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Research Article

Extraction and antitumor activity evaluation of small molecular weight collagen peptides from Chinese giant salamander (*Andrias davidianus*) skin

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Abstract: The skin of the Chinese giant salamander (*Andrias davidianus*) is a rich source of collagen. However, current research indicates limited efficiency and activity in collagen protease hydrolysis from this source. Further enzymatic hydrolysis to produce small molecular weight active peptides and their antitumor potential remain underexplored. This study employed a two-step enzymatic hydrolysis method to prepare small molecular weight collagen peptides (SMWCPs), followed by ultrafiltration separation. The extraction process was optimized using single-factor and response surface methodology. The amino acid composition of SMWCPs was analyzed, and their antitumor activity was assessed *in vitro* through cell migration, cell cycle, and apoptosis assays. Results revealed a negative correlation between collagen peptide molecular weight and antitumor activity, with optimal antitumor activity observed for peptides with a molecular weight of 300–1 000 Da (neutral protease hydrolyzed Chinese giant salamander skin collagen peptides (NP-GSKCP)). Optimal extraction conditions were: enzyme dosage 8 000 U/g, enzymatic hydrolysis temperature 52.6 °C, enzymatic hydrolysis pH 7.1, and enzymatic hydrolysis time 4.3 h, yielding 87.82% NP-GSKCP-IV. Notably, NP-GSKCP-IV contained 55.31% hydrophobic amino acids. Treatment of A549 cells with 5 mg/mL NP-GSKCP-IV significantly altered cell morphology, inhibited migration, arrested the cell cycle at G0/G1 phase, and induced early apoptosis. This study contributes to the development of efficient extraction techniques for antitumor active ingredients from Chinese giant salamander skin and informs the potential development of medicinal functional foods.

Keywords: *Andrias davidianus*; two-step enzymatic hydrolysis method; ultrafiltration separation; collagen peptide; antitumor activity

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1 Introduction

Amphibian skin and its secretions, particularly from edible species such as frogs and the Chinese giant salamander (*Andrias davidianus*), have garnered significant research interest as exogenous sources of bioactive peptides, exhibiting promising biological and medical potential[1]. Nevertheless, these potential resources have been largely neglected and underutilized, despite the notable bioactive peptides they contain, which have been reported to possess antioxidant, antimicrobial, anticancer, and antidiabetic activities, among others[2–3]. The Chinese giant salamander, also known as the Sichuan salamander, is a rare and among the largest amphibian species globally[4]. Renowned for its nutritional richness, this species boasts high-quality proteins, fats, minerals, and various vitamins. Notably, its skin is abundant in 17 amino acids, comprising 21.86% of the total amino acid content, with 5.91% being essential for human nutrition, indicating its substantial nutritional value[5]. Furthermore, the skin is rich in proteins, accounting for approximately 30% of its dry weight, equivalent to over 90% of the total skin protein content[6]. In traditional Chinese medicine, the skin is believed to nourish Qi and blood, enhance cognitive function, and aid in treating conditions like neurasthenia, anemia, dysentery, and malaria[7].

In recent years, the Chinese giant salamander has garnered widespread attention due to its unique biological characteristics, particularly the abundance of collagen in its skin tissue. Collagen, a crucial structural protein found in skin, bones, muscles, and connective tissues, exhibits diverse biological activities[8]. Studies have demonstrated that artificially bred Chinese giant salamanders possess abundant collagen in their skin[9]. Utilizing low-temperature enzymatic hydrolysis, Li[10] achieved a collagen peptide extraction rate of 26.7%, identifying it as type I collagen. Zhang et al.[11] further optimized this process, achieving an extraction rate of 44.43%, with amino acid analysis revealing high levels of glycine, glutamic acid, proline, and hydroxyproline, underscoring its nutritional significance. Moreover, the skin and muscles of the Chinese giant salamander harbor various bioactive substances, including peptides, glycopeptides, and polysaccharides, which exhibit antioxidative, anti-aging, immune-regulatory, and antimicrobial effects, suggesting potential medical applications[12–13]. For instance, mucous protein extracts from its skin have demonstrated anti-lung cancer activity, offering potential therapeutic avenues for lung cancer[14]. Additionally, peptides extracted from its skin have shown antioxidative and antimicrobial properties, paving the way for the development of natural health products and antimicrobial agents[15]. Given these attributes, the development of the Chinese giant

salamander as an edible and medicinal resource is gaining momentum. Collagen in its skin not only serves a vital role in its survival and life habits, but also presents potential biomedical and bioengineering applications. Consequently, research on the extraction process and activity analysis of collagen from its skin has become a focal point. Notably, studies have established a correlation between the activity of collagen peptides and their molecular weight[16–17], emphasizing the need to investigate this relationship in the context of the Chinese giant salamander.

However, existing research on Chinese giant salamander skin collagen has primarily focused on antioxidant activity, with limited exploration into the extraction, preparation, and anticancer activity analysis of peptides of varying molecular weights. This study aims to extract collagen peptides of different molecular weights from the skin of the Chinese giant salamander using enzymatic hydrolysis and ultrafiltration techniques, and subsequently analyze their anticancer activity. By optimizing the extraction process, we aim to select collagen peptides with optimal anticancer properties. This endeavor is expected to contribute scientific insights into the deep processing of Chinese giant salamander resources and the preparation of anticancer collagen peptides, while also offer novel perspectives and opportunities for the comprehensive utilization of this valuable resource. More concretely, in this study, enzymatic hydrolysis was initially employed to degrade collagen in the skin of the Chinese giant salamander, followed by the use of ultrafiltration separation technology to prepare collagen peptides. Subsequently, rigorous research was conducted on the anticancer activity of the collagen peptides, evaluating its impact on tumor cells. This comprehensive investigation aims to unravel the underlying anticancer mechanisms of these peptides, thereby providing fresh insights and directions for cancer treatment research and development. Furthermore, this study contributes to a deeper understanding of the conservation and sustainable utilization of the Chinese giant salamander.

