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Study on antioxidant synergistic anti-inflammatory peptide from Giant Salamander (*Andrias davidianus*): functional mechanism and delivery by nano-starch

Jun-qi Zhan[a,b], Qiao-bo Zhang[a,b], Si-yan Mei[a,b], Jun-xin Wu[d], Yan-wei Yuan[a,b], Gaoshang Li[c], Jing-jing Fu[a,b], Yue-wen Chen [a,b,*]

[a] *School of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou, Zhejiang 310035, China*

[b] *Zhejiang Provincial Collaborative Innovation Center of Food Safety and Nutrition, Zhejiang Gongshang University, Hangzhou, Zhejiang 310035, China*

[c] *School of Food Science and Engineering, Ningbo University, Ningbo 315800, Zhejiang, China*

[d] *Zhejiang Shanding Biotechnology Co., Ltd, Zhejiang, Lishui, 323000, China*

ABSTRACT: In this study, we explored the anti-inflammatory and antioxidant characteristics of the giant salamander peptide P3. We found that P3 enhances the resistance of RAW264.7 cells to Lipopolysaccharide (LPS) by modulating the Mitogen-Activated Protein Kinase / Nuclear Factor kappa-light-chain-enhancer of activated B cells (MAPK/NF-κB) signaling pathway. Specifically, P3 boosted the activation of mitogen-activated protein kinase, interleukin-6 (IL-6), and tumor necrosis factor alpha, while decreasing levels of inflammatory factors like Interleukin-1β (IL-1 β) and nitric oxide (NO). Given its potent antioxidant properties, we further examined impact of P3 in the Keap1-ARE-Nrf2 signaling pathway and its products. P3 increased the levels of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase, while reducing the concentrations of oxidation products like malondialdehyde and catalase. During *in vitro* digestion, the functional efficacy of P3 diminished. However, when combined with nano-starch (NS), NS-P3 demonstrated preserved functionality. Our findings indicate that P3 not only exhibits anti-inflammatory effects but also mitigates chronic oxidative stress damage. NS-P3 effectively safeguards the antioxidant capabilities of P3 during simulated digestion.

Key words: Antioxidant peptides; Anti-inflammatory peptides; Nano starch; *in vitro* digestion

1. Introduction

In recent years, foodborne antioxidant products have garnered significant attention due to their potential to combat oxidative damage and degenerative diseases caused by oxidative stress, which has been extensively researched. Free radicals and other reactive oxygen species (ROS) are inevitably formed during metabolic processes and can damage biomolecules due to various exogenous factors [1]. Oxidative stress and resulting neuronal damage have been linked to various mechanisms of Alzheimer's disease [2]. Research on antioxidants has revealed that free radical inhibitors (antioxidants) react with free radical molecules in multiple chain

***Corresponding author**
chenyw@zjgsu.edu.cn

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reactions, forming stable products [3]. Peptides, as bioactive substances, possess strong antioxidant activity and hydrophobic properties. Their amphiphilic nature enhances peptide solubility, reduces interactions with free radical species, and enhances antioxidant activity, a phenomenon extensively studied [4].

Vascular inflammation-induced cytokines contribute to excessive ROS production, reducing nitric oxide (NO) bioavailability, leading to endothelial dysfunction and potential blood pressure elevation [5]. Inflammatory factors accumulate in connective tissue, triggering inflammatory signaling pathways and related adverse effects and complications [6]. Both animal and plant protein hydrolysates exhibit anti-inflammatory effects in different cell models. While only a few peptides have been identified as functional, peptides possess potent activity and are promising candidates for development [7].

Animal and plant proteins serve as potential sources of bioactive peptides. Hydrolysis of proteins from various sources, such as soy, rice, corn, wheat, sunflowers, eggs, milk, fish, and meat, yields peptides with diverse biological effects, including anticancer, antithrombotic, angiotensin-converting enzyme inhibition, anti-inflammatory, antioxidant, DPP-IV inhibition, metal binding, and immune regulation properties [8]. Bioactive peptides, embedded within precursor proteins, exhibit biological activity upon release. Concentrated products containing bioactive peptides have the potential to prevent or treat human diabetes or cardiovascular diseases [9].

