

Original Article

Pearl peptides (*Hyriopsis Cumingii*) as tyrosinase inhibitors: identification, characterization, inhibitory activity, conformational change and mechanism

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Abstract

Pearl protein extracted from freshwater mussels has been shown to inhibit tyrosinase activity. However, the unclear inhibitory mechanism and associated conformational changes limit their practical applications. In this study, peptides from the crude extract of pearl powder are analyzed via liquid chromatography-tandem mass spectrometry (LC-MS/MS). These peptides are subsequently assessed for their tyrosinase inhibitory potential via molecular docking techniques. Notably, experiments reveal that a small-molecule peptide effectively prevents the interaction between 3,4-dihydroxyphenylalanine (L-DOPA) and tyrosinase, impeding the formation of dopaquinone. The peptide GLGGGLAGAGDGA (95P, 1099.54 Da) shows particular promise, with synthesized 95P inhibiting both the monophenolase and diphenolase activities of tyrosinase, with IC₅₀ values of 30.97 ± 0.75 μM and 64.62 ± 3.08 μM, respectively. 95P inhibits tyrosinase activity in a mixed competitive and reversible manner. Molecular dynamics simulations suggest that 95P stabilizes 5M8N, impeding structural contraction identified by model number 5M8N. In conclusion, 95P is a powerful and stable tyrosinase inhibitor with the potential to be used as a skin whitening agent in the pharmaceutical field and an anti-browning agent in the food industry.

Key words tyrosinase inhibitory, molecular dynamics simulations, enzyme kinetics

Introduction

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase, is an enzyme that is extensively found in microorganisms, animals, and plants. As the key catalyst for melanin biosynthesis, its dysregulation contributes to dermatological hyperpigmentation disorders, including freckles, chloasma, and senile plaques [1,2]. Moreover, abnormal melanin levels can cause the enzymatic browning of fruits and vegetables, affecting their sensory qualities and nutritional value [3,4]. Common tyrosinase inhibitors, including hydroquinone, ascorbic acid, arbutin, and kojic acid, are widely used in the pharmaceutical, food, and cosmetics sectors [5]. However, these inhibitors are associated with serious adverse effects, such as cytotoxicity, dermatitis, skin cancer, and neurological diseases [6,7]. Therefore, the search for new, safe, and naturally sourced tyrosinase inhibitors remains an essential challenge for these industries.

Tyrosinase inhibitors are broadly categorized into polyphenols,

quinones, terpenoids, alkaloids, heterocyclic compounds, polysaccharides, synthetic small molecules, peptides, metal complexes, nucleic acid aptamers, and nanocarrier systems. The mechanisms of action and critical analysis for each category are presented in Table 1. The advantages of natural products include biodegradability, environmental compatibility, and non-toxicity [25–27]. Among these, peptide inhibitors have been extensively studied because of their ultrahigh tyrosinase inhibitory activity and superior biosafety. For instance, peptides isolated from rice bran, one of which has the sequence "SSEYGGEGSSSEQGYGEG", demonstrated both copper chelating and tyrosinase inhibitory activities [28]. Deng *et al.* [29] identified two inhibitory peptides from Chinese papaya seed proteins, NYRRE and RHAKF, which also disrupt tyrosinase function.

The freshwater mussel (*Hyriopsis cumingii*), known for its economic value, contains pearls that are primarily composed of calcium carbonate, proteins, amino acids, trace elements, and

Table 1. Classification, mechanisms of action, and critical analysis of tyrosinase inhibitors

Category	Representative substances	Inhibitory mechanism	Advantage	Disadvantage	Reference
Polyphenols	Resorcinol, curcumin, resveratrol, <i>etc.</i>	Competitive inhibition	Ability to chelate Cu ²⁺ and induce conformational changes, high efficacy (IC ₅₀ down to μM level) Natural and safe	Poor stability (susceptible to oxidation), low water solubility	[8,9]
Quinones	Bis-thiourea derivatives, enzyme-catalyzed quinones, <i>etc.</i>	Allosteric inhibition, blocking substrate channels, precise feedback regulation	Precise feedback regulation	Potential cytotoxicity	[10,11]
Terpenoids	Citral, geraniol, arbutin, <i>etc.</i>	Non-competitive inhibition	Hydrophobic binding, excellent skin permeability, multi-component synergy	Low single-component potency (IC ₅₀ up to mM level)	[12,13]
Alkaloids	Berberine, caffeine, <i>etc.</i>	Mixed-type inhibition	Dual functionality (enzyme inhibition + free radical scavenging), indirect antioxidant effects	Moderate solubility issues, medium potency (IC ₅₀ >50 μM)	[14]
Polysaccharides	Lentinan, sulfated algal polysaccharides, <i>etc.</i>	Non-specific binding	Downregulate MITF signaling pathway, excellent biocompatibility	Weak inhibition (IC ₅₀ up to mM level)	[15,16]
Synthetic small molecules	Cefotaxime, kojic acid derivatives, <i>etc.</i>	Non-competitive allosteric inhibition	Block hydrophobic pockets, well-defined structures, high potency (IC ₅₀ down to μM level)	Potential side effects (e.g., antibiotic resistance)	[17,18]
peptides	Walnut polypeptides, egg-white peptides, <i>etc.</i>	Cu ²⁺ chelation, substrate channel blocking, conformational disruption	Ultra-high activity (IC ₅₀ down to μM level), high biosafety	Ultra-high activity (IC ₅₀ down to μM level), high biosafety	[19,20]
Heterocyclic compounds	Coumarin derivatives, thiazolinones, <i>etc.</i>	Reversible binding	Induce conformational unfolding, high efficacy (IC ₅₀ = 3–10 μM), easy structural optimization	Complex synthesis, partial cytotoxicity	[21,22]
Metal complexes	Cu(II)-Quercetin complex, Zn-Resveratrol complex, <i>etc.</i>	Enhanced Cu ²⁺ chelation	Synergistic effects (5–10 × activity boost)	Potential metal toxicity	[5]
Nucleic acid aptamers	Anti-tyrosinase DNA/RNA aptamers, <i>etc.</i>	High-specificity binding to allosteric sites	Precise targeting, programmable design	Poor <i>in vivo</i> stability, extremely high synthesis costs	[23]
Nanocarrier systems	Liposome-encapsulated polyphenols, peptide-loaded gold nanoparticles, <i>etc.</i>	Enhanced penetration, sustained release	Protect active ingredients, solve transdermal/stability issues, improved bioavailability	Complex manufacturing processes	[24]

vitamins. These components impart various health benefits, such as enhancing immunity, supplementing calcium, delaying aging, improving sleep, and supporting skin repair [30]. Pearl powder has been shown to inhibit melanin production and promote collagen cell regeneration, indicating its potential therapeutic applications [31]. Dai *et al.* [32] found that crude protein extract from pearls markedly stimulated fibroblast proliferation and collagen synthesis. Additionally, in studies employing mouse skin melanoma cells (B16F10) and L-DOPA as the tyrosinase substrate, Yang *et al.* demonstrated that pearl protein extract is non-toxic to B16F10 cells and effectively inhibits tyrosinase activity and melanin production [33,34]. This practical significance is particularly valuable given the demand for natural, non-toxic tyrosinase inhibitors to replace traditional synthetic agents which often have adverse side effects.

The objectives of this study were to identify bioactive peptides from pearl powder peptide extracts that significantly inhibit

tyrosinase activity and to investigate the mechanism of tyrosinase inhibition and its impact on the conformational changes of tyrosinase. The innovative aspects of this research lie in enhancing the extraction efficiency of pearl peptides and identifying a highly efficient natural peptide with potent tyrosinase inhibitory activity from the extracts, thereby improving the utilization of pearl powder. Furthermore, research on screening tyrosinase inhibitors from pearl powder has not been reported previously. Specifically, reverse-phase high-performance liquid chromatography (RP-HPLC) and gel permeation chromatography (GPC) were employed to determine the purity, molecular weight (Mw) distribution, and secondary structure of peptide extracts derived from pearl powder. The peptide 95P with the highest binding energy to 5M8N was screened by molecular docking. The inhibitory mechanism of 95P on tyrosinase was investigated through its enzyme kinetics, interaction with copper ions, and effect on the oxidation of L-DOPA. Fluorescence spectroscopy, circular dichroism analysis, and mole-

cular dynamics simulations were used to investigate the conformational changes of 95P-5M8N. These findings provide an important reference for the practical application of 95P in the later stage and provide a theoretical basis for the coupling of 95P with other drug modifications to further improve its activity.

Materials and Methods

Materials

Nanosized pearl powder was supplied by Zhejiang Fenix Health Science and Technology (diameter <100 nm; Zhuji, China). Tyrosinase (derived from *Agaricus bisporus*, EC 1.14.18.1, 128 kDa, 25 kU) was obtained from Sigma-Aldrich (St. Louis, USA). The product specifications were as follows: tyrosinase, Cas: 9002-10-2; product code: T3824; lyophilized powder, >1000 units/mg solid; and stored at -20°C. Mouse melanoma cells (B16F10) were acquired from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Fetal bovine serum (FBS) was obtained from Grand Island Biological (Thermo Fisher Scientific, Sydney, Australia). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco (Thermo Fisher Scientific). MTT, α -melanocyte-stimulating hormone (α -MSH), tretinoin, and kojic acid were purchased from Beyotime Biotechnology (Shanghai, China). Product specifications: MTT, product code: ST316, lyophilized powder, stored at 4°C. Product specifications: α -MSH, product code: Y211288, lyophilized powder, stored at -20°C. Product specifications: tretinoin, Cas: 302-79-4; product code: ST1627, lyophilized powder, stored at -20°C. Product specifications: kojic acid, Cas: 501-30-4; product code: Y263092, lyophilized powder, stored at 4°C.

Isolation and purification

Ten grams of pearl powder was suspended in 300 mL of ultra-pure water and stirred continuously for 10 h at 25°C. The mixture was then subjected to immersion in a 40 kHz ultrasonic water bath for 1 h. Subsequently, the supernatant was filtered through a 0.45- μ m filter, dialyzed (cutoff = 500 Da), and lyophilized. To analyze the structure of the samples, the products were separated via RP-HPLC and analyzed via a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, USA) with the detection method set to positive ion mode. Separation was performed on an Agilent PLgel 5 μ m MIXED-C column (300 $\times$ 7.5 mm) at 30°C with deionized water as the eluent (1.0 mL/min), with the detection wavelength set at 254 nm. The data were processed via MaxQuant software version 1.5.5.1 to perform protein identification and quantitative analysis against an appropriate database. To determine the molecular weight distribution of the sample, gel permeation chromatography (GPC) was conducted on a Waters1525 RP-HPLC system (Waters, Milford, USA), which employs a series of ultrahydrogel columns (500, 250, and 120 $\times$ 7.8 $\times$ 300 mm) (Waters). The mobile phase (0.2 M NaNO₃ and 0.01 M NaH₂PO₄) was pumped at 1.0 mL/min. UV detection: 254 nm. Column temperature: 30°C. The peptide 95P used was chemically synthesized for experimental testing. 95P with a purity >96% was synthesized by AtaGenix Laboratories (Wuhan, China).