2 Materials and methods

2.1 Materials and reagents

Healthy, second-generation Chinese giant salamanders, aged four years, with a mean weight of 5.21 kg and an average length of 72.31 cm, were generously provided by Guizhou Xiangsheng Chinese Giant Salamander Breeding Co., Ltd. (Guiyang, China). The A549 cell line was procured from the Cell Bank of the Chinese Academy of Sciences. The F12K medium and annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis assay kit were sourced from Wuhan Boster Biotechnology Co., Ltd. (Wuhan, China), whereas fetal bovine serum (FBS) was obtained from Biological Industries (Haemek, Israel). Penicillin-streptomycin solution (100 ×) was acquired from Xinsaimei Biotechnology Co., Ltd. (Suzhou, China). Additionally, the TransDetect® cell counting kit (CCK) was purchased from Beijing Quanshi Jin Biological Co., Ltd. (Beijing, China), the cell apoptosis positive control kit and cell cycle and cell apoptosis assay kit were sourced from Shanghai Biyun Tian Biotechnology Co., Ltd. (Shanghai, China). Furthermore, neutral protease (50 000 U/g), pepsin (1:10 000), trypsin (1:250), and alkaline protease (200 000 U/g) were all purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

2.2 Preparation of Chinese giant salamander skin collagen peptides

Chinese giant salamanders were anesthetized in a solution comprising 0.6 g/L MS-222 under 12–16 °C, subsequently dissected to procure their skin, which was then cut into small, approximately 5 mm × 5 mm pieces. The prepared skin underwent defatting and decolorization processes. Digestion was facilitated with 0.2% pepsin, and the pH of the filtrate was initially adjusted to 8, followed by readjustment to its original pH using 0.5 mol/L acetic acid. Subsequent to pH adjustment, the supernatant was centrifuged (8 000 r/min, 15 min), and a 5 mol/L NaCl solution was added. Centrifugation was repeated to retrieve the flocculent material, which underwent dialysis and freeze-drying in a freeze dryer (Triacl 2.5 L, Labconco Co., USA), resulting in the isolation of collagen. The extracted Chinese giant salamander skin collagen was solubilized in a 0.5 mol/L acetic acid solution to prepare the collagen solution. Four distinct proteases were individually added to this solution for enzymatic hydrolysis, with the conditions outlined in Table 1. The enzymatic conditions were selected within the optimal range for each respective enzyme, ensuring consistent enzyme activity.

Following a 10-minute enzyme inactivation period and neutralization of the pH, the supernatant was collected via centrifugation. The crude extract of Chinese giant salamander skin collagen peptides was then filtered through a 0.45 μm membrane, diluted with ultrapure water, and introduced into an organic membrane separation system. Sequential filtration through membranes with molecular weight cut-offs of 5 000, 3 000, 1 000, and 300 Da was performed. The filtrate from each step was retained, and the collagen peptides of varying molecular weights, hydrolyzed by the four proteases, were freeze-dried: neutral protease hydrolyzed Chinese giant salamander skin collagen peptides (NP-GSKCP), alkaline protease hydrolyzed Chinese giant salamander skin collagen peptides (AP-GSKCP), pepsin hydrolyzed Chinese giant salamander skin collagen peptides (P-GSKCP), and trypsin hydrolyzed Chinese giant salamander skin collagen peptides (T-GSKCP). The collagen peptide extraction rate was calculated using the following formula:

$$\text{Extraction rate}\ (\%) = \frac{m}{m_0} \times 100$$

Where m was the mass of collagen peptides after freeze-drying (g); m_0 was the mass of collagen (g).

Table 1 Extraction conditions of collagen peptide from Chinese giant salamander skin with different proteases.

Enzyme type	Enzymatic hydrolysis time (h)	Enzymatic hydrolysis temperature (°C)	Enzymatic hydrolysis pH	Enzyme dosage (U/g)	Material-to-liquid ratio (g/mL)
Neutral protease	4	50	7.0	6 000	0.1
Pepsin	4	37	2.0	6 000	0.1
Alkaline protease	4	50	8.0	6 000	0.1
Trypsin	4	40	8.0	6 000	0.1

2.3 Determination of A549 cells viability after Chinese giant salamander skin collagen peptides treatment

According to CCK-8 assay, logarithmic phase A549 cells were harvested and subsequently allocated into control and experimental groups. Varying concentrations (0.2, 0.6, and 1.0 mg/mL) of collagen peptide solutions with distinct molecular weights were introduced into the experimental groups to assess the impact of the collagen peptide extraction on cell viability, referencing relevant parameters. The experimental protocol adhered strictly to the manufacturer's instructions. Following incubation, the optical density (OD) was measured at a wavelength of 460 nm using a microplate reader (SpectraMAX M2, Molecular Devices, USA). Relative cell viability can be calculated by deducting the OD of the blank group from that of the experimental group and then dividing the result by the OD of the negative control group minus the OD of the blank. Based on the results, the most efficacious fraction, spanning a molecular weight range of 300 to 1 000 Da, derived from NP-GSKCP (designated as NP-GSKCP-IV), was chosen for further process optimization experiments.

2.4 Single-factor experiments of the extraction of collagen peptides from Chinese giant salamander skin

2.4.1 Enzyme dosage

To investigate the influence of enzyme dosage on the extraction rate of collagen peptides from Chinese giant salamander skin, a series of experiments were conducted under optimized conditions: enzymatic hydrolysis temperature, pH, and time of 47 °C, 7.0, and 4 h, respectively. The enzyme dosage was varied across levels of 2 000, 4 000, 6 000, 8 000, and 10 000 U/g to identify the optimal amount for maximizing extraction rate.

2.4.2 Enzymatic hydrolysis temperature

With an enzyme dosage fixed at 8 000 U/g, a pH maintained at 7.0, and an enzymatic hydrolysis time of 4 h, the enzymatic hydrolysis temperature was varied across 27, 37, 47, 57, and 67 °C to assess its impact on the extraction rate of collagen peptides from Chinese giant salamander skin. This optimization aimed to determine the optimal temperature for enhancing the extraction process.

2.4.3 Enzymatic hydrolysis pH

Under conditions of 47 °C, an enzyme dosage of 8 000 U/g, and an enzymatic hydrolysis time of 4 h, the pH was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 to study its effect on the extraction rate of collagen peptides from Chinese giant salamander skin. This experiment aimed to identify the optimal pH level that maximized the extraction efficiency.

2.4.4 Enzymatic hydrolysis time

With a temperature set at 47 °C, an enzyme dosage of 8 000 U/g, and a pH of 7.0, the enzymatic hydrolysis time was varied from 1.0 to 5.0 h in increments of 1.0 h to investigate its influence on the extraction rate of collagen peptides from Chinese giant salamander skin. This optimization sought to determine the optimal duration of enzymatic hydrolysis for maximizing the extraction rate.

2.5 Box-Behnken response surface experiment

Based on the outcomes of the single-factor experiments conducted for the preparation of collagen peptides from Chinese giant salamander skin, three significant factors influencing the extraction rate were identified: enzymatic hydrolysis temperature (X_1), enzymatic hydrolysis pH (X_2), and enzymatic hydrolysis time (X_3). A three-level Box-Behnken response surface methodology (RSM) was employed, utilizing the extraction rate (Y) of NP-GSKCP-IV as the response variable. This design encompassed 17 independent experiments to optimize the extraction process. The factor levels for the response surface design are presented in Table 2.

Table 2 Factors and levels of Box-Behnken scheme design.