Biological peptides are easily degraded in complex digestive reactions in the body. The delivery system that protects biological peptides can protect the activity and structure of peptides. Hou et al. [10] reported a starch-based nano-capsules for colon-targeting delivery of peptides, which was an effective insulin delivery system. Wang et al. [11] reported a dual-modified starch micelles for efficient encapsulation and controlled release of walnut-derived active peptides.

This study explores the utilization of giant salamander peptides to peptize nano-starch. We investigate the antioxidant and anti-inflammatory properties of the compounds and their resistance to gastric digestion. This research presents a novel peptide delivery approach to prevent the loss of functional properties during gastric digestion.

2. Materials and methods

2.1 Materials

Giant salamander was obtained from Zhejiang Shan ding Salamander R&D Co., Ltd. (Qingyuan, Zhejiang). We chose giant salamanders that had been raised for approximately 1 year and had a body length of over 1 meter as our samples. The giant salamanders were slaughtered and eviscerated before reaching the laboratory. Giant salamanders were stored in biological storage tanks, and transported to the laboratory. In the laboratory, we removed the bones, fat and skin of the giant salamander and further studied the giant salamander meat.

Sephadex G-15, ethanol (95%), $K_2S_2O_8$ (99%), 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), Acetate (99.8%), Ammonium acetate (AR), Phosphate Buffered Saline (PBS), Dulbecco's Modification of Eagle's Medium

(DMEM, High Glucose, L-glutamine, Sodium Pyruvate, Sodium Bicarbonate, Phenol Red) and other reagents were purchased from Macklin (Shanghai Macklin Biochemical Technology Co., Ltd, Shanghai, China)

Caco-2 cell and RAW 264.7 cell (mouse leukemia cells of monocyte macrophage) was purchased from Nanjing Cobioer Biosciences Co., LTD. (Nanjing, Jiangsu, China)

Reactive oxygen species (ROS) detection kit, Human Interleukin 1, 6, 8, 10, NF-κB Kit and Human mitogen activated protein kinase kit (MAPK ELISA) (Wuhan Saipei Biotechnology Co., Ltd, Wuhan, Hubei, China)

2.2 Instruments

UV detector (HD-3000, Shanghai Jiapeng Technology Co., Ltd, Shanghai, China), Nano High performance liquid chromatography (Nano HPLC) (EASYnLC1200, Thermo Scientific, USA), Cell incubator (YAN-80N, Shanghai Yuyan Scientific Instrument Co., Ltd, Shanghai, China), Fluorescence Microscope (Leica DM IL LED, Leica Microsystems GmbH, Wetzlar, Germany), Water bath shaking (SHZ-88, Changzhou Jintan Wanhua Experimental Instrument Factory, Jinhua, Zhejiang, China)

2.3 Methods

2.3.1 Enzymatic hydrolysis of Giant Salamander meat

According to the method from Zhan et al. [12] and improved. We stripped the fat off the meat of the giant salamander. Afterwards, giant salamander meat was feezed dry and crushed. We mixed giant salamander meat powder with water for a 1:20 solution and added 1% (w/v) alkaline protease. This solution was heated to 45 ℃ at pH 8.0 for 5 h. This solution became enzymatic hydrolysates of giant salamander meat.

2.3.2 Isolation and purification of enzymatic hydrolysates from Giant Salamander meat

According to the method from Zhan et al. [12] and improved. We centrifuged the enzymatic hydrolysate of giant salamander meat and retained the supernatant. We pour Sephadex G-15 gel into the chromatographic column (Φ 1.6 cm * 100 cm). Afterwards, we used pure water with a flow rate of 1 ml/min as the mobile phase to separate peptides from the enzymatic hydrolysate of giant salamanders.

2.3.3 Preparation of peptidized Nano starch (NS)

We mixed 10 g rice starch with 100 g pure water, stirred thoroughly, and heated the mixture in a 95 ℃ water bath for 1 h. Afterwards, we added 200 ml of alcohol to the rice starch-water solution and homogenized it for 5 mins using a cell crusher. Nano-starch was obtained by centrifuging the mixture at 5000 r/min for 10 mins followed by freeze-drying.

We combined 10 mg of NS and 20 mg of giant salamander peptide in 10 ml of water, then stir at 200 r/min for 1 h in a shaker at 37 ℃. Next, we added 200 ng of Fluorescein Isothiocyanate (FITC) and continued stirring for another 1 h. We captured fluorescence images of the peptide-bound NS using an inverted fluorescence microscope.