Cell viability assay (MTT assay)

B16F10 mouse melanoma cells were cultured in DMEM containing 10% FBS at 37°C in a 5% CO₂ constant-temperature incubator. The cells were inoculated at a density of 1×10^5 cells per well and cultured in 96-well plates for 24 h. To induce tyrosinase production,

0.1 μ M α -MSH was added, and the mixture was pretreated for 24 h. Subsequently, 95P (0, 10, 20, 30, 40 μ g/mL, dissolved in deionized water) or 1 mM tretinoin (positive control for the tyrosinase inhibitory activity assay) was added, and the mixture was incubated for 24, 48, and 72 h, respectively [35].

Cell viability was assessed via MTT assay [36]. At the end of the treatment, 10 μ L of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 h at 37°C in the dark. The medium was aspirated, 100 μ L of DMSO was added, and the absorbance was measured at 570 nm via a microplate reader (CLARIOstar Plus; Thermo Fisher Scientific, Waltham, USA). The cell viability (%) was calculated as follows:

$$Viability(\%) = \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100 \quad (1)$$

where OD_{control} represents the absorbance of cells treated with only α -MSH and 0.1% DMSO (0 μ g/mL compound) and where OD_{blank} represents the absorbance of a mixture of medium and MTT without cells. Data with a cell viability less than 90% were excluded (to avoid cytotoxic interference).

Tyrosinase activity assay

Tyrosinase activity was determined via a modified L-DOPA oxidation assay [37]. The cells treated with 95P and kojic acid as in the cell viability assay were washed twice with pre-cooled PBS and lysed for 30 min at 4°C with 100 μ L of lysis buffer. The lysate was centrifuged at 12,000 $\times$ g for 10 min at 4°C, and the supernatant was collected. Subsequently, 50 μ L of the supernatant was mixed with 50 μ L of 2 mM L-DOPA solution (dissolved in 50 mM phosphate buffer, pH 6.8) and incubated for 30 min at 37°C in the dark, after which the absorbance at 475 nm was measured via a microplate reader. Tyrosinase inhibition (%) was calculated as follows:

$$Inhibition(\%) = 1 - \frac{OD_{sample} - OD_{blank}}{OD_{negative\ control} - OD_{blank}} \times 100 \quad (2)$$

The OD_{negative control} was the absorbance of α -MSH-stimulated but uninhibited cells, and the OD_{blank} was the absorbance of the cell-free L-DOPA and lysate mixture.

Enzyme activity analysis

Monophenolase activity

Different concentrations of pearl peptide inhibitors (0, 10, 20, 30, 40, and 50 μ M) and 0.2 mg/mL of tyrosinase were added to deionized water. L-tyrosine (6 mM) was used as the substrate and dissolved in 0.05 mol/L (3 mL, pH 6.8) phosphate buffer. Various concentrations of 95P (0.1 mL) were added, followed by 2.85 mL of L-tyrosine and 0.05 mL of tyrosinase. The mixture was mixed immediately, and the final concentration of tyrosinase was 3.33 μ g/mL. The assay was performed at a UV wavelength of 475 nm, with the reaction proceeding for 10 min in a water bath set at 30°C. Monophenolase activity was determined by calculating the slope of the linear portion of the reaction curve, whereas the lag time was assessed by the intercept on the horizontal axis [38,39].

Diphenolase activity

Different concentrations of peptide inhibitors (0, 1, 2, 3, 4, 5, and 6 μ M) and 0.2 mg/mL tyrosinase were added to deionized water. Various concentrations of 95P (0.1 mL) were added, followed by 0.05 mL of tyrosinase and 2.85 mL of L-DOPA (50 mM L-DOPA solution with 50 mM phosphate buffer, pH 6.8). The mixture was

mixed immediately, and the final concentration of tyrosinase was 3.33 µg/mL. The assay was performed at a UV wavelength of 475 nm, with the reaction proceeding for 10 min in a water bath set at 30°C. The relative residual activity of the enzyme was plotted against the concentration of the inhibitor. The IC₅₀ value, the concentration at which 50% of the residual activity of the enzyme was inhibited, was determined. Kojic acid served as a positive control in this setup.

$$\text{Relative activity(\%)} = \left(\frac{B_2 - B_1}{A_2 - A_1} \right) \times 100\% \quad (3)$$

Here, A₁ and A₂ denote the absorbance readings for the blank at 0 and 10 min, whereas B₁ and B₂ represent the absorbance readings for the sample at the same time points.

Inhibitory type

Different concentrations of 95P (0, 10, 20, 30, 40, and 50 µM) and different concentrations of tyrosinase (0, 1.665, 3.33, 4.995, 6.66, and 8.325 µg/mL) were used for this experiment. L-DPOA (0.5 mM) was used as the substrate and dissolved in 0.05 mol/L (3 mL, pH 6.8) phosphate buffer. Various concentrations of 95P (0.1 mL) were added, followed by 2.85 mL of L-DOPA and 0.05 mL of tyrosinase. The reaction conditions were the same as those in monophenolase activity. This was done to examine the impact of different inhibitor concentrations on the initial rate of enzyme catalysis and then to determine the relationship between the enzyme concentration and the initial reaction rate [40].

Enzyme kinetics

Different concentrations of 95P (0, 20, 40, 60, and 80 µM) and 0.2 mg/mL tyrosinase were used for the experiment. L-DOPA at 0.25, 0.5, 1, 2, and 4 mM was used as the substrate and dissolved in 0.05 mol/L (3 mL, pH 6.8) phosphate buffer. Various concentrations of 95P (0.1 mL) were added, followed by 2.85 mL of L-DOPA and 0.05 mL of tyrosinase. The reaction conditions were the same as those in monophenolase activity [39]. Kojic acid served as a positive control in this setup. Employing the Lineweaver-Burk equation, the models for mixed inhibition are constructed as follows:

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_I} \right) \frac{1}{[S]} + \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_{IS}} \right) \quad (4)$$

$$\text{Slope} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_I} \right) \quad (5)$$

$$\text{intercept} = \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_{IS}} \right) \quad (6)$$

Where, K_m (Michaelis-Menten constant), V_{max} (maximal reaction velocity), and v (reaction velocity) are represented. The notation [S] and [I] denote the concentrations of the substrate and inhibitor, respectively. K_I (inhibition constant) represents the dissociation constant for the binding of an inhibitor to the free enzyme (E), forming the enzyme-inhibitor complex (EI). K_{IS} denotes the dissociation constant for the binding of an inhibitor (I) to the enzyme-substrate complex (ES). This reflects the ability of the inhibitor to bind to the ES complex.

Interaction with copper ions

The 95P solution was prepared with deionized water and mixed with different concentrations of CuSO₄, and the final concentrations of Cu²⁺ were 5 µM, 10 µM, 15 µM, and 20 µM. The concentration of 95P in the final reaction system was 20 µM, and the reaction system was incubated for 1 min at room temperature and scanned with a spectrophotometer in the range of 200–500 nm [1].

Fluorescence spectral analysis

A 2 mM 95P solution and 2.5 mL of tyrosinase solution (0.2 mg/mL) were used for the fluorescence spectral experiment, and different volumes of 95P solution were then added to the tyrosinase solution to obtain different concentrations of 95P (0, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, and 0.7 mM) mixed with tyrosinase. The excitation wavelength was set at 280 nm, and the bath temperatures were controlled at 298 K, 303 K, and 310 K, with a scanning wavelength range of 290 nm to 500 nm. Both the excitation and emission bandwidths were maintained at 2.5 nm [5,41]. The inner filter effect necessitated the adjustment of all the fluorescence readings via the following correction formula:

$$F_{\text{cor}} = F_{\text{cor}} e^{(A_1 + A_2)/2} \quad (7)$$

where F_{cor} represents the corrected fluorescence intensity and where F_{obs} denotes the initially measured fluorescence intensity. A₁ is the absorption value of the inhibitor at the excitation wavelength, and A₂ is the absorption value at the emission wavelength.

The Stern-Volmer formula was applied to calculate the K_{sv} and k_q values [42]:

$$\frac{F_0}{F} = 1 + K_{SV}[P] = 1 + k_q \tau_0 [P] \quad (8)$$

The apparent binding constant (K_b) was determined via the following equation [43]:

$$\log \frac{F_0}{F} = \log K_b + n \log [P] \quad (9)$$

where F₀ is the fluorescence intensity of tyrosinase or the inhibitor at baseline, and F is the respective fluorescence intensity in the presence of the inhibitor. K_b represents the apparent binding constant, K_{sv} represents the quenching constant, [P] represents the concentration of the inhibitor, τ₀ represents the average fluorescence lifetime of tyrosinase without a quenching agent (10⁻⁸ s), -k_q represents the quenching rate constant, and n represents the number of binding sites. The variable n indicates the number of binding sites involved.

Changes in thermodynamic parameters

Temperature influences the binding interactions between inhibitors and tyrosinase, and estimating thermodynamic parameters helps elucidate the nature of these interactions. The enthalpy (ΔH°) and entropy (ΔS°) serve as key indicators for gauging the binding strength in the tyrosinase-inhibitor reaction [5]. Moreover, the Gibbs free energy (ΔG°) is the principal indicator for determining the spontaneous nature of the reaction. The values of ΔH°, ΔS° and ΔG° were determined from the parameters (K_b and T) obtained via fluorescence spectroscopy. The following formula was used to calculate ΔH°, ΔS°, and ΔG° [44]:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (10)$$

$$\ln K_b = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (11)$$

In the equation, R denotes the gas constant with a value of 8.314 J/(mol·K), and T represents the temperature measured in Kelvin (K).

Molecular docking analysis

The crystal structure of the protein complex was retrieved from the RCSB Protein Data Bank (<http://www.rcsb.pdb>). The receptor domain encompasses the entirety of the target protein, identified by the PDB ID: 5M8N [3]. Pymol 0.99 (Schrodinger, San Diego, USA) was used to remove water molecules from the protein crystals,

and hydrogen was added. AutoDock Vina 4.2.6 was employed to conduct molecular docking simulations and determine the binding affinity of the peptide PP to the tyrosinase protein. The grid box dimensions were set to 47.25 Å × 47.25 Å, with coordinates on the x-axis at -25.082, the y-axis at 4.234, and the z-axis at 10.407, facilitating the calculation of the root mean square deviation (RMSD) for each compound with a docking grid spacing of 0.375 Å [4,45]. Discovery Studio Visualizer software was used to visualize the docking results.

Oxidation of L-DOPA influenced by 95P

The 3-mL reaction system consisted of L-DOPA (2.7 mL at 2.5 mM), an inhibitor solution (0.2 mL at 1 mM), and tyrosinase (0.1 mL at 0.2 mg/mL) and was mixed at room temperature. The samples were subsequently analyzed at wavelengths ranging from 400 nm to 700 nm at 0, 5, 10, 15, and 20 min, respectively. Kojic acid served as a positive control in this setup [41].

Circular dichroism spectroscopy

Circular dichroism spectra were obtained using a circular dichroism spectropolarimeter (Applied Photophysics Ltd., Leatherhead, UK) and a 1.0-mm quartz cuvette at 298 K. Circular dichroism solutions were made by combining a specific quantity of tyrosinase with anthraquinone. The measurements were taken at wavelengths ranging from 190 nm to 260 nm, with a scanning speed of 100 nm min⁻¹ and an interval of 1 nm. A solution of 285 µL of tyrosinase (0.01 mM) was incubated with 15 µL of 95P (0.01 mM, 0.02 mM, and 0.04 mM) at different concentrations (the 95P/tyrosinase molar ratios were 0:1, 1:1, 2:1, 4:1, and 1:0) at 25°C for 1 min, and the circular dichroism spectra were subsequently scanned at 25°C. The circular dichroism spectral data were smoothed and preprocessed via OMNIC software and saved for uploading at <http://dichroweb.cryst.bbk.ac.uk>, and the wave number range was selected from 190 to 240 nm and validated via the Contin algorithm to assess the relative content of the secondary structure [3].