Level	X_1 Enzymatic hydrolysis temperature (°C)	X_2 Enzymatic hydrolysis pH	X_3 Enzymatic hydrolysis time (h)
−1	37	5.0	3
0	47	6.0	4
1	57	7.0	5

2.6 Analysis of free amino acid and hydrolyzed amino acid composition of optimized NP-GSKCP-IV

To the collagen peptide powder sample, 0.05 g/mL sulfosalicylic acid was added in a 1:4 (g/mL) ratio to precipitate the protein. The mixture was centrifuged at 18 000 r/min using a high-speed refrigerated centrifuge (Allegra X-30R, Beckman Coultre Ltd., Burea, USA) for 30 min. The supernatant was collected, filtered through a 0.45 μm membrane, and subsequently analyzed using an amino acid analyzer (L8900, Biochrom Ltd., UK). The determination of hydrolyzed amino acid content adhered to the GB 5009.124–2016 *National standard for food safety-determination of amino acids in food*, which outlines a standardized method for amino acid quantification in food products.

2.7 Analysis of effect of optimized NP-GSKCP-IV on A549 cells

2.7.1 Morphology

A549 cells in logarithmic phase were seeded into 6-well plates at a density of 2 mL per well. After cell adherence, the culture medium was replaced, and experimental groups (0.1, 0.5, 1.0, 3.0, and 5.0 mg/mL NP-GSKCP-IV) and a negative control group were established, with three replicates per group. Cells were incubated for 24 h, and morphological changes were observed using an inverted fluorescence microscope (DMIL LED, Leica, Germany).

2.7.2 Migration ability

A549 cells in logarithmic phase were seeded in 6-well plates for a scratch test. Following the scratch, the medium was replaced with fresh culture medium (10% FBS) for the control group and NP-GSKCP-IV-containing media (0.1, 0.5, 1.0, 3.0, and 5.0 mg/mL) for the treatment groups. The scratch area was photographed at 0 h and again after 48 h of incubation in a CO_2 incubator (Thermo Fisher Scientific, USA). The area of cell scratch healing rate was analyzed using ImageJ software.

2.7.3 Cell cycle distribution

The influence of collagen peptides on the A549 cells cycle was assessed using a cell cycle and cell apoptosis assay kit, following the manufacturer's protocol. Experimental groups (0.1, 0.5, 1.0, 3.0, and 5.0 mg/mL NP-GSKCP-IV) and a negative control group were established, with three replicates per group. After 24 h of incubation, cells were fixed, stained with PI, and analyzed using a flow cytometer (NovoCyte 2040R, ACEA Biosciences Inc., USA).

2.7.4 Cell apoptosis

Apoptosis induced by collagen peptides was detected using an annexin V-FITC/PI cell apoptosis assay kit, according to the manufacturer's instructions. Experimental groups with varying concentrations of NP-GSKCP-IV (1.0, 3.0 and 5.0 mg/mL), a blank control, PI single-stain, and annexin V-FITC single-stain groups were established, with three replicates per group. After 24 h of incubation, cells were collected, stained, and analyzed using a flow cytometer (NovoCyte 2040R, ACEA Biosciences Inc., USA).

2.8 Statistical analysis

All experiments were performed independently at least three times, and results are presented as mean ± standard deviation. Data were analyzed using SPSS 26.0 for one-way analysis of variance (ANOVA), and graphs were generated using Sigma Plot 12.5 software. The response surface design and analysis were conducted using Design Expert 10.0 software. Statistical significance was determined at $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the control group.

3 Results and discussion

3.1 Effect of different enzymatic hydrolysis of Chinese giant salamander skin collagen peptides on the relative viability of A549 cells

The data presented in Figure 1A underscore the significant inhibitory effect of P-GSKCP on the relative viability of A549 cells, particularly at a concentration of 1.0 mg/mL. Notably, the inhibitory potency augmented inversely with the decrease in molecular weight of P-GSKCP, indicating a pronounced molecular weight-dependent response. When subjected to varying concentrations (0.2, 0.6 and 1.0 mg/mL) of P-GSKCP within the molecular weight range of 300 to 1 000 Da, the relative viability of A549 cells diminished progressively from (102.36 ± 2.13)% to (67.76 ± 3.09)% and (47.20 ± 1.80)%. This phenomenon suggested a dose-gradient effect, where increased concentrations of P-GSKCP resulted in heightened inhibition. As depicted in Figure 1B, T-GSKCP with a molecular weight range of 3 000 to 5 000 Da exhibited the most pronounced effect at 1.0 mg/mL, with relative cell viability reducing to (67.60 ± 2.42)%. Figure 1C highlights the potent inhibitory activity of AP-GSKCP within the 300 to 1 000 Da on A549 cells relative viability. Across concentrations of 0.2, 0.6, and 1.0 mg/mL, the relative cell viability declined sequentially from (69.13 ± 3.45)% to (47.40 ± 0.26)% and ultimately to (30.45 ± 0.18)%, emphasizing a concentration-dependent inhibition. The analysis in Figure 1D reveals the optimal bioactivity and dose-responsive nature of NP-GSKCP. Among them, the 300–1 000 Da fraction exhibited the highest inhibitory activity against A549 cells. At concentrations of 0.2, 0.6, and 1.0 mg/mL, the relative cell viability declined progressively from (58.46 ± 1.10)% to (40.90 ± 1.57)% and (25.38 ± 0.76)%, respectively. Consequently, NP-GSKCP, particularly NP-GSKCP-IV, was chosen for further optimization of the extraction process and preliminary *in vitro* antitumor activity assessments due to its notable inhibitory effect on A549 cells viability.

3.2 Single-factor experimental outcomes

3.2.1 Effect of enzyme dosage on NP-GSKCP-IV extraction rate

Figure 2A illustrates that within an enzyme dosage range of 2 000–8 000 U/g, optimal enzyme-collagen contact is achieved, facilitating a thorough reaction. Consequently, there was a pronounced surge in the extraction rate of NP-GSKCP-IV. This upward trend moderated as the enzyme dosage reached 8 000–10 000 U/g, prompting the subsequent experiments to adopt a fixed enzyme dosage of 8 000 U/g.