2.3.4 Determination of antioxidant capacity

2.3.4.1 ABTS assay

The experiment was carried out by improving the experimental method of GB39100-2020 [13]. We prepared 7.4 mmol/L ABTS solution and 2.6 mmol/L potassium persulfate solution for ABTS stock solutions. We mixed 88 μL potassium persulfate solution with 5 mL ABTS solution for 12-16 h to make ABTS working solution. The absorbance of the resulting working solution was adjusted to 0.7 ± 0.02 by diluting it with PBS. Next, 200 μL of the diluted working solution was mixed with 10 μL of the sample, and the absorbance was immediately measured after allowing the mixture to stand in the dark for 6 minutes. The ABTS free radical scavenging rate was calculated as follows:

$$\text{ABTS free radical scavenging rate (\%)} = (\text{ODWorking fluid} - \text{ODSample})/\text{ODWorking fluid} * 100\%$$

2.3.4.2 DPPH assay

The experiment was carried out by improving the experimental method of GB39100-2020 [13]. We diluted 100 mmol/L DPPH solution with ethanol to prepare 2 mmol/L DPPH solution. For absorbance measurements, 100 μL of the 2 mmol/L DPPH solution mixed with 100 μL of ethanol was recorded as A_0. A mixture of 100 μL of the 2 mmol/L DPPH solution and 100 μL of the sample solution was recorded as A_1. Additionally, A_2 was recorded for a mixture of 100 μL of the sample solution and 100 μL of ethanol. The DPPH free radical scavenging rate was calculated as follows:

$$\text{DPPH free radical scavenging rate}(\%) = \frac{(A1 - A2)}{(A1 - A0)} * 100\%$$

2.3.4.3 Ferric ion reducing antioxidant power (FRAP) assay

The FRAP analysis involved the method of Benzie et al. [14], and was expressed in μmol ferrous equivalent per gram fraction.

2.3.5 In vitro digestion experiment

According to the method of Li G. et al. [15] with some modifications. The pH of a 5 ml peptide solution (1 mg/mL) was adjusted to 2.0 ± 0.3. Next, 9,600 U/mL pepsin was added for initial digestion at 120 r/min for 1 h. Following this, the mixture was adjusted to pH 7.0 ± 0.3, and 12,000 U/mL trypsin was introduced for further digestion at 120 r/min for 2 h. After digestion, the enzymes were deactivated by placing the mixture in a boiling water bath for 15 mins. Samples after digestion were used in following experiments.

2.3.6 Cell experiment

2.3.6.1 Anti-oxidant cell experiment

According to the method of Li et al. [16], HepG2 cells were cultured in DMEM containing 20% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin at 37 ℃, 95% humidified air and 5% CO_2. When the cells grow to more than 80% fusion, the cells are passaged every 1-2 d. Randomly select one well of cells from a 6-well cell culture plate and transfer them to a 96 well cell culture plate for incubation for 2 h.

Determine the cell survival rate and absorbance values (450nm) of DMEM medium containing different amounts of H_2O_2 (0.05-10mmol/L) by adding the Cell Counting Kit-8 (CCK-8) kit to ensure the half inhibitory concentration (IC_{50}) of H_2O_2.

2.3.6.2 Anti-inflammatory cell experiment

According to the method from Zhang H et al. [17] and improved. The CO_2 concentration in the cell culture chamber was set to 5%. We added pure water to the beaker and put it in the cell culture chamber, when set its temperature at 37 ℃. We seeded and cultured 5×10^4 RAW264.7 cells per well in 96 well plates for 24 h. Then, we replaced the old culture medium with 100 μL culture medium contains different concentrations (1, 2, 4, 8, 16, 32 mg/mL) of Lipopolysaccharide (LPS) for 12 h. 10 μL CCK-8 was added into every well. After 3 h, we measured the absorbance of each well at 450nm. The absorbance of the solution containing cells, LPS, culture medium, and CCK-8 in the well is recorded as B_1. The absorbance of the solution containing cells, culture medium, and CCK-8 in the well is recorded as B_2. The absorbance of the solution containing culture medium and CCK-8 in the well is recorded as B_0. Macrophage viability assay was counted as follow:

$$\text{Cell viability } (\%) = \frac{(B1 - B0)}{(B2 - B0)} * 100\%$$

We conducted anti-inflammatory cell experiments based on the results of the anti-inflammatory cell experimental model. We seeded and cultured 5×10^5 RAW264.7 cells per well in 6 well plates for 48 h. Then, we replaced the old culture medium with 2 ml culture medium contains 8 mg/mL LPS for 12 h. After that, we removed and lysed the cells. After centrifuging at 10000 r/min for 10 min, we stored the supernatant in a -20 ℃ frozen.