Molecular dynamics simulations

Molecular dynamics simulations were performed via GROMACS 2016 software (<https://www.gromacs.org>; developed by the GROMACS development team at schools and universities worldwide). The force field interactions between the ligand and protein were modeled via the Charmm36 force field alongside the CHARMM General Force Field (CGenFF). A standard cubic box with 8-nm sides was constructed around the protein 5M8N. The peptide was randomly placed inside the predefined box. To replicate the behavior of water molecules, the Transferable Intermolecular Potential 3 Point (TIP3P) model was utilized. Ionic balance within the system was achieved by adding Na⁺ and Cl⁻ ions. The pressure of the system and temperature were regulated at 1 bar and 297 K via the V-rescale and Parrinello-Rahman methods, respectively. An equilibration process spanning 150 ps was conducted under NVT (constant number of particles, volume, and temperature) and NPT (constant number of particles, pressure, and temperature) ensembles [46,47].

Statistical analysis

The mean and standard deviation (SD) were calculated on the basis of three individual measurements ($n = 3$). The data were analyzed

via GraphPad Prism (GraphPad Software, La Jolla, USA).

Results

Purity assessment and structural characterization of pearl peptides

The yield of pearl peptides extracted *via* water was $0.52 \pm 0.11\%$ (520 mg/kg), which was notably higher than the 0.23% yield reported by Zhang *et al.* [30]. As depicted in Figure 1A and B, the refractive index chromatogram and ultraviolet-visible chromatogram from RP-HPLC exhibited distinct signal peaks at 11.68 min (96.72% area) and 11.53 min, respectively. While weak absorption signals were present from 5.79–10.63 min in the UV chromatogram, there were negligible absorption peaks in the differential chromatogram, indicating the presence of minimal protein in the outflow samples. The above results corresponded to $93.07 \pm 1.26\%$ of the detection results of the protein kit. Furthermore, GPC analysis revealed that peptides less than 5000 Da constituted 99.38% of the total peptides (Figure 1C). LC-MS/MS analysis identified a total of 178 unique peptides. Seven peptides that demonstrated the most favorable docking results contained 13–15 amino acid residues, with molecular weights ranging from 905.40 to 1532.92 Da (Supplementary Table S1).

95P-5M8N binding interactions analysis

The peptide bound to 5M8N in a variety of binding poses and with different binding energies, but the binding position was always in the active pocket of 5M8N. The sequence Gly-Leu-Gly-Gly-Gly-Leu-Ala-Gly-Ala-Gly-Gly-Ala-Asp-Gly-Ala had the best affinity of -9.8 kcal/mol (GLGGGLAGAGGADGA, 1099.54 Da, referred to as 95P) (Figure 1D). Interactions between the ligand 95P and the amino acid residues of the receptor 5M8N, including its secure binding within the hydrophobic pocket of 5M8N, are illustrated in Figure 2A and B. Higher binding energy values typically suggest better receptor-ligand affinity. Additionally, as demonstrated in Figure 2C, the main forces driving the binding of 95P to 5M8N were hydrogen bonding, van der Waals forces, electrostatic interactions, and hydrophobic effects. In total, 95P interacted with 27 amino acid residues of 5M8N, forming eleven hydrogen bonds-six conventional (Thr391, Tyr348, Asn350, and Gly388) and five carbon-hydrogen bonds (Glu210, Gly388, His215, and His377). Positively charged residues (His215, His377, and Arg321) and negatively charged residues (Glu210) may exert electrostatic influences, affecting ligand binding [12].

95P inhibits tyrosinase activity in B16F10 cells

MTT assay was employed to assess the cytotoxic effects of 95P (a synthetic peptide) on B16F10 cell viability (Figure 2D). Although a statistically significant reduction ($P < 0.01$) was observed at higher concentrations, cell viability remained above 90% in all cases, indicating low cytotoxicity. A tyrosinase activity assay confirmed the anti-tyrosinase properties of 95P (Figure 2E), as evidenced by a 58.33% increase in tyrosinase production following treatment with 0.1 µM α-MSH alone. The intracellular tyrosinase activity in cells was significantly suppressed by 95P supplementation. The tyrosinase activity in the untreated blank group was set to 100%. Compared with the blank group (activity: $158.33 \pm 5.77\%$), the groups treated with 10 µg/mL, 20 µg/mL, 30 µg/mL, and 40 µg/mL 95P presented reductions in activity of $14.90 \pm 5.77\%$, $25.65 \pm 1.71\%$, $27.77 \pm 1.72\%$, and $56.42 \pm 1.61\%$, respectively. The

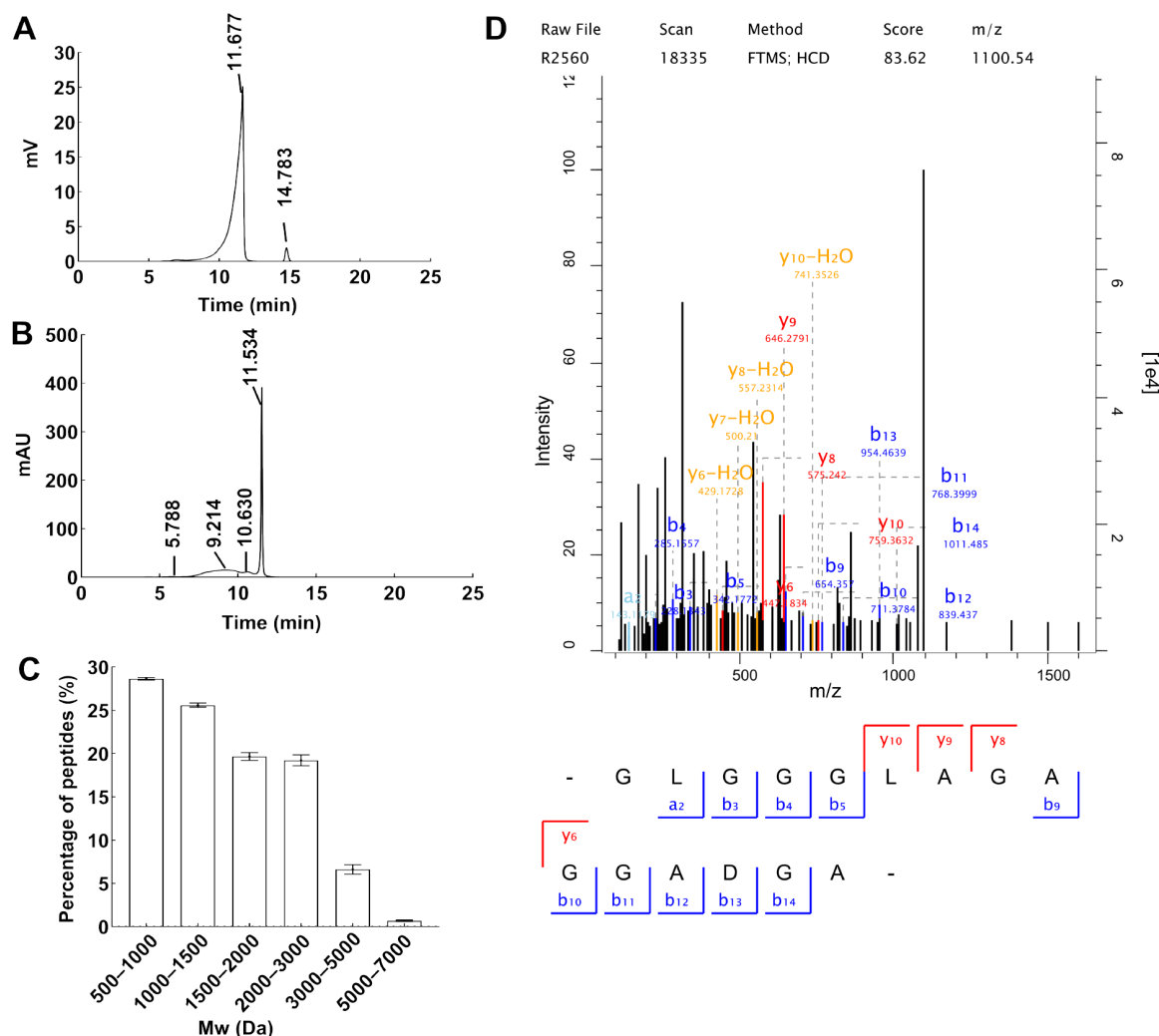


Figure 1. Composition analysis of PP and structural characterization of 95P (A) UV chromatography of PP was performed *via* RP-HPLC. (B) Differential chromatogram of PP identified *via* RP-HPLC. (C) The Mw distribution of PP was detected by GPC. (D) Q Exactive LC-MS/MS chromatogram of 95P.

tyrosinase activity of 95P (40 $\mu\text{g/mL}$) was $69.00 \pm 3.61\%$, which was not significantly different from the $68.22 \pm 1.36\%$ of the positive control kojic acid. The above results indicated that 95P can inhibit tyrosinase activity and is non-toxic, allowing for further research.

Effects of 95P on monophenolase and diphenolase activities

L-tyrosine served as the substrate, and various concentrations of 95P functioned as inhibitors to assess the inhibitory effect of 95P on tyrosinase monophenolase activity. As depicted in Figure 3A, the product concentration increased with reaction time, and the IC_{50} value for monophenolase was determined to be $30.27 \pm 1.59 \mu\text{M}$. Furthermore, increasing concentrations of 95P extended the lag phase and diminished the steady-state velocity of tyrosinase. By utilizing L-DOPA as a substrate and kojic acid as a positive control, Figure 3B and C confirmed that 95P significantly hindered tyrosinase diphenolase activity, achieving an IC_{50} value of $2.05 \pm 0.07 \mu\text{M}$, which was considerably less than that of the control ($38 \pm 4.06 \mu\text{M}$).