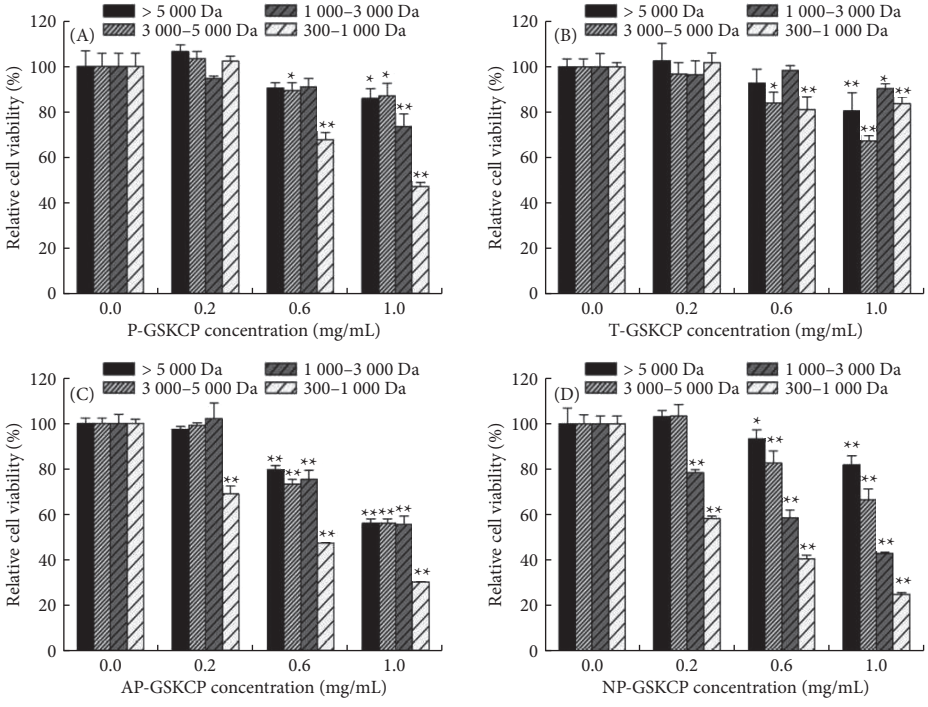

Figure 1 Effects of (A) P-GSKCP, (B) T-GSKCP, (C) AP-GSKCP, and (D) NP-GSKCP on the relative cell viability of A549 cells. Compared to the control group, $^{*}P < 0.05$, $^{**}P < 0.01$.

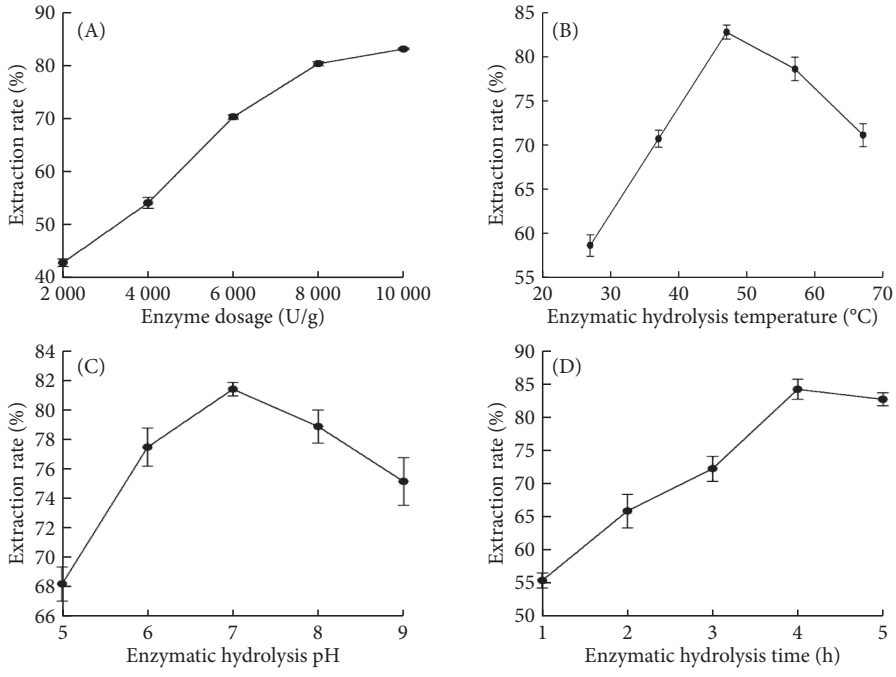

Figure 2 Effect of (A) enzyme dosage, (B) enzymatic hydrolysis temperature, (C) enzymatic hydrolysis pH, and (D) enzymatic hydrolysis time on NP-GSKCP-IV extraction rate.

3.2.2 Effect of enzymatic hydrolysis temperature on NP-GSKCP-IV extraction rate

The influence of enzymatic hydrolysis temperature on extraction rate is evident in Figure 2B. When temperatures ranged from 27 to 47 °C, an elevation in temperature augmented the activity of hydrolytic enzyme, significantly enhancing NP-GSKCP-IV extraction rate. Conversely, temperatures exceeding 47 °C exerted a detrimental effect on enzyme activity, leading to a gradual decline in NP-GSKCP-IV extraction rate. Hence, 47 °C was deemed the optimal temperature for subsequent enzymatic hydrolysis experiments.

3.2.3 Effect of enzymatic hydrolysis pH on NP-GSKCP-IV extraction rate

The extraction rate of NP-GSKCP-IV, as depicted in Figure 2C, initially rised and then declined with increasing pH. Specifically, within a pH range of 5.0 to 7.0, the NP-GSKCP-IV extraction rate escalated proportionally with pH. However, upon surpassing a pH of 7.0, the extraction rate gradually diminished. Consequently, a pH of 7.0 was established as the optimal hydrolysis pH for subsequent enzymatic hydrolysis procedures.

3.2.4 Effect of enzymatic hydrolysis time on NP-GSKCP-IV extraction rate

The duration of hydrolysis exerted a direct influence on the NP-GSKCP-IV extraction rate, as illustrated in Figure 2D. Within the first 4.0 h of enzymolysis, the extraction rate progressively increased with time. Nevertheless, beyond 4.0 h, the extraction rate begins to decline. Therefore, a hydrolysis duration of 4.0 h was chosen as the optimal timeframe for subsequent enzymatic hydrolysis experiments.

3.3 Results of RSM for extraction process optimization

3.3.1 Design of response surface experiments and variance regression analysis

Based on the outcomes of preliminary single-factor experiments, three pivotal factors influencing the extraction rate of collagen peptides were identified: enzymatic hydrolysis temperature (X_1), enzymatic hydrolysis pH (X_2), and enzymatic hydrolysis time (X_3). To determine the optimal combination of these factors, a Box-Behnken design with 17 experimental runs was implemented. The experimental matrix and corresponding results are summarized in Table 3.

Table 3 Test design and results of Box-Behnken scheme.