2.3.7 Determination of anti-inflammatory capacity

According to the instructions from manufacturer, human ELISA kits were used to determine the absorbance of inflammatory pathway marker NF- κB and MAPK at 450nm.

2.4 Statistical analysis

All tests were performed thrice parallelly, and the results were expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA), and significant differences were analyzed by Duncan multiple comparisons ($P < 0.05$) using software SAS8.1 (SAS Campus Drive, Cary, NC USA). The results were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1 Preparation of Giant Salamander Peptide

In Figure 1, giant salamander peptides are categorized into four groups based on their molecular weights, with Group 1 having the highest and Group 4 the lowest. Each group was individually assessed for antioxidant and anti-inflammatory properties. Notably, Group 3 (P3) exhibited the highest ABTS and DPPH clearance rates, while Group 2 (P2) showed the highest in the t-AOC antioxidant activity. Moreover, P3 demonstrated

the most prominent anti-6inflammatory properties. The sequence of P3 was further analyzed by mass spectrometry.

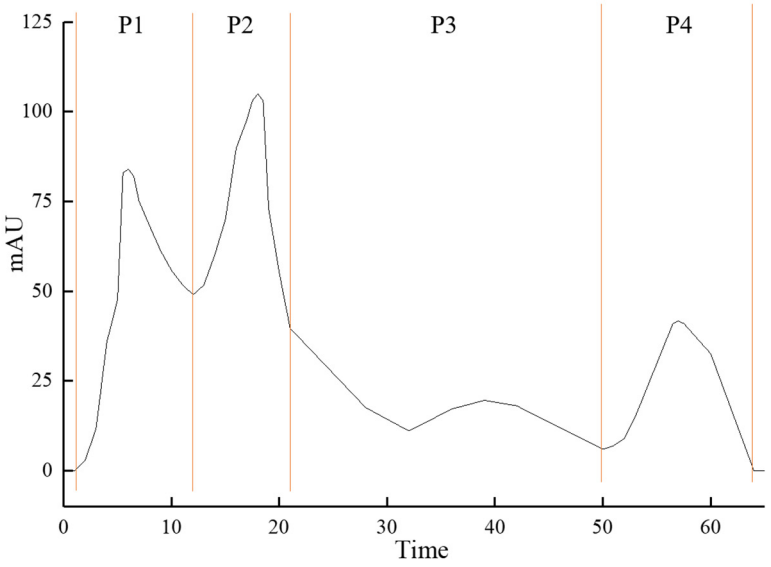


Figure 1 Isolation of salamander peptides by gel chromatography ($P< 0.05$)

3.2 Preparation of NS

The diameters of both NS and peptidized NS were determined using a particle size analyzer. As depicted in Figure 2, the diameter of NS is approximately 600 nm, while that of (nano-starch-P3) NS-P3 is around 800 nm. To mitigate the digestion of P3 by gastric enzymes and acid, we formed NS-polypeptide complexes by combining P3 with NS and embedding the peptides into the starch. Initially, NS was prepared using the anti-solvent extraction method [18], followed by freeze-drying and preservation.

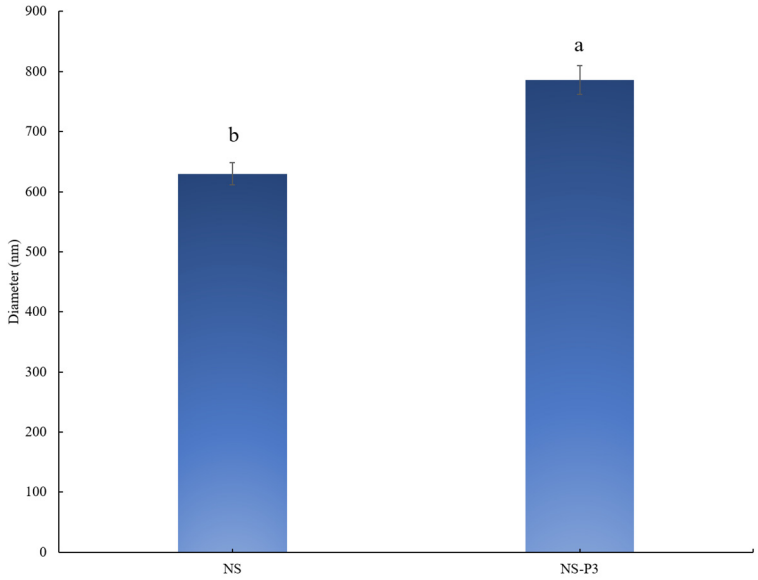


Figure 2 Determination of size of salamander peptide P3 and nano starch-P3 (NS-P3) by particle size analyzer