Reversibility and inhibition type of tyrosinase by 95P

The reversibility and inhibition type of 95P were analyzed as follows: an increase in 95P concentration resulted in all five lines passing through the origin with diminished slopes, demonstrating the reversible inhibition of tyrosinase by 95P (Figure 3D). This indicated that 95P restricted the activity of the enzyme rather than reducing the quantity of the enzyme [41,48]. A Lineweaver-Burk plot was used to analyze the inhibition type of 95P on bisphenolase activity. As presented in Figure 3E (I), the plot of $1/v$ versus $1/[S]$ yielded five linear lines with varying slopes intersecting in the second quadrant, implying that the inhibition of tyrosinase by 95P was a mixed-type inhibitor. In other words, 95P can bind to both the free enzyme and the enzyme-substrate complex, thereby competing with L-DOPA for tyrosinase binding [49,50]. The consistent linear relationship between the slope and the Y-intercept demonstrated in Figure 3E (II)–(III) indicated that 95P likely interacted with the same family of binding sites or possessed a single binding site on tyrosinase. This finding aligns with the studies by Nie *et al.* and Yan *et al.* [50,51]. On the basis of slope and Y-intercept analysis, the K_i and K_{is} values for 95P were determined to be $0.036 \pm 0.002 \text{ mM}$ and

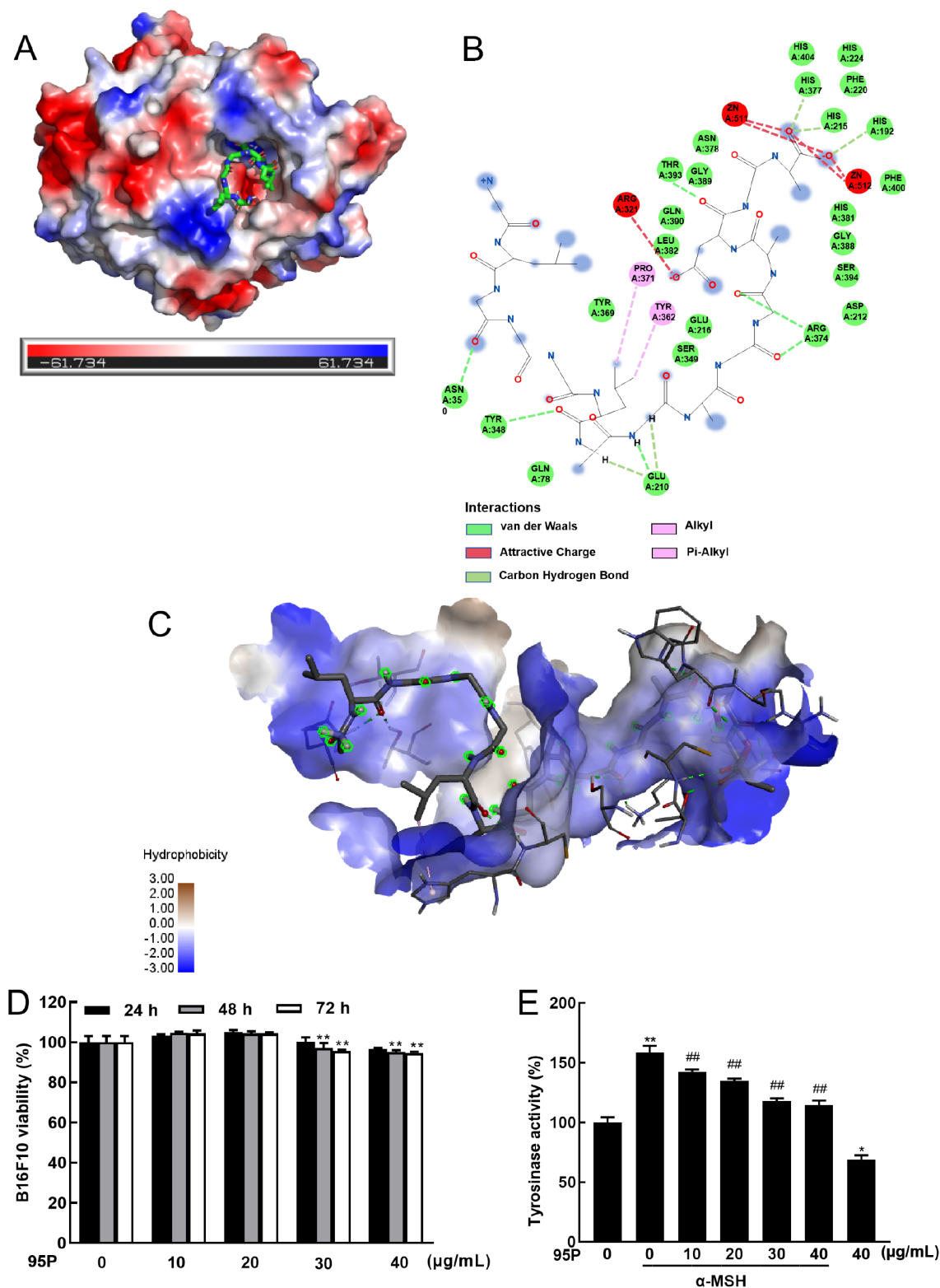


Figure 2. Molecular docking analysis of the interaction between 5M8N and the inhibitor (A) Three-dimensional (3D) structure surface model. (B) Two-dimensional structure (2D) showing the interaction between 95P and amino acids. (C) The binding of the 95P and 5M8N hydrophobic interfaces. 95P inhibited melanogenesis activated by α -MSH in B16F10 melanocytes. (D) The impact of 95P (0–40 μ g/mL, 24, 48, and 72 h) on B16F10 cell viability. (E) Tyrosinase activity was quantified by pretreating B16F10 cells with 95P (0–40 μ g/mL), followed by treatment with α -MSH (0.1 μ M, 24 h). Kojic acid (1 mM) was used as a positive control. The means $\pm$ SD ($n=3$) were used to express the results. ANOVA was employed to determine the statistical significance. * $P < 0.05$ and ** $P < 0.01$ compared with untreated control cells, # $P < 0.05$ and ### $P < 0.01$ compared with α -MSH-stimulated cells.

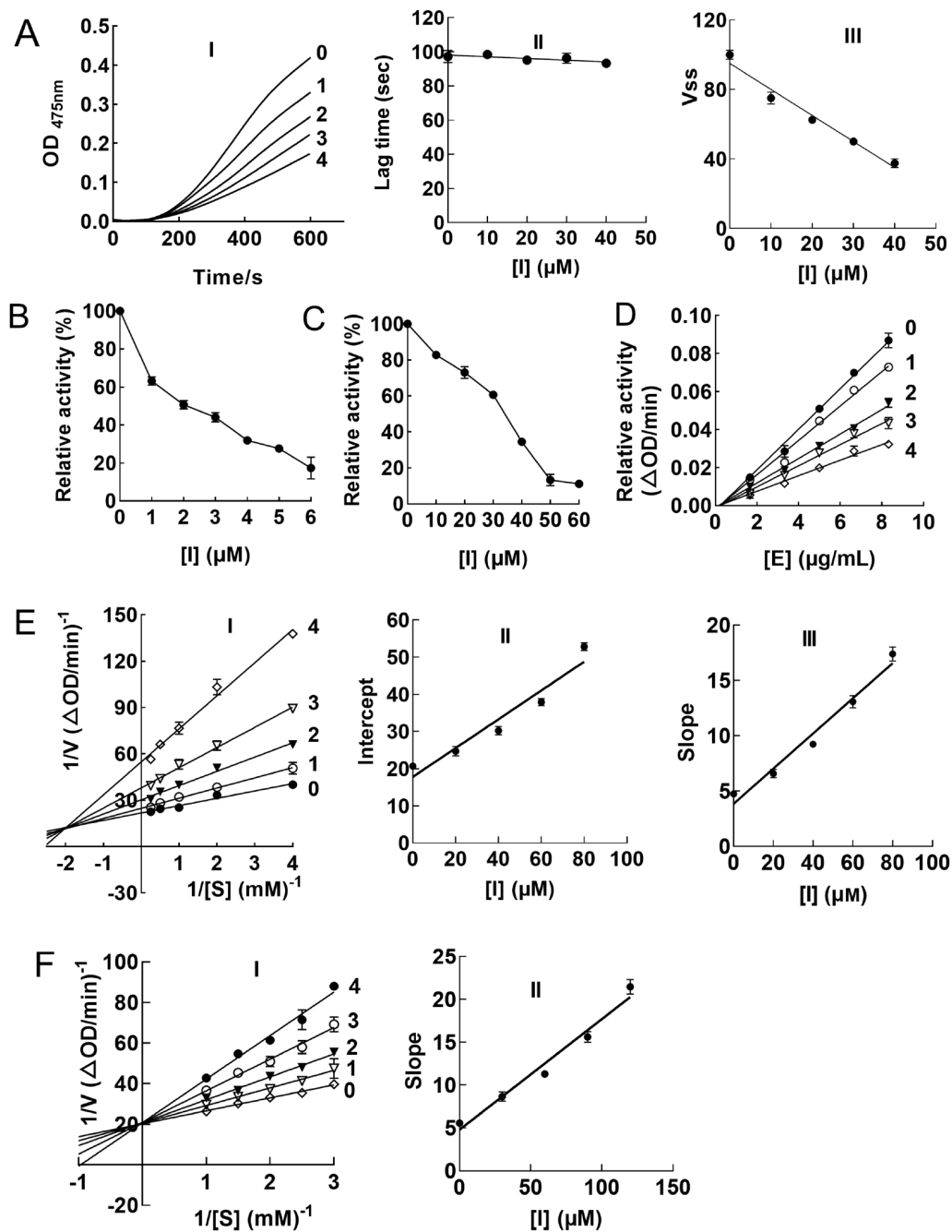


Figure 3. Tyrosinase monophenolase and diphenolase activities, reversibility and inhibition type of 95P (A) Tyrosinase monophenolase activity of 95P. (I) Progress curve for the oxidation of L-tyrosine by the enzyme. c (L-tyrosine) = 6 mM; c (tyrosinase) = 0.2 mg/mL; c (95P) = 0, 10, 20, 30, 40, and 50 μ M for curves 0 $\rightarrow$ 4. (II) Effects of 95P on the lag time of tyrosinase. (III) Effects of 95P on the steady-state rate of monophenolase. (B) Tyrosinase diphenolase activity of 95P. c (tyrosinase) = 0.2 mg/mL, c (L-DOPA) = 0.5 mM; c (95P) = 0, 1, 2, 3, 4, 5, and 6 μ M, respectively. (C) Tyrosinase diphenolase activity of kojic acid. c (kojic acid) = 0, 10, 20, 30, 40, 50, and 60 μ M, respectively. (D) Identification of the inhibitory mechanism of 95P. c (tyrosinase) = 0, 1.665, 3.33, 4.995, 6.66, and 8.325 μ g/mL, respectively. c (L-DOPA) = 0.5 mM; c (95P) = 0, 10, 20, 30, 40, and 50 μ M for curves 0 $\rightarrow$ 4. (E) Identification of the inhibitory type of 95P. (I) Diphenolase activity plotted via Lineweaver-Burk plots. (II) Plot of slope versus the concentration of 95P for calculating the inhibition constant K_i . (III) Plot of the intercept versus the concentration of 95P for determining the inhibition constant K_{is} . c (tyrosinase) = 0.2 mg/mL; c (L-DOPA) = 0.25, 0.5, 1, 2, and 4 mM, respectively. c (95P) = 0, 20, 40, 60 and 80 μ M for curves 0 $\rightarrow$ 4, respectively. (F) Identification of kojic acid's inhibitory effect. (I) Diphenolase activity plotted via Lineweaver-Burk plots. (II) Plot of the intercept versus the concentration of 95P for determining the inhibition constant K_{is} . c (tyrosinase) = 0.2 mg/mL; c (L-DOPA) = 0.33, 0.40, 0.50, 0.67, and 1.00 mM; c (kojic acid) = 0, 30, 60, 90, and 120 μ M for curves 0 $\rightarrow$ 4, respectively.