Experiment No.	X_1 Enzymatic hydrolysis temperature (°C)	X_2 Enzymatic hydrolysis pH	X_3 Enzymatic hydrolysis time (h)	Y Extraction rate (%)
1	57	6	4	80.37 ± 0.52
2	37	8	4	70.34 ± 1.03
3	47	8	5	81.83 ± 0.80
4	57	8	4	82.43 ± 0.80
5	47	6	5	79.24 ± 0.70
6	47	7	4	85.33 ± 0.82
7	37	7	3	69.42 ± 0.69
8	47	7	4	86.32 ± 1.12
9	47	7	4	84.92 ± 0.40
10	57	7	3	82.58 ± 1.16
11	47	7	4	86.67 ± 0.78
12	47	8	3	75.48 ± 1.05
13	47	6	3	74.84 ± 0.49
14	57	7	5	84.54 ± 0.70
15	37	7	5	72.22 ± 0.59
16	37	6	4	68.38 ± 0.71
17	47	7	4	85.38 ± 0.73

Utilizing the Design Expert 10.0 software, a regression analysis was conducted on the experimental data obtained from the RSM, with the extraction rate of NP-GSKCP-IV serving as the dependent variable (Y), and the aforementioned factors as independent variables. This analysis yielded a quadratic regression equation that governed the relationship between Y and the factors: $Y = 85.754 + 6.195X_1 + 0.906X_2 + 1.939X_3 + 0.025X_1X_2 - 0.210X_1X_3 + 0.488X_2X_3 - 5.516X_1^2 - 4.858X_2^2 - 3.048X_3^2$. The variance analysis of the response surface model is presented in Table 4. The model's high significance

Table 4 Regression model analysis of variance table.

Parameter	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value	Significance
Model	640.46	9	71.16	68.42	< 0.000 1	**
X_1	307.02	1	307.02	295.22	< 0.000 1	**
X_2	6.57	1	6.57	6.32	0.040 2	*
X_3	30.07	1	30.07	28.91	0.001 0	**
X_1X_2	0.002 5	1	0.002 5	0.002 4	0.962 3	
X_1X_3	0.176 4	1	0.176 4	0.169 6	0.692 8	
X_2X_3	0.95	1	0.95	0.914 1	0.370 9	
X_1^2	128.10	1	128.10	123.17	< 0.000 1	**
X_2^2	99.38	1	99.38	95.56	< 0.000 1	**
X_3^2	39.12	1	39.12	37.62	< 0.000 5	**
Residual	7.28	7	1.04			
Lack-of-fit	4.81	3	1.60	2.59	0.189 9	
Pure error	2.47	4	0.62			
Sum	647.74	16				
		R^2 = 0.988 8		R^2_{adj} = 0.974 3		

Note: *P < 0.05, **P < 0.01.

was evidenced by a P < 0.000 1. Furthermore, the lack-of-fit term's non-significance (P > 0.05) attested to the model's robust fit. The determination coefficient (R^2 = 0.988 8) and adjusted determination coefficient (R^2_{adj} = 0.974 3) underscored the model's ability to accurately predict and explain 97.43% of the variability in the response, respectively. Consequently, the model's factor ranking in terms of their influence on the extraction rate was established as X_1 (enzymatic hydrolysis temperature) > X_3 (enzymatic hydrolysis time) > X_2 (enzymatic hydrolysis pH). Notably, all factors exhibited a statistically significant effect on the extraction rate, as indicated by their respective P values (< 0.05).

3.3.2 Optimal extraction conditions and verification

Optimal extraction conditions were identified through the response surface optimization process: an enzymatic hydrolysis temperature of 52.6 °C, an enzymatic hydrolysis pH of 7.1, and an enzymatic hydrolysis time of 4.3 h, leading to an expected extraction rate of 87.82% for NP-GSKCP-IV. This prediction was validated through three independent experiments, which yielded an average extraction rate of (86.76 ± 0.45)%, confirming the model's reliability and the feasibility of the RSM for optimizing the NP-GSKCP-IV extraction process.

3.4 Composition of free amino acid and hydrolyzed amino acid in optimized NP-GSKCP-IV

The optimized NP-GSKCP-IV was analyzed for its free amino acid content, revealing the presence of 17 amino acids. Among these, hydrophobic amino acids constituted 38.84%, while essential amino acids accounted for 30.13%. Notably, tyrosine was the most abundant amino acid, representing 40.05% of the total, followed by isoleucine, methionine, phenylalanine, and others. The hydrolyzed amino acid profile of the optimized NP-GSKCP-IV was determined, with glycine emerging as the most prevalent amino acid (17.04%). Other notable amino acids included proline, methionine, isoleucine, glutamic acid, alanine, and valine, all of which mirrored the amino acid composition of optimized NP-GSKCP-IV. Hydrophobic amino acids comprised 55.31% of the total, while essential amino acids accounted for 29.35% (Table 5).

Table 5 Free amino acid and hydrolyzed amino acid composition of optimized NP-GSKCP-IV.

Amino acid	Content (mg/g)	
	Free amino acid	Hydrolyzed amino acid
Aspartic acid	0.079 ± 0.007	1.894 ± 0.055
Threonine	0.051 ± 0.005	0.848 ± 0.023
Serine	0.028 ± 0.004	1.838 ± 0.014
Glutamic acid	0.028 ± 0.005	3.220 ± 0.120
Glycine	0.009 ± 0.002	7.855 ± 0.058
Alanine	0.007 ± 0.002	3.139 ± 0.091
Cysteine	0.079 ± 0.006	1.325 ± 0.032
Valine	0.095 ± 0.007	3.667 ± 0.080
Methionine	0.118 ± 0.008	5.501 ± 0.042
Isoleucine	0.172 ± 0.008	4.416 ± 0.003
Leucine	0.056 ± 0.008	2.581 ± 0.020
Tyrosine	0.662 ± 0.002	0.419 ± 0.006
Phenylalanine	0.114 ± 0.004	0.841 ± 0.017
Histidine	0.047 ± 0.003	0.422 ± 0.010
Lysine	0.010 ± 0.003	1.174 ± 0.021
Arginine	0.018 ± 0.003	1.601 ± 0.018
Proline	0.080 ± 0.017	5.346 ± 0.142
Sum	1.653 ± 0.015	46.087 ± 0.475

3.5 Antitumor activity of NP-GSKCP-IV

3.5.1 Effect of optimized NP-GSKCP-IV on the proliferation of A549 cells

The antiproliferative activity of the optimized NP-GSKCP-IV was evaluated against A549 cells. As depicted in Figure 3, the optimized

NP-GSKCP-IV exhibited a pronounced inhibitory effect on A549 cells proliferation, with relative cell viability decreasing to (56.86 ± 2.20)%, (40.67 ± 1.79)%, and (29.46 ± 0.59)% at concentrations of 0.2, 0.6, and 1.0 mg/mL, respectively.