3.3 Microstructure of Peptidized NS

Bright green fluorescence can be observed under a fluorescence microscope when peptidylated NS is combined with FITC. Through fluorescence microscopy, we determined that the giant salamander peptide and NS were bound through the C-terminus, and FITC was bound to the giant salamander peptide at the

N-terminus. This is similar to Li's fluorescence image [13] of NS, which he combined with poly-lysine to bridge FITC. FITC can not only combine with poly-lysine, but also with ornithine [15]. This indicates that the salamander peptide may have a lysine or ornithine terminal and connect to FITC. We combined P3 with FITC to obtain fluorescent P3 (FITC-P3). Then combine FITC-P3 with NS. After cleaning excess starch and P3, we took fluorescence images. In Figure 3, it can be seen that NS was successfully combined with P3.

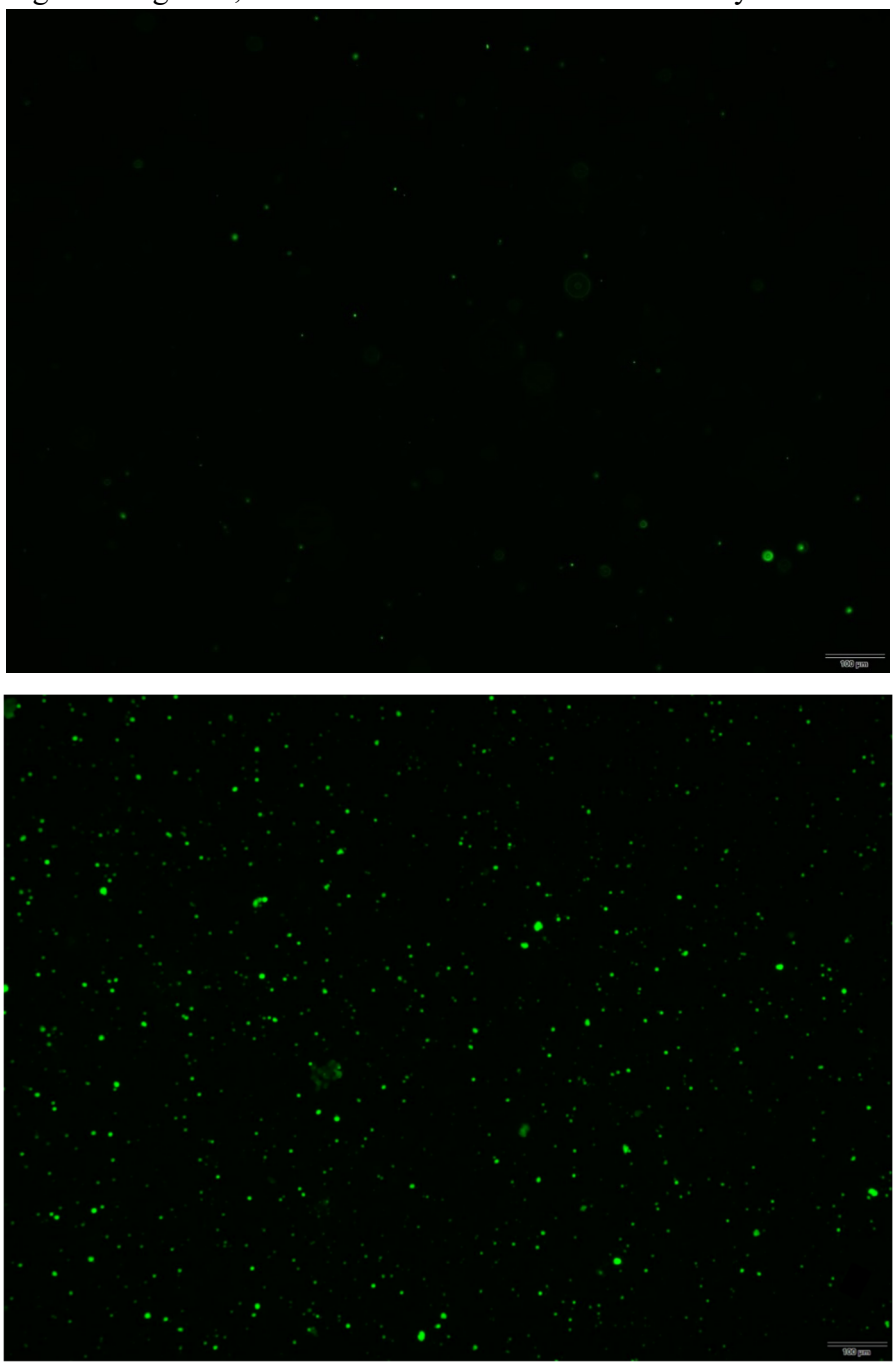


Figure 3 Photographing the combination of FITC and NS-P3 by Fluorescence microscope. (A) 5 mg/ml NS combined with 2.5 μg/ml FITC. (B) 5 mg/ml NS-P3 combined with 2.5 μg/ml FITC.

3.4 Detection of antioxidant properties and mechanism of action

3.4.1 ABTS、DPPH、FRAP assay

In the DPPH and ABTS free radical scavenging experiments, both ABTS free radicals and DPPH free radicals lacked electron radicals. In the experiment, the absorbance before and after the combination of free