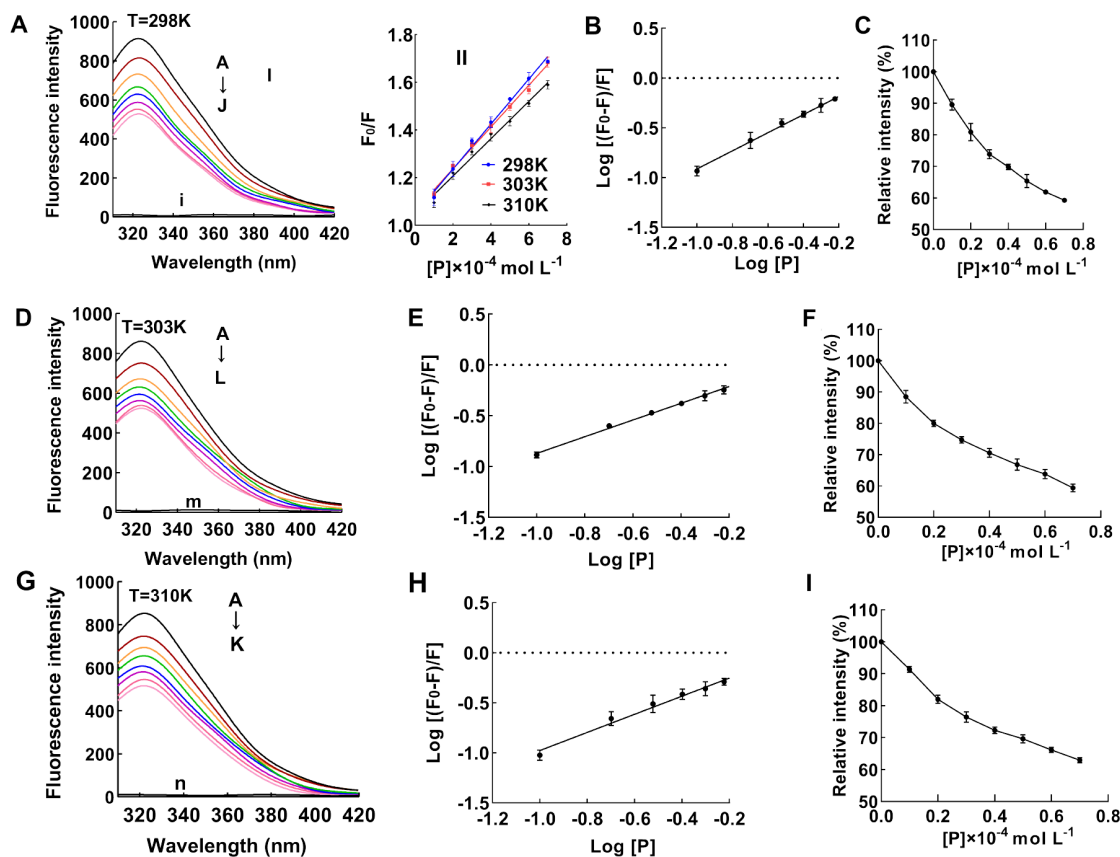


Figure 4. Determination of fluorescence quenching and thermodynamic parameters (A) Fluorescence spectrum of tyrosinase and/or 95P ($T = 298$ K). (B) Plot of $\log [(F_0 - F)/F]$ against $\log [Q]$ at 298 K. c (tyrosinase) = 0.02 mg/mL, and c (95P) = 0, 1, 2, 3, 4, 5, 6, and 7×10^{-4} mol/L for curves A $\rightarrow$ J, A $\rightarrow$ L and A $\rightarrow$ K, respectively; curves i, m and n solely show the 95P emission spectrum. c (95P) = 1 mM. Stern-Volmer plots of tyrosinase fluorescence quenching by 95P. (C) Changes in the maximum fluorescence intensity at 298 K. (D) Fluorescence spectrum of tyrosinase and/or 95P ($T = 303$ K). (E) Plot of $\log [(F_0 - F)/F]$ against $\log [Q]$ at 303 K. (F) Changes in the maximum fluorescence intensity at 310 K. (G) Fluorescence spectrum of tyrosinase and/or 95P ($T = 310$ K). (H) Plot of $\log [(F_0 - F)/F]$ against $\log [Q]$ at 310 K. (I) Changes in the maximum fluorescence intensity at 310 K.

0.102 ± 0.08 mM, respectively. Remarkably, the K_i value for 95P was lower than that of kojic acid (0.067 ± 0.003 mM) (Figure 3F).

Analysis of the change in tyrosinase conformation based on fluorescence

As depicted in Figure 4A, a pronounced fluorescence emission peak appeared at 322 nm in the tyrosinase solution. As the 95P concentration increased, the intensity of tyrosinase fluorescence decreased, although the time of peak emergence remained relatively stable. This suggested that 95P may induce a change in the conformation of tyrosinase [52,53]. The fluorescence quenching mechanism typically comprises static and dynamic quenching. This study conducted fluorescence experiments at temperatures of 298 K (Figure 4A–C), 303 K (Figure 4D–F), and 310 K (Figure 4G–I) to differentiate these quenching types. The Stern-Volmer plots revealed a single quenching mechanism with a robust linear relationship within the 0–0.7 mM concentration range. The decreasing K_{sv} values with increasing temperature led to the classification of tyrosinase quenching by 95P as static (Table 2). The K_{sv} values decreased with increasing temperature, indicating that the stability of the complex decreased with increasing temperature. The k_q values obtained at 298 K, 303 K, and 310 K were $9.45 \pm 0.01 \times 10^{12}$ L/mol, $8.76 \pm 0.01 \times 10^{12}$ L/mol, and $7.83 \pm$

0.01×10^{12} L/mol, respectively, all exceeding the maximum collision quenching constant (2.0×10^{10} L/mol/S) for various quenchers and biopolymers, further attesting to the static nature of quenching by 95P [54]. The n values at all three temperatures were close to 1 (Table 2), indicating that the interaction between them occurred through a specific site [55,56].

Oxidation of L-DOPA influenced by 95P

In this study, the spectral data of L-DOPA and tyrosinase solutions are shown in Figure 5A, revealing an absorbance of 1.40 at 475 nm (P_{475}) after 20 min. The optimal absorbance of dopaquinone was concentrated at approximately 475 nm, and the reaction system of tyrosinase, L-DOPA, and inhibitors usually stabilized at 20 min. The scanned spectra of the reaction system after the addition of kojic acid and 95P are displayed in Figure 5B and C, respectively. As the reaction time increased, the absorbance at P_{475} decreased to 0.62 and 0.51 after 20 min, respectively. The reduction in absorbance suggested that the yield of the final oxidation product was substantially lower with 95P than without 95P, indicating that 95P inhibited tyrosinase activity, thus partially inhibiting the oxidation of L-DOPA and finally hindering the formation of dopaquinone.

Full-wavelength scanning (200–500 nm) was used to detect the

Table 2. Effect of 95P on the binding constants, binding sites and thermodynamic parameters at 298 K, 303 K, and 310 K

T (K)	K_{SV} ($\times 10^4$ L/mol)	R^a	k_q ($\times 10^{12}$ L/mol·s)	K_b ($\times 10^3$ L/mol)	n	R^b	ΔH° (kJ/mol)	ΔG° (kJ/mol)	ΔS° (J/mol·K)
298	9.45±0.01	0.9927	9.45±0.01	1.01±0.05	0.91±0.018	0.9959	15.52±0.25	-39.47±0.23	184.53±0.15
303	8.76±0.01	0.9942	8.76±0.01	1.12±0.08	0.93±0.033	0.9968	/	-40.73±0.37	185.64±0.42
310	7.83±0.01	0.9861	7.83±0.01	1.18±0.12	0.91±0.025	0.9851	/	-41.98±0.17	185.48±0.20

interaction between 95P and copper ions (Figure 5D). The spectral impact of the inhibitor on the Cu²⁺ solution was inferred from the redshift or blueshift observed when the copper ion formed a complex with the inhibitor, which is a characteristic feature of such chelation products. The peak absorption wavelength shifted from 214 nm to 228 nm as the Cu²⁺ concentration reached 20 mM

(redshift), suggesting that 95P can disrupt the structure and function of the catalytic site of tyrosinase through copper chelation, thereby reducing the catalytic efficiency of tyrosinase.

Effect of 95P on the secondary structure of tyrosinase
To further reveal the effect of 95P on the conformation of tyrosinase,

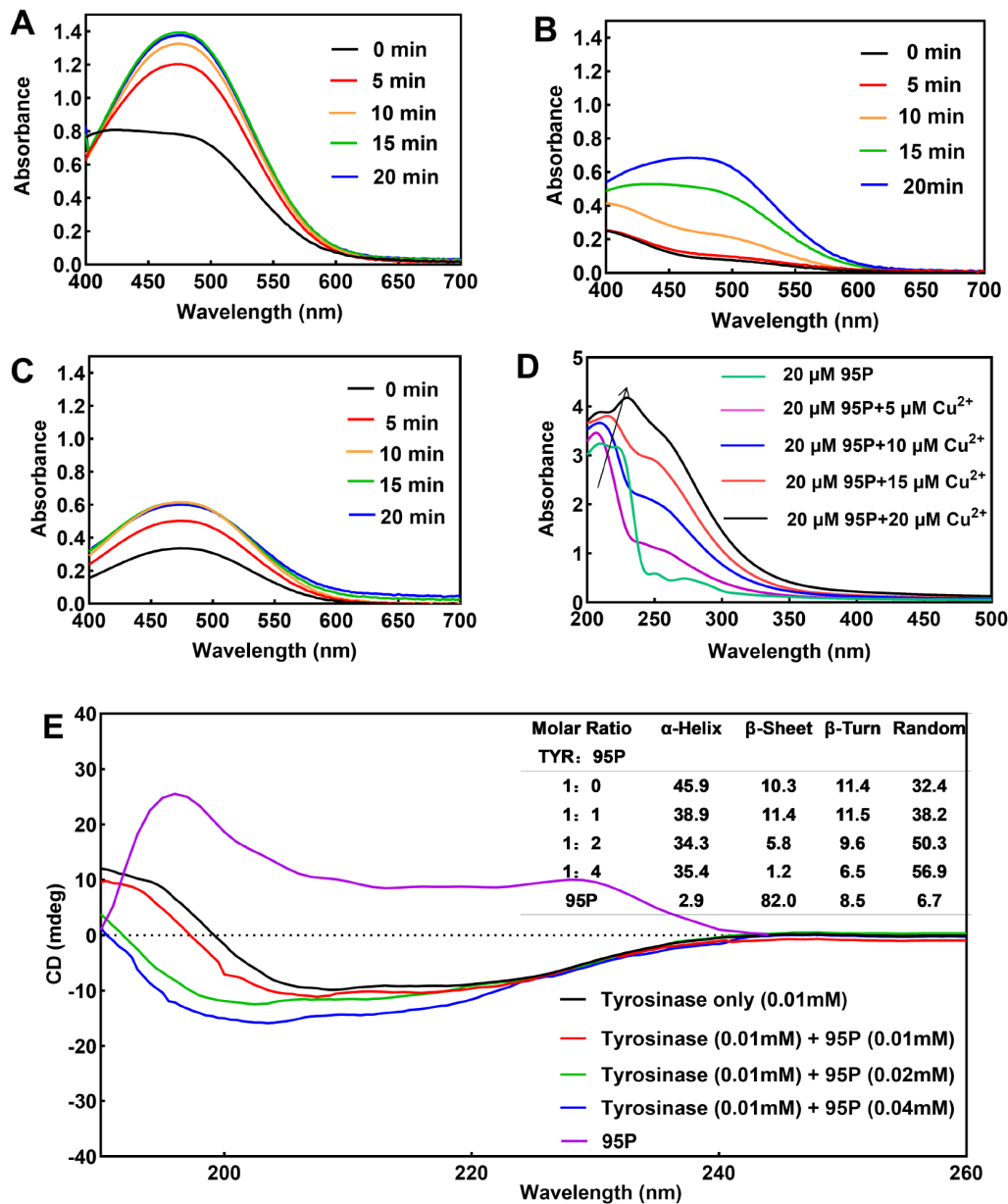


Figure 5. Changes in the spectra obtained during the oxidation of L-DOPA with 95P by tyrosinase and the copper chelation activity of 95P (A) L-DOPA reacted with tyrosinase. (B) Kojic (1 mM) acid was added to the reaction mixture of L-DOPA with tyrosinase. (C) 95P (1 mM) was added to the reaction mixture of L-DOPA with tyrosinase. (D) UV-vis spectrum of the compound produced by adding different amounts of CuSO₄ to 95P. (E) Circular dichroism spectra of tyrosinase in the absence and presence of 95P.