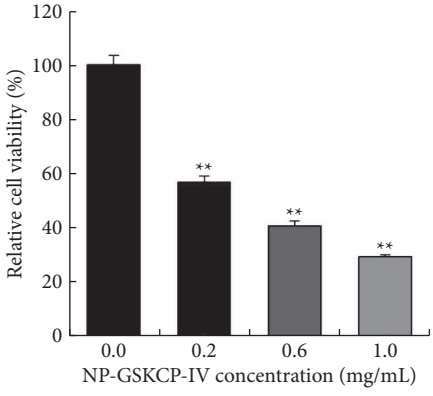

Figure 3 Effect of optimized NP-GSKCP-IV on the relative cell viability of A549 cells. Compared to the control group, $^{**}P < 0.01$.

3.5.2 *Effect of optimized NP-GSKCP-IV on the morphology of A549 cells*

The results of the influence of optimized NP-GSKCP-IV on the morphology of A549 cells are presented in Figure 4. In the blank control group, A549 cells exhibited good growth status, with firm adhesion to the substrate and close interconnection between cells. Morphologically, they appeared as regular long fusiform shapes. As the concentration of optimized NP-GSKCP-IV increased, the number of cells significantly decreased, and the cells gradually transformed from a long fusiform shape to a rounded shape, undergoing shrinkage, loose arrangement, and an increase in intercellular spaces.

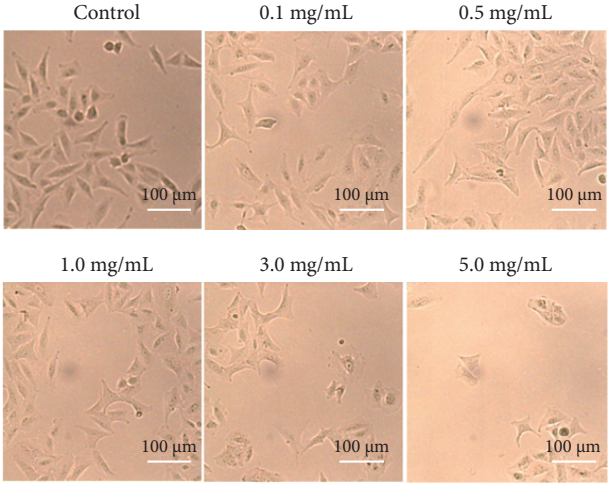

Figure 4 Effect of NP-GSKCP-IV on the morphology of A549 cells. Magnification: 100 ×.

3.5.3 *Effect of optimized NP-GSKCP-IV on the migration of A549 cells*

As shown in Figure 5, the optimized NP-GSKCP-IV significantly impeded the migration of A549 cells. At a concentration of 5.0 mg/mL, the wound healing rate was reduced to (36.61 ± 4.88)% after 48 h, indicating a marked suppression of cell motility.

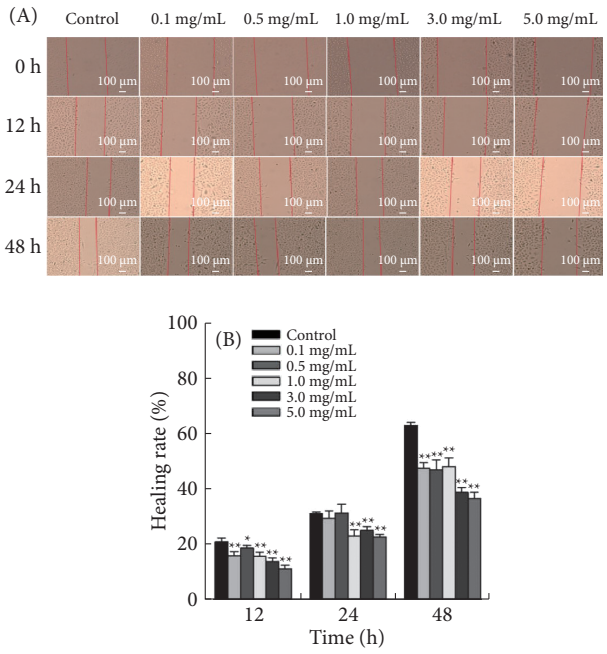

Figure 5 Effect of optimized NP-GSKCP-IV on the (A) migration and (B) healing rate of A549 cells. Magnification: 40×. Compared to the control group, $^{*}P<0.05$, $^{**}P < 0.01$.

3.5.4 *Effect of optimized NP-GSKCP-IV on the cycle distribution of A549 cells*

As depicted in Figure 6, the optimized NP-GSKCP-IV altered the cell cycle distribution of A549 cells, causing an increase in the G0/G1 phase population and a corresponding decrease in the S phase population. Specifically, the G0/G1 phase population increased from (30.28 ± 2.55)% to (54.31 ± 1.88)%, while the S phase population decreased from (36.14 ± 0.30)% to (24.75 ± 1.88)% at a concentration of 5.0 mg/mL.

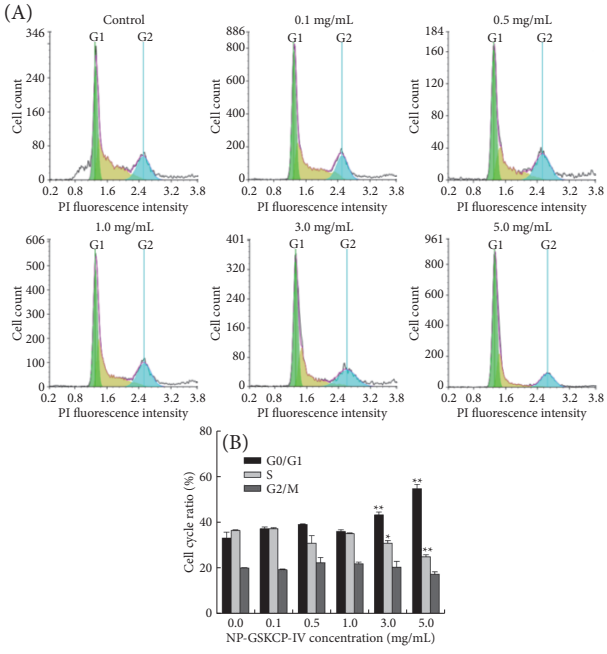

Figure 6 Effects of optimized NP-GSKCP-IV on A549 cells cycle. (A) Flow cytometry for cell cycle analysis; (B) Ratio of cell cycle phases. Compared to the control group, $^{*}P < 0.05$, $^{**}P < 0.01$.

3.5.5 *Effect of optimized NP-GSKCP-IV on the apoptosis of A549 cells*

The results presented in Figure 7 demonstrated that the optimized NP-GSKCP-IV effectively induced apoptosis in A549 cells. Compared to the control group (apoptosis rate (4.63 ± 0.64)%), the apoptosis rate increased to (7.49 ± 0.81)%, (15.98 ± 1.35)%, and (29.16 ± 2.21)% at concentrations of 1.0, 3.0, and 5.0 mg/mL, respectively.