radicals and antioxidant substances was detected. Although both follow the reaction mechanism in which free radicals are neutralized after electron transfer mixed with hydrogen transfer, the reaction ratios and reaction rates followed by the two are different. Generally, electron transfer is very fast, while hydrogen transfer is relatively slow [19]. Hydrogen atom transfer is not affected by pH, but is strongly affected by solvent and inhibited by hydrogen bonding solvents such as alcohols. These differences actually have a critical impact on the role of antioxidants in complex multiphase systems [20].

3.4.1.1 ABTS assay

ABTS•+ is a radical monocation generated by the pre-reaction of potassium persulfate oxidation of 2,2-azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [21]. ABTS radicals are widely used to test the antioxidant properties of peptides. In the study of salamander peptide P3, the scavenging ability of P3 on ABTS free radicals reached (79.72%). After gastric digestion, the antioxidant properties of P3 decreased, but after intestinal digestion, the antioxidant properties of P3 increased slightly. However, as shown in the Figure 4, after *in vitro* digestion, the ABTS free radical scavenging rate of P3 was significantly reduced.

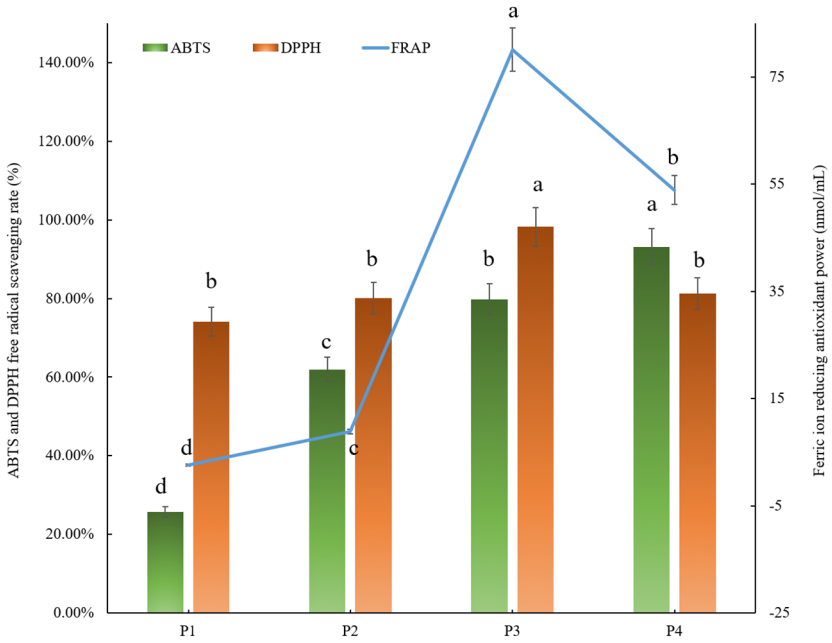


Figure 4 ABTS free radical scavenging rate, DPPH free radical scavenging rate and FRAP of giant salamander peptide P3