we measured the circular dichroism spectra of tyrosinase both in the absence and presence of 95P, and the results are shown in [Figure 5E](#), where tyrosinase showed two significant negative peaks at 208 and 222 nm, which suggested that tyrosinase possessed many α -helices in its structure. This result was confirmed by the crystal structure of tyrosinase [3]. However, upon the addition of 95P, the shape of the circular dichroism spectrum of tyrosinase changed significantly, indicating a change in its secondary structure. The calculation of the relative content of the secondary structure was based on the methods of Zeng and Cen [3,57]. As the concentration of 95P increased from 0.01 mM to 0.04 mM, the content of α -helices in tyrosinase decreased from 45.9% to 35.4%, the content of β -sheet decreased from 10.3% to 1.2%, and the content of random coils increased from 32.4% to 56.9%. These findings provide strong evidence that 95P altered the tyrosinase structure and inhibited its activity.

Thermodynamic parameters

The interaction forces between small molecules and biomolecules are often characterized by thermodynamic parameters, including enthalpy (ΔH), entropy (ΔS), and free energy change (ΔG) [57]. Ross and Subramania established a correlation between the signs of thermodynamic parameters and the possible interactions: (A) When $\Delta H < 0$ and $\Delta S < 0$, Van der Waals force and hydrogen bonds constitute the main factors; (B) when $\Delta H > 0$ and $\Delta S > 0$, the binding is formed mainly by hydrophobic interactions; (C) if $\Delta H < 0$ and $\Delta S > 0$, the charge interaction is the main factor. The changes in ΔH , ΔS , and ΔG for the interaction of 95P with tyrosinase are listed in [Table 2](#). The van't Hoff equation was fitted to the different values of K and T in [Table 2](#) of the fluorescence test, which in turn gave values of ΔH and ΔS ([Supplementary Figure S1](#)). When both ΔH and ΔS are positive, hydrophobic interactions are predominant in the binding of 95P to tyrosinase. The negative change in ΔG suggested that the binding of 95P with tyrosinase was spontaneous [40]. The term “entropy-driven” referred to a molecular binding process that occurred spontaneously ($\Delta G < 0$), primarily driven by entropy gain ($\Delta S > 0$, endothermic) rather than being dominated by enthalpy changes ($\Delta H < 0$, exothermic). Spontaneous binding is a core characteristic of drug molecules for achieving efficient and stable interactions. The advantages of the development of tyrosinase inhibitors include rapid onset, long-lasting inhibition, low dosage requirements, and high safety profiles [8,10]. Combined with the properties of static quenching and single binding sites, these findings could further optimize drug design and advance its applications in fields such as dermatological therapy and the food industry.

Analysis of the binding mechanism and stability of 95P-5M8N

The stability and mechanism of action of 95P binding to 5M8N were investigated via molecular dynamics simulations. RMSD, RMSF, and Rg represent the root mean square deviation, root mean square fluctuation, and radius of gyration, respectively, which are the parameters used in molecular simulations to describe the structural and dynamic properties of molecular simulations [4]. In this study, the molecular mechanics generalized Born surface area (MM-PBSA) method was used to calculate the free energy of binding between 95P and 5M8N, and the results are listed in [Supplementary Table S2](#). Comparing the measured values with the fluorescence binding

energies (-39.47 ± 0.23 kJ/mol to -41.98 ± 0.17 kJ/mol), the calculated binding energies were closer to the actual values (-129.43 ± 29.28 kJ/mol), with electrostatic interactions (-1877.35 ± 100.15 kJ/mol) being the binding energy that dominated. The polar solvation energy (2059.96 ± 94.53 kJ/mol) had a strong shielding effect on the electrostatic interaction. In the MM-PBSA analysis, the negative values of van der Waals forces (-271 kJ/mol) and SASA energy (-41 kJ/mol) supported the hydrophobic effect. $\Delta S > 0$ indicated that the binding was primarily entropy driven (dominated by the hydrophobic effect), whereas $\Delta H > 0$ suggested a weak net enthalpic contribution from direct intermolecular interactions (van der Waals forces, hydrogen bonding) or the need to overcome energy barriers [46,58]. In summary, the binding of 95P to 5M8N was primarily entropy driven (hydrophobic effect dominated), supplemented by van der Waals forces and electrostatic interactions.

The root mean square deviation (RMSD) is used to measure the degree of change in the molecular conformation, indicating the deviation between the molecule and the reference or the average conformation. A smaller RMSD indicates a more stable molecular conformation that is closer to the reference or average conformation [58]. The RMSD results for the 5M8N and 5M8N-95P complexes are shown in [Figure 6A](#). The RMSD values for 5M8N tended to increase from 1–15 ns and fluctuated within the 15–36 ns range. The overall trend indicated minimal change in the RMSD (< 0.1 nm), indicating that the structural configuration of 5M8N remained stable [59]. The 5M8N-95P complex displayed a similar trend, with the fluctuation range of RMSD values narrowing to less than 0.05 nm between 28–38 ns, which suggested that 95P might restrict the conformational freedom of 5M8N. These observations suggest that a relatively stable composite system formed between 5M8N and 95P.

The root mean square fluctuation (RMSF) reflects the fluctuation amplitude of the individual atoms or groups in the molecule, namely, their variation range in the time series. The RMSF determines which parts are more flexible and which parts are relatively fixed, thus providing important information for understanding the dynamic behavior of molecules [45]. Higher RMSF values in amino acids typically reflect increased flexibility during the binding process. The effect of 95P on amino acid residues was assessed via RMSF values. In agreement with the RMSD findings, the RMSF values for 5M8N were low (< 0.3 nm), indicating stability. [Figure 6B](#) reveals that the most dynamic amino acids were located at the protein extremities (20–80 and 465–470). Notably, the interaction with 95P led to an overall decrease in the RMSF values for the initial 15 ns. Moreover, residues such as Arg49, Pro50, His51, and Pro52 experienced substantial reductions in RMSF values (by more than 0.12 nm). These changes likely resulted from specific and hydrophobic interactions between the 95P and 5M8N residues, combined with the formation of hydrogen bonds with the hydrophilic residues of 5M8N, causing structural alterations. Consequently, it was hypothesized that these residues serve as binding sites for the 5M8N-95P interaction.

The radius of rotation (Rg) describes the degree of compactness or size of the molecule. A smaller Rg value indicates a more compact molecule, whereas a larger Rg value indicates a more extended molecule [58]. This study used the Rg value to assess the influence of 95P on the structural integrity of 5M8N. As shown in [Figure 6C](#) 5M8N contracted gradually in an aqueous environment. Between 5–25 ns, the Rg value for the 5M8N-95P complex was lower than

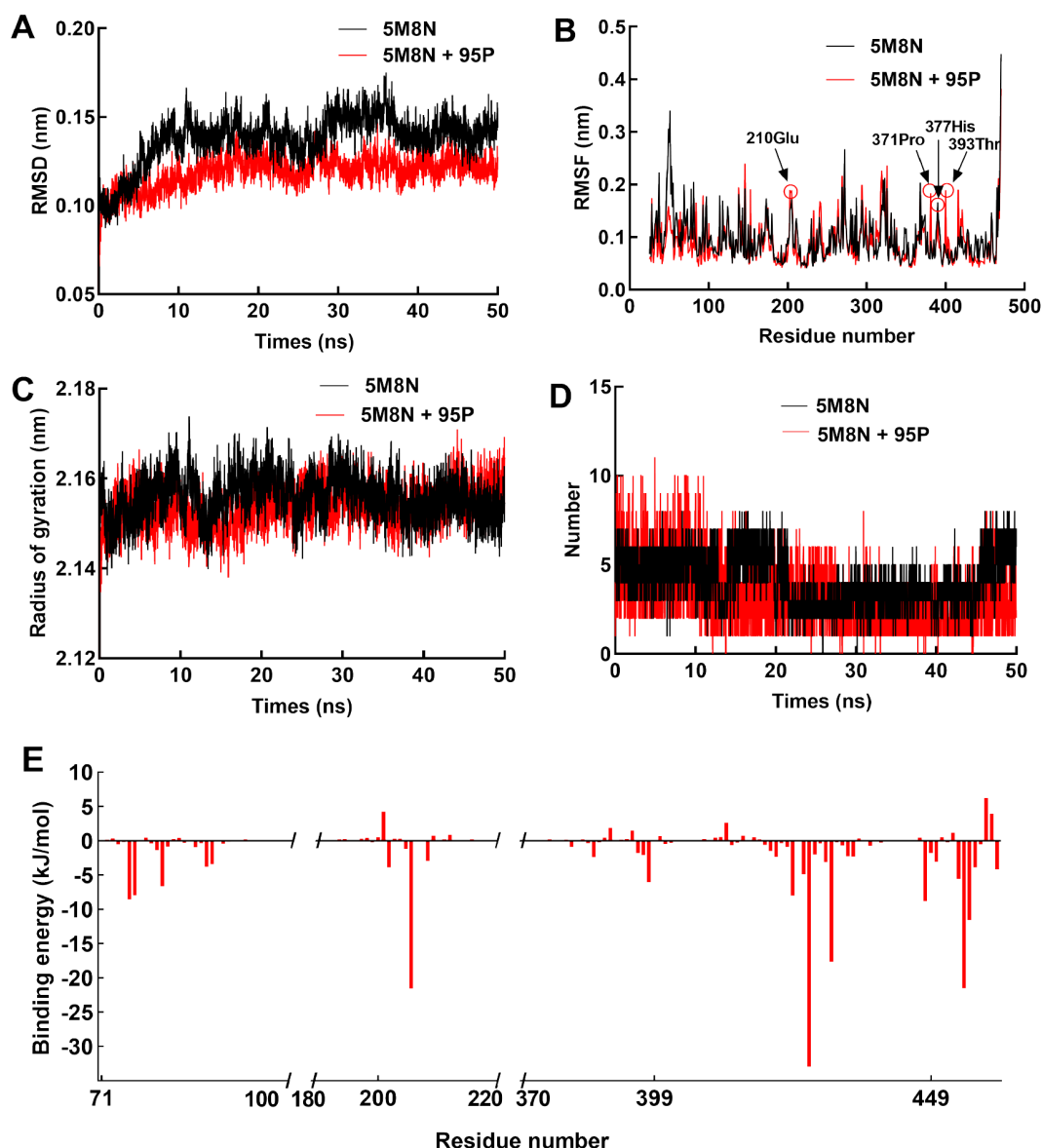


Figure 6. Molecular dynamics simulation analysis (A) The time-varying RMSD values of 5M8N and/or 95P. (B) RMSF values of 5M8N and/or 95P. (C) The time-varying Rg values of 5M8N and/or 95P. (D) The hydrogen bond number values of 5M8N and/or 95P. (E) Contribution of amino acid residues to binding energy.

that for 5M8N alone. Upon stabilization after 30 ns, the addition of 95P constrained the contraction of 5M8N, thus stabilizing its structure. These conclusions were in accordance with the RMSD and RMSF data, suggesting that including 95P promoted the stability of the 5M8N complex.