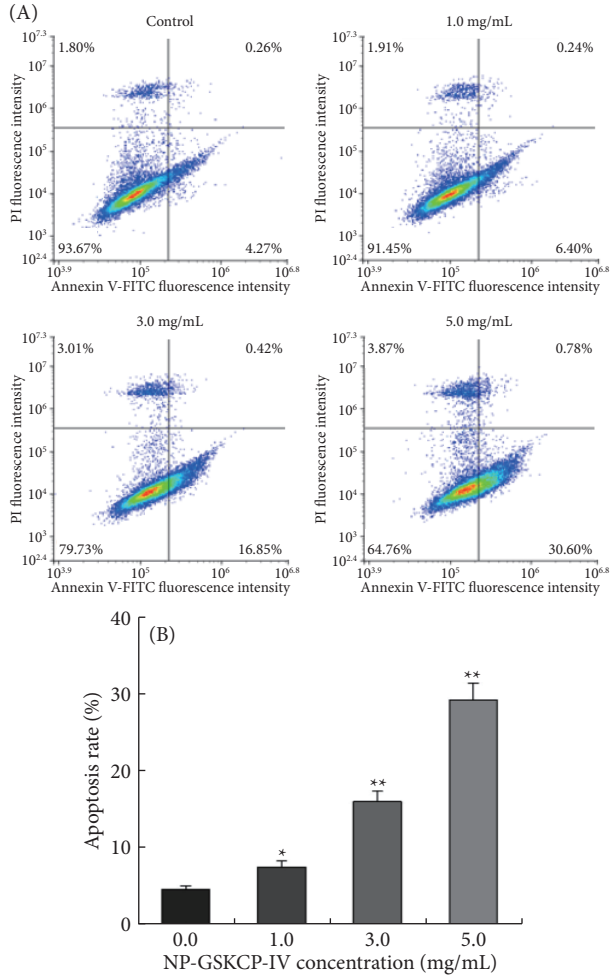

Figure 7 Effects of optimized NP-GSKCP-IV on apoptosis of A549 cells. (A) Flow cytometry for cell apoptosis; (B) Ratio of apoptotic cells. Compared to the control group, $^{*}P < 0.05$, $^{**}P < 0.01$.

4 Discussion

Amphibian collagen peptides exhibit promising applications across diverse sectors, including food, pharmaceuticals, and consumer goods. The present study capitalized on the untapped potential of discarded Chinese giant salamander skin, optimizing its collagen peptide extraction process, thereby contributing vital scientific insights into the sustainable development and utilization of this resource. Initially, the Chinese giant salamander skin collagen was subjected to hydrolysis using neutral protease, enabling the isolation of a spectrum of collagen peptides tailored for specific purposes via ultrafiltration technology. Subsequently, to evaluate the *in vitro* antitumor potential of these enzymatic hydrolysis products, we employed the proliferation inhibition activity against A549 cells as a benchmark. Our findings revealed that collagen peptides of smaller molecular weights, derived from neutral protease hydrolysis, demonstrated the most potent inhibition of A549 cells proliferation. This corroborates prior research, which underscores neutral protease's superiority in generating small molecular peptides and its superior hydrolytic efficiency compared to alternative enzymes[18]. Furthermore, the integration of ultrafiltration technology facilitated the precise segregation of polypeptide fragments within the hydrolysis products based on their molecular weights, facilitating the isolation of bioactive peptides with targeted functionalities. Overall, this study underscores the potential of Chinese giant salamander skin as a valuable source of bioactive collagen peptides, with implications for advancing healthcare, nutrition, and potentially other industries.

Small molecular active peptides are usually between the relative molecular mass of proteins and free amino acids, which are more easily absorbed and utilized by the body[19], thus showing higher bioactivity. The results of this study further verified that there is a negative correlation between the antitumor activity of collagen peptides and molecular weight. The specific mechanism behind the observed negative correlation between the molecular weight of collagen peptides and their antitumor activity is a multifaceted phenomenon that involves several biological processes. Smaller collagen peptides may exhibit superior cellular penetration capabilities. Their reduced size facilitates interactions with cell surface receptors and enables easier translocation across cell membranes. This enhanced permeability can lead to more direct interference with tumor cell growth, proliferation, and metastatic processes. For instance, they may modulate intracellular signaling pathways, induce apoptosis, or inhibit angiogenesis, all of which contribute to antitumor activity. However, it is essential to acknowledge that antitumor activity is not solely determined by molecular weight. Other factors, including the source, purity, specific structural features, dosage, and route of administration of collagen peptides, also play crucial roles. Furthermore, the biological responses to these peptides can vary significantly across different tumor types, which may account for discrepancies in observed antitumor effects. In summary, the negative correlation between collagen peptide molecular weight and antitumor activity likely arises from a complex interplay of factors that influence their bioavailability, cellular penetration, and mode of action. Further research is needed to fully elucidate the specific mechanisms underlying this phenomenon and to optimize collagen peptide-based therapies for cancer treatment. Relevant studies have also pointed out that purification after ultrafiltration can effectively prepare small molecular weight active peptides[20]. In a study, using mice as an animal model, the effects of different molecular weights of skate skin collagen peptides on lipid metabolism in the liver and adipose tissue of mice were studied, and the results showed that the lower the molecular weight of collagen peptides, the better the effect[16]. Another study separated the collagen peptides of squid hydrolyzed by protease through ultrafiltration, and the results showed that the bioactivity of the squid protease hydrolyzed collagen peptides was enhanced after ultrafiltration[17]. These studies are consistent with our research results and support the advantages of small molecular weight collagen peptides in antitumor activity.

Upon comprehensive comparison, it was discovered that pepsin, alkaline protease, and neutral protease, when utilized for hydrolyzing Chinese giant salamander skin collagen peptides, all elicited a noteworthy inhibitory impact on the vitality of A549 cells. Notably, the overall inhibitory potency of the products derived from neutral protease hydrolysis emerged as the most pronounced.

This observation suggests that under proteolytic action, collagen generates polypeptide fragments that possess antitumor activities. Among these enzymes, neutral protease stands out as a non-specific endonuclease, characterized by a greater abundance of proteolytic sites compared to the other enzymes in this study. Consequently, neutral protease generates a higher yield of bioactive fragments, accounting for its superior performance in inhibiting A549 cells vitality. The optimization of peptide size and molecular weight, as demonstrated in studies utilizing enzymatic hydrolysis and membrane ultrafiltration, has revealed that certain peptide fractions exhibit heightened inhibitory effects. However, the molecular weight distribution of collagen peptides after different protease treatments is a highly variable outcome that depends on several factors, including the type of protease used, its specificity, reaction conditions (such as temperature, pH, and duration of treatment), and the initial collagen source and structure. This will be considered in future studies, where the structure-function relationships of collagen peptides should be investigated in greater depth.

