstream melanogenic signal.

Notably, the exact post-transcriptional mechanisms, particularly the specific phosphorylation levels of ERK and other key proteins in the MAPK pathway, require further comprehensive verification via Western blotting to fully solidify the regulatory network. Additionally, the proposed novel pathways, such as cAMP degradation, necessitate future functional assays (e.g., intracellular cAMP quantification) for definitive confirmation. Furthermore, while preliminary *in vivo* efficacy of the hydrolysates was demonstrated in zebrafish, the *in vivo* performance of the pure synthetic Pep2—particularly its gastrointestinal stability for functional foods and transdermal absorption for cosmetic applications in mammalian models—requires further systematic investigation. Furthermore, while this study successfully identified optimal proteases and peptides under fixed baseline conditions, systematic optimization of hydrolysis parameters (such as time,

temperature, and substrate ratio) using response surface methodology (RSM) will be conducted to maximize peptide yield. Future research will focus on these aspects, along with structural modification and synergistic effects with other ingredients, to promote its application in food, cosmetics, and pharmaceuticals.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data reported in this article will be shared upon reasonable request.

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