The variations in hydrogen bonding over 50 ns are depicted in Figure 6D. The structure of 95P includes carboxyl, amino, and isopentyl groups capable of forming hydrogen bonds with the hydrophilic region of 5M8N. The hydrogen bonding count between 5M8N and 95P ranged from 2 to 10 during the initial 10 ns, with the frequency stabilizing thereafter, which indicated a more secure complex. In comparison, in the absence of 95P, the number of hydrogen bonds in 5M8N varied considerably within the range of 2–8. Overall, the 5M8N-95P complex exhibited greater stability, as evidenced by an increased number of hydrogen bonds, a finding

that aligned with the RMSF data.

The energy contribution distribution of amino acid residues (Figure 6E) revealed that 95P mainly interacted with the amino acid residues of 5M8N in the range of 370–485 and was distributed mainly near the 5M8N hydrophobic pocket [57]. The above results indicated that the 95P-5M8N complex was more stable and that 95P limited the contraction of 5M8N and suppressed the activity of 5M8N.

Discussion

The water-ultrasound technique can effectively retain the original structure of the extract and facilitate further structural identification [31–33]. The increase in the extraction rate may be due to the addition of ultrasonic extraction in this method, whereas Zhang *et al.* only used stirring extraction, which may not have fully released

proteins from the powder. The ultraviolet–visible detector can identify only chromophores at specific wavelengths, whereas the differential refractive index detects all substances, including interferences without UV absorption. Simultaneous analysis of the two profiles can reveal which proteins are target proteins and which interferences are foreign. Research has suggested that peptides less than 5000 Da in size are readily absorbed by the human body and demonstrate potent tyrosinase inhibitory and antioxidant activities [60]. Zhuang *et al.* [61] demonstrated that zein-derived peptides with molecular weights <10 kDa exhibited strong antioxidant and copper chelation activities. Wu *et al.* [62] reported significant tyrosinase inhibitory activity in sericin protein hydrolysates within a molecular weight range of 250–4000 Da. Therefore, we hypothesized that pearl peptides substantially contribute to tyrosinase inhibitory activity, which provides a basis for further activity investigations. In summary, the water-ultrasonic extraction method has been validated as an efficient technique for obtaining bioactive pearl peptides.

TYRP1 has been utilized as a model in multiple tyrosinase inhibition studies [63–65]. The core scientific rationale for selecting TYRP1 (PDB: 5M8N) as the receptor model in this study is as follows: owing to the lack of a complete high-resolution crystal structure for human tyrosinase (TYR, the current best fragment, 5M8Q, has only 3.5 Å resolution), TYRP1 (5M8N) is the sole available full-length high-resolution structure (2.8 Å). Its catalytic domain exhibits high structural conservation with TYR, featuring 40–50% sequence similarity, 100% identical copper-coordinating residues, and an active site RMSD of only 1.2 Å. Although TYRP1's active site contains Zn²⁺ rather than Cu²⁺, key functional regions (e.g., substrate channels and hydrophobic pockets) show >85% spatial overlap with TYR. This model has been extensively applied in melanogenesis mechanism research and melanoma-targeted therapy development, fully validating its reliability as a surrogate for TYR. A variety of screening methods exist for functional peptides, and virtual screening based on computational tools has been widely used. The advantages of this method are high efficiency and high accuracy, and it is suitable for initial screening [49,66]. The exterior of 5M8N is a hydrophilic environment. However, a hydrophobic cavity is formed inside its active center, and amino acids containing hydrophobic side chains can bind more easily in the hydrophobic pocket near the active site, thereby providing better inhibitory activity against 5M8N [1,3,4]. Research by Ochiai *et al.* suggested that peptides with consecutive identical amino acid residues may have enhanced tyrosinase inhibition capabilities [67]. The results revealed that 95P contained several adjacent Gly residues in the sequence; moreover, it possessed two Leu residues and four Ala residues. In addition, Honisch *et al.* [68] demonstrated that the Gly residue improved the water solubility and flexibility of the peptide. Hydrophobic amino acids can provide electrons to free radicals, prevent macromolecular oxidation, and facilitate ligand–receptor interactions. Surprisingly, 95P contains both Leu and Ala hydrophobic amino acids. The substitution of Glu with Ala at the C-terminus in the corn-derived tetrapeptide Leu-Asp-Tyr-Glu (yielding Leu-Asp-Tyr-Ala) enhanced the hydrophobic binding ability, resulting in tyrosinase inhibitory activity that was 1.25-fold greater than that of arbutin. Furthermore, the carboxyl group of the C-terminal Ala may participate in Cu²⁺ coordination, directly interfering with Cu²⁺ redox cycling. Notably, 95P features Ala at its C-terminus. Therefore, we speculated that 95P has strong tyrosinase

inhibitory activity [69].

Van der Waals forces are correlated with molecular polarity and electrostatic interactions, which increase with increasing polar amino acid charge [70,71]. The active site of 5M8N, which comprises two densely packed copper ions, each coordinated by three His residues, was observed to interact with 95P. Notably, hydrophobic interactions were also important; the oxygen atom on the carboxyl group of 95P engaged in a π -cation interaction with a copper ion, whereas the 1-hydroxyl group aligned with Zn511 and Zn512, which are critical for activity within the hydrophobic cavities. Pro371 and Tyr362 contribute to the binding between 95P and 5M8N through alkyl and π -alkyl forces [1]. The three histidine residues in 5M8N interact with peptide 95P through C–H bonds. The role of these bonds in enzymatic catalysis is not singular; hydrogen bonding and electrostatic interactions dominate the binding, whereas hydrophobic interactions and van der Waals forces play auxiliary roles. Consequently, 95P was synthesized in batches for subsequent validation assays.

Tyrosinase has a dual catalytic function, functioning as both a monophenolase and diphenolase. As a monophenolase, tyrosinase can catalyze the hydroxylation of monophenol and convert it to diphenol. This represents the first step in melanin synthesis and provides the necessary intermediates for subsequent reactions. As a diphenolase, tyrosinase can catalyze the oxidation of o-diphenol to produce o-diquinone [6,33]. This is the second step of melanin synthesis and one of the key steps in the process of tyrosinase catalysis; details of the reaction process are shown in [Supplementary Figure S2](#). The IC₅₀ values of monophenolase and diphenolase were inversely proportional to the inhibitory effect of tyrosinase. The inhibitory effect of 95P on tyrosinase was validated by evaluating its influence on both monophenolase and diphenolase activities via the use of a synthetic peptide with an identical sequence. Additionally, the IC₅₀ values for 95P were lower than those reported for the peptides DGL (0.47 ± 0.01 μM), GAR (1.13 ± 0.04 μM), and SDW (2.08 ± 0.01 μM) from donkey collagen [72] and marginally surpassed those of the peptide RHAKF (1.14 μM) from Chinese quince seed protein and the peptide SSEYYGGEGSS-SEQYYGEG (0.63 μM) derived from hydrolyzed rice bran albumin [11,12]. A distinct study reported that the IC₅₀ values for the monophenolase and bisphenolase activities of peptide FPY from defatted walnut meal were 1.11 ± 0.05 mM and 3.22 ± 0.09 mM, respectively [68]. Nie *et al.* [50] identified a novel peptide (IQSPHFF) isolated from a phage display library with IC₅₀ values for monophenolase and bisphenolase activities of 1.7 mM and 4.0 mM, respectively. In comparison, the IC₅₀ values for monophenolase (30.97 ± 0.75 μM) and diphenolase (2.05 ± 0.07 μM) activities for 95P were notably lower than those reported for other peptides, indicating the substantial potential of 95P for tyrosinase inhibition.

In comprehensive comparisons with other peptide inhibitors, the superiority of 95P originated from its potency, mechanism of action, structural characteristics, and biocompatibility (Table 3). The potency advantage (diphenolase IC₅₀ 2.05 ± 0.07 μM) was rooted in its capacity to chelate Cu²⁺, precise blockade of oxygen transfer pathways, and unique flexibility of its consecutive Gly backbone penetrating hydrophobic channels to obstruct substrate-binding sites. Crucially, it induced conformational changes in tyrosinase that disrupted the central geometry—a feature absent in mono-mechanism peptides. Structurally, the flexible consecutive Gly

Table 3. Molecular mechanisms of tyrosinase inhibition by representative peptides

Peptides	Source	Diphenolase IC ₅₀	Mechanism of Action	Conformational change	Key binding forces	Deficiencies	Reference
95P (GLGGGLA-GAGGADGA)	Pearl	2.05 μ M	Cu ²⁺ chelationConformational disorder, substrate channel blockage	α -Helix $\downarrow$ Random coil $\uparrow$	Hydrogen bonds, van der Waals forces, electrostatic interactions	Lack of <i>in vivo</i> metabolic and transdermal kinetic data	/
SSEYYGGEGSS-SEQGYGEG	Hydrolyzed rice-bran albumin	0.63 μ M	Tyr-OH chelation to Cu ²⁺	/	Hydrogen bonds, π - π stacking	Low transmembrane efficiency, metabolic instability	[11]
RHAKF	Chinese quince seed protein	1.14 μ M	Strong Cu ²⁺ chelation by His imidazole ring	α -Helix $\downarrow$	Coordination bonds, hydrophobic interactions	Short-chain limitation, lacks hydrophobic module, unable to block substrate channel	[12]
DGL GAR SDW	Donkey collagen	0.47 mM 0.11 mM 2.08 mM	Arg ⁺ charge interference with substrate binding, Trp hydrophobic stacking, weak Asp electrostatic effects	/	Hydrogen bonds, hydrophobic interactions, electrostatic interactions	Same as above	[62]
DEK	Egg white	0.205 mM	Electrostatic interaction dominance	/	Salt bridges, hydrogen bonds	Same as above	[24]
DGE	Coconut protein	9.58 mM	Dicarboxylate Cu ²⁺ chelation (Asp/Glu)	/	Entropy-driven ($-T\Delta S$ predominates)	Same as above	[25]
FPY	Walnut	3.22 $\pm$ 0.09 mM	Weak Tyr phenolic hydroxyl chelation, Phe hydrophobic stacking	/	π - π stacking, weak Cu ²⁺ coordination	Same as above	[56]

chain of 95P overcomes the rigidity constraints imposed by Pro in FPY, enabling deep spatial penetration, whereas the N-terminal Ala synergizes with His in 5M8N to chelate Cu²⁺, significantly increasing complex stability and overcoming the likelihood of dissociation of short peptides (e.g., DGE, DEK) due to entropically driven binding in aqueous environments [24,25]. Concurrently, its 1.3 kDa molecular weight and lack of aromatic residues conferred greater stability than RHAKF (which is protease-labile) and SSEYY (whose Tyr residue is oxidation sensitive).