This study optimized the extraction process of NP-GSKCP-IV, utilizing both single-factor experimentation and RSM. The optimal extraction conditions were established as follows: enzyme dosage of 8 000 U/g, enzymatic hydrolysis temperature of 52.6 °C, enzymatic hydrolysis pH of 7.1, and enzymatic hydrolysis time of 4.3 h. Under these optimized conditions, the extraction rate of NP-GSKCP-IV reached 87.82%. In contrast, previous research by Ye et al.[21] employed a compound extraction approach, prioritizing protein recovery rate. Their optimal conditions were a hydrolysis time of 7 h, a temperature of 55 °C, and a solid-to-liquid ratio of 1:4 (g/mL), yielding a protein recovery rate of 78.9%, with 46.86% of the peptides having a molecular mass below 5 000 Da. Meanwhile, Li et al.[22] adopted the central composite design, using SAS software to investigate the effects of solid-to-liquid ratio, hydrolysis time, and pressure. Their response surface optimization resulted in an optimal process with a solid-to-liquid ratio of 1:2 (g/mL), a processing time of 25 min, and a pressure of 0.075 MPa, achieving an extraction rate of 80.76% for collagen peptides. Compared to these existing extraction methods for Chinese giant salamander skin collagen peptides, the present study innovatively introduced a two-step hydrolysis process. This approach not only offers milder processing conditions but also imposes lower demands on hydrolysis equipment, simplifying the overall process while enhancing the extraction efficiency.

The analysis of the amino acid composition of NP-GSKCP-IV reveals an abundant presence of essential amino acids, suggesting the substantial nutritional value and promising potential of NP-GSKCP-IV for functional food development. Notably, the content of hydrophobic amino acids comprises a significant proportion, accounting for 55.31% of the total hydrolyzed amino acids. This high proportion of hydrophobic amino acids facilitates the peptides' efficient traversal of the cell membrane's phospholipid bilayer, enabling their intracellular entry and subsequent bioactivity manifestation. Consistent with this observation, Jin et al.[23] reported that neutral protease-hydrolyzed sea cucumber collagen displayed the highest activity and contained an elevated number of hydrophobic amino acid sequences, compared to those obtained through papain, pepsin, and trypsin hydrolysis. Furthermore, extensive research underscores the strong antitumor activity often associated with small molecular weight peptides rich in hydrophobic amino acids. Additional studies corroborate the close link between antitumor activity and hydrophobic amino acid content in peptides. Zhang et al.[24], for instance, isolated antitumor peptides from grass carp meat, where two-step hydrolysis products contained 32.92% hydrophobic amino acids. Similarly, Shi et al.[25] identified three antitumor peptides from Chinese soft-shelled turtle meat, featuring leucine, glycine, lysine, alanine, isoleucine, valine, and cysteine as the most abundant amino acids, with respective hydrophobic amino acid proportions of 47.83%, 48.74%, and 49.15%. Collectively, these findings underscore the favorable antitumor activity of NP-GSKCP-IV, which can be attributed, in part, to its high content of hydrophobic amino acids. This characteristic not only enhances the peptides' cellular penetration but also contributes to their therapeutic potential, positioning NP-GSKCP-IV as a promising candidate for further exploration in functional food and biomedical applications.

This study further delved into the preliminary *in vitro* antitumor activity of NP-GSKCP-IV through a comprehensive suite of experiments, including cell scratch assays, cell cycle analysis, and apoptosis induction tests. The microscopic examination of cell morphology post-treatment revealed that NP-GSKCP-IV significantly altered the appearance of A549 cells. Specifically, the cell scratch assay demonstrated that collagen peptides hydrolyzed by neutral protease effectively decelerated the closure rate of scratches, a phenomenon also observed in other studies where high antitumor activity small molecular peptides inhibited cancer cell migration[26]. Furthermore, cell cycle analysis illuminated that NP-GSKCP-IV arrested the progression of A549 cells in the G0/G1 phase, effectively hindering cellular proliferation and reinforcing its antitumor efficacy. This finding aligns with previous research, which highlights the ability of small molecular active peptides to impede cell growth by perturbing the cell cycle[27]. Lastly, the apoptosis induction experiment revealed that NP-GSKCP-IV treatment triggered apoptosis in A549 cells, displaying a clear dose-dependent relationship. This underscores the peptide's capacity to suppress cellular proliferation via apoptotic mechanisms, echoing similar discoveries in which small molecular active peptides inhibited cancer cell growth by inducing apoptosis[28]. Collagen peptides have emerged as candidates displaying anticancer properties, as evidenced by several *in vitro* investigations revealing their potential against various cancer cell lines[29]. Specifically, peptides extracted from the skin of unicorn leatherjacket fish (*Aluterus monoceros*) exhibited inhibitory effects on COLO 320 cells in controlled laboratory settings[30]. Baehaki et al.[31] further hydrolyzed milk fish (*Chanos chanos*) skins utilizing collagenase derived from *Bacillus licheniformis* F11.4, demonstrating optimal activity against HeLa cells at 60 min and HCT-166 cells at 30 min. The byproduct of barred mackerel was processed with a two-step enzymatic hydrolysis involving actinidin and alcalase, yielding peptides within the 3–10 kDa. At a concentration of 1 mg/mL, these peptides exhibited robust antioxidant (67.75%) and anticancer (96.93%) activities[32]. Yaghoubzadeh et al.[33] adopted membrane ultrafiltration in conjunction with alcalase and flavourzyme enzymes to isolate bioactive peptides from rainbow trout skin, revealing that collagen peptides with molecular weights below 3 kDa possessed the highest inhibitory potency. Collectively, these studies underscore the remarkable anticancer potential of collagen peptides *in vitro*. Nevertheless, to fully comprehend the underlying mechanisms and health benefits associated with these peptides, it is imperative to conduct comprehensive *in vivo* studies that delve into the intricate relationship between their chemical structure and anticancer

activity. Such endeavors will pave the way for the development of targeted therapeutic strategies leveraging the anticancer properties of collagen peptides.

5 Conclusion

In summary, we optimized the extraction process of Chinese giant salamander skin collagen peptides hydrolyzed by neutral protease through single-factor and RSM, determined the optimal process conditions, and the extraction rate reached 87.82%. This study also analyzed the amino acid composition of NP-GSKCP-IV and found that it has a high content of essential amino acids, indicating its high nutritional value and potential for the development of functional foods. In addition, the NP-GSKCP-IV screened in our study has strong antitumor activity and can inhibit the growth and migration of tumor cells through various mechanisms. In conclusion, this study provides important scientific evidence for the development and application of Chinese giant salamander skin collagen peptides and provides valuable references for further research and development of small molecular active peptides with antitumor activity.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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