95P is a reversible mixed-type inhibitor, and its concentration-dependent inhibitory effect is a key advantage. This property allows it to maintain basal metabolic requirements while avoiding the long-term toxicity (e.g., leukoderma) associated with irreversible inhibitors [9]. Furthermore, mixed-type inhibitors establish a dual-action barrier by simultaneously occupying both the active site (competitive inhibition) and the allosteric site (noncompetitive inhibition). This mechanism achieves multitarget effects against drug resistance and reduces the likelihood of mutation-induced drug escape. The synergistic effects of mixed-type inhibitors enable target inhibition rates to be achieved at lower doses while also decreasing production costs and potential adverse effects [48].

A smaller K_i value indicates a stronger binding affinity (higher affinity) between the inhibitor and the enzyme. Similarly, a lower K_{is} value indicates stronger binding to the ES complex and a more pronounced inhibitory effect. In contrast, Nie *et al.* [50] reported that the peptide IQSPHFF, with a K_i value of 0.765 mM, exhibited reversible and competitive inhibition of tyrosinase, and Si *et al.* [42] reported that hesperidin also inhibited tyrosinase in a competitive and reversible manner, with a K_i of 4.03 ± 0.26 mM. The smaller the

K_{is} , the more tightly the inhibitor binds to the enzyme (higher affinity), meaning that a small amount of inhibitor can significantly inhibit enzyme activity. Moreover, the observed $K_{is} > K_i$ suggested a greater likelihood of 95P binding to tyrosinase over the enzyme-substrate complex. Thus, 95P inclusion may reduce the affinity of tyrosinase for L-DOPA.

Fluorescence spectroscopy can detect changes in overall conformation stability and binding affinity, and fluorescence quenching experiments are commonly used to study ligand–protein interactions [56]. The fluorescence burst experiments were performed according to the methods of Megias and Zhang [53,56]; specifically, the samples were usually scanned at temperatures of 298 K, 303 K, and 310 K to obtain the values of K_{sv} , k_q , and n , thereby determining the type of fluorescence quenching and binding site constant. A fluorescence peak within the 300–500 nm range was observed at an excitation wavelength of 280 nm when the tyrosinase solution with and without 95P was scanned. This occurred because the excitation wavelength of the fluorescence spectrum of the proteins was 280 nm, around which the tryptophan, tyrosine, and phenylalanine residues in the proteins were able to efficiently absorb photons and emit fluorescence. Static quenching indicated that 95P directly binds to tyrosinase to form a stable complex rather than through dynamic collision mechanisms (e.g., energy transfer or solvent effects). The value of $n \approx 1$ suggested a 1:1 stoichiometric binding ratio between 95P and tyrosinase, implying that each enzyme molecule possesses only one primary binding site. These findings provide critical insights for targeted drug design, dose optimization, and mechanistic studies of 95P.

Tyrosinase can catalyze L-tyrosine to produce L-DOPA and then

catalyze L-DOPA to produce dopaquinone (Supplementary Figure S2). The ability of 95P to inhibit tyrosinase activity was verified by observing the production of dopaquinone. The formation and conformational changes of the tyrosinase-peptide complex can be efficiently analyzed via the visible spectrum [73]. Copper chelation significantly reduces the catalytic efficiency of tyrosinase by competitively depriving the enzyme's active center of an essential cofactor (Cu^{2+}) and disrupting the structure and function of the catalytic site. For example, copper chelators such as tretinoin and vitamin C derivatives inhibit tyrosinase activity via the above mechanism, combining both reducing and chelating abilities [39]. These observations were consistent with studies showing significant copper chelation activity in protein hydrolysates of chickpea species [74]. Similarly, whey protein, milk protein, protein hydrolysates, and amino acid derivatives such as hydroxypyridones also demonstrated copper chelation activities [75,76]. The nitrogen atom of the His imidazole ring and the thiol group of Cys can chelate Cu^{2+} . However, 95P (GLGGGLAGAGGADGA) contains Asp, whose side-chain β -carboxyl group dissociates into $-\text{COO}^-$ and coordinates with Cu^{2+} via oxygen atoms. The $-\text{COO}^-$ group of Asp in peptides results in significantly greater Cu^{2+} binding affinity than do neutral amino acids (e.g., Asp, Lys, and Leu). Crucially, the positioning of Asp at the solvent-exposed C-terminus provided substantial spatial freedom, facilitating Cu^{2+} binding. Adjacent Gly residues further enhanced the orientational flexibility of the Asp side chain through backbone plasticity. This led to the conjecture that the tyrosinase inhibition mechanism of 95P might be related to copper chelating activity.

As indicated by the RMSF analysis, the overall structure of 5M8N remained stable after binding with 95P, while significant local dynamic changes were observed. The analysis of RMSD and Rg data from molecular dynamics simulations indicated that the 95P-5M8N complex exhibited greater global stability compared to the 5M8N structure. However, detailed RMSF analysis revealed that this global stability coincided with marked alterations in the conformational dynamics of key local regions. Notably, upon 95P binding, residues such as Glu210, Pro371, His377, and Thr393 presented significantly increased RMSF values. These residues were located within or near the active site of 5M8N or its critical secondary structural elements. The elevated flexibility in these local regions led to destabilization and restructuring of secondary structures, directly accounting for the reduction in α -helix and β -sheet contents and the increase in random coil signals observed via circular dichroism spectroscopy.

Conversely, several residues at the binding interface showed markedly reduced flexibility, with RMSF values decreasing by more than 0.12 nm, indicating that 95P binding simultaneously stabilized the interface and disrupted local structural stability near the active center. The residuewise binding energy contribution analysis (Figure 6E) further revealed that 95P primarily interacted with residues 370–485 of 5M8N, particularly around the hydrophobic pocket, supporting the stability of the complex and suggesting that 95P inhibited 5M8N activity by constraining its structural contraction.

Circular dichroism spectroscopy confirmed that 95P binding induced structural alterations in tyrosinase. Molecular docking and dynamics simulations indicated that secondary structure changes occurred mainly near the active center, especially around the Cu^{2+} -chelating site, likely due to disruption of the hydrogen-bonding network by 95P. The binding was found to be thermodynamically spontaneous ($\Delta G < 0$), driven primarily by van der Waals interac-

tions. Decreased RMS fluctuations reflected enhanced global conformational rigidity, and reduced Rg values confirmed increased compactness of the overall structure. Together, these results demonstrated the high stability of the complex, wherein local conformational disturbances were compensated by global compaction, leading to the enhanced stability observed in the simulations.

The disparity between intracellular and acellular enzyme inhibitory activities lies in the direct interaction of inhibitors with tyrosinase in acellular systems, which eliminates the influence of biological barriers such as cell membrane penetration and metabolic degradation. This enabled the acquisition of definitive parameters, including binding sites in molecular docking, mixed competitive inhibition type, reversible inhibition, reaction spontaneity, and fluorescence quenching type. In intracellular systems, inhibitory activity directly reflects the outcome of 95P suppressing tyrosinase activity but is confounded by barrier interference (e.g., compound transmembrane efficiency), precluding direct equivalence to 95P's intrinsic inhibitory potency. The connection between intracellular and acellular enzyme inhibitory activity was mechanistically consistent, as evidenced by the alignment of dose-dependent trends across both experimental paradigms (Supplementary Figure S3). These differences reflect the complexity of biological systems, collectively substantiating 95P's core mode of action.

Relationship between complex structure and inhibitory activity: the presence of multiple consecutive Gly residues, N-terminal Ala, and a high proportion of hydrophobic amino acid residues in 95P are critical factors for its inhibitory activity. 95P exhibited significantly stronger tyrosinase inhibition than the positive control (kojic acid), and no cytotoxic side effects were observed in the cell-based assays. The results from fluorescence spectroscopy and molecular dynamics simulations confirmed that the primary binding forces between 95P and tyrosinase included hydrogen bonds, van der Waals forces, electrostatic interactions, and hydrophobic interactions. Enzyme kinetics revealed that 95P acted as a reversible mixed-type inhibitor, with the advantages of concentration-dependent inhibitory effects and the ability to achieve target inhibition rates at lower dosages. Fluorescence quenching analysis classified the interaction as static quenching, indicating direct binding between 95P and tyrosinase to form a stable complex with a 1:1 stoichiometric ratio ($n \approx 1$). The inhibition mechanism was further corroborated by the blockade of L-DOPA oxidation by 95P and its ability to chelate copper ions in tyrosinase. Molecular dynamics and circular dichroism analyses demonstrated that 95P enhanced the stability of the tyrosinase complex and induced conformational changes in tyrosinase. These experimental results collectively validated the mechanism of 95P-mediated tyrosinase inhibition. However, the specific extent of inhibition and the underlying pathways require further elucidation through *in vivo* experiments, which will be addressed in future studies.

The efficiency of inhibitor screening was indisputable in this study, yet certain limitations exist. Some peptides capable of inhibiting tyrosinase activity might have been overlooked, and further investigation is needed to determine whether the synergistic effects of multiple peptides are more pronounced than those of individual peptides. The synthetic peptide 95P might lack the multicomponent synergistic interactions observed in natural extracts, such as the complex effects between calcium ions and peptides in pearl powder. Future experiments should focus on

refining structural analyses and functional studies of other components. The strength of this study lies in its ability to rapidly screen tyrosinase-inhibitory peptides and elucidate their inhibition mechanisms through multispectral analysis and molecular dynamics simulations. The experimental design included structural analysis, conformational changes, kinetic profiling, and quenching mechanisms, thereby addressing the knowledge gap in understanding how pearl peptides inhibit melanogenesis. This work established a foundation for industrial applications of pearl peptides. In terms of practical prospects, 95P, as a precisely engineered multifunctional synthetic peptide, has demonstrated unique potential in two fields: skin whitening and food antibrowning. It could serve as a medical-grade skin-whitening dressing to replace traditional components such as hydroquinone and kojic acid. In the food industry, 95P is applicable in fresh-cut fruit/vegetable color-preserving solutions and healthy baked goods additives, aligning with the clean-label trend.

In conclusion, this study identified purified pearl peptide fractions and screened peptides with significant tyrosinase inhibitory activity through molecular docking, obtaining the peptide "GLGGGLAGAGGADGA" (95P) with the highest binding energy. The molecular interactions between 95P and tyrosinase involve primarily hydrogen bonds, van der Waals forces, electrostatic interactions, and hydrophobic interactions. 95P inhibited dopaquinone formation, confirming its ability to suppress tyrosinase activity and L-DOPA oxidation. The synthetic peptide 95P inhibited both monophenolase and diphenolase activities, with IC_{50} values of $30.97 \pm 0.75 \mu M$ and $64.62 \pm 3.08 \mu M$, respectively. Enzyme kinetic analysis revealed that 95P reversibly inhibits tyrosinase via mixed competitive inhibition. Copper ion chelation assays confirmed that 95P also reduced tyrosinase catalytic efficiency by chelating copper ions. Additionally, fluorescence quenching assays and molecular dynamics simulations demonstrated that 95P inhibited tyrosinase activity by altering the enzyme conformation through noncovalent bonds. These findings indicate that 95P is a potent and stable tyrosinase inhibitor with potential applications as a skin-whitening agent in pharmaceuticals and an antibrowning agent in the food industry.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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