3.4.1.2 DPPH assay

The free radical DPPH• is a relatively stable substance that has been widely used to determine the free radical scavenging activity of natural compounds [21]. Although it has been reported that DPPH free radicals are not sensitive to the antioxidant binding sites of peptides [22]. But the DPPH free radical is one of the most stable free radicals. The DPPH radical scavenging assay is a simple and fast method to test the ability of a compound to act as a hydrogen donor [23]. In the study of giant salamander peptide P3, the scavenging ability of P3 on DPPH free radicals reached 98.26%. After gastric digestion, the antioxidant properties of P3 decreased, but after intestinal digestion, the antioxidant properties of P3 increased slightly. However, as

shown in the Figure 4, after *in vitro* digestion, the DPPH free radical scavenging rate of P3 was significantly reduced.

3.4.1.3 FRAP assay

For iron ion, antioxidant activity was measured by a single electron transfer-based assay [24]. In Figure 4, the scavenging capacity of salamander peptide P3 for iron ions reaches 0.0801 μmol/mL. Compared with surimi peptide [15], salamander peptide P3 shows stronger antioxidant properties. However, after *in vitro* digestion, the antioxidant properties of P3 decreased slightly. After *in vitro* digestion, the integrity of the peptides decreases and becomes easily degraded and absorbed by the intestines [25]. The antioxidant properties of the peptides may also be affected and reduced.

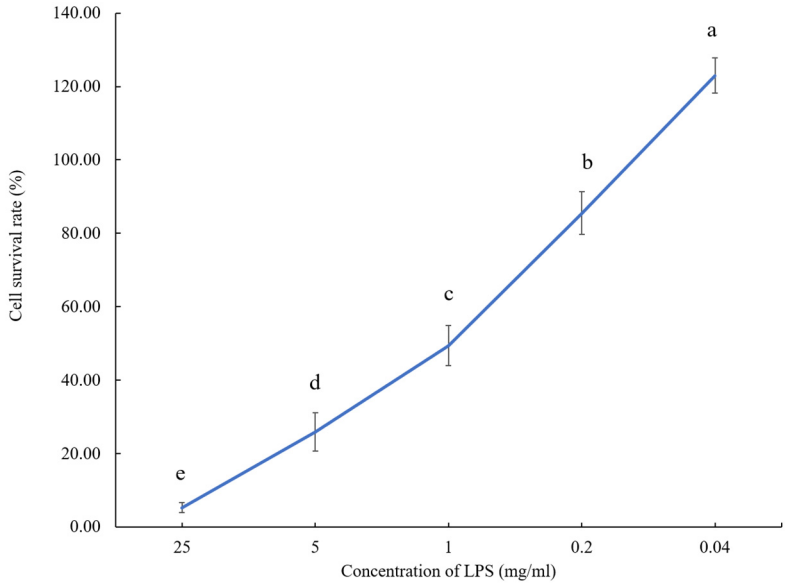
3.4.2 Salamander peptide P3 affects the Keap1-Nrf2 antioxidant signaling pathway

3.4.2.1 Construction of Caco-2 cell antioxidant model and peptide cellular absorption rate

Caco-2 cells were cultured in Permeable Supports. The Pore Size of Permeable Supports can ensure that cells grow in the upper compartment. The culture medium and samples in the culture medium can enter the lower compartment through the Pore. After Caco-2 cells were continuously cultured for 21 d, Hank's Balanced Salt Solution (HBSS) containing 1 mg/mL salamander peptide was added. In Figure 5a, after further incubation for 3 h, the peptide content in HBSS dropped to 490.67 μg/mL. Therefore, Salamander peptide P3 can be absorbed by cells without digestion. When cells were cultured for 4 h, the protein and peptide content in the HBSS increased, which may be due to cell death caused by long-term serum-free culture. Intracellular materials were released into the HBSS.

To induce oxidative stress, we supplemented the culture medium with H_2O_2 (8 μmol/mL). The viability of Caco-2 cells was assessed by measuring absorbance after the addition of CCK-8 to serum-free medium. Following incubation with 8 μmol/mL H_2O_2 for 40 minutes, the cell survival rate approached 50%, registering at 48.52%.

a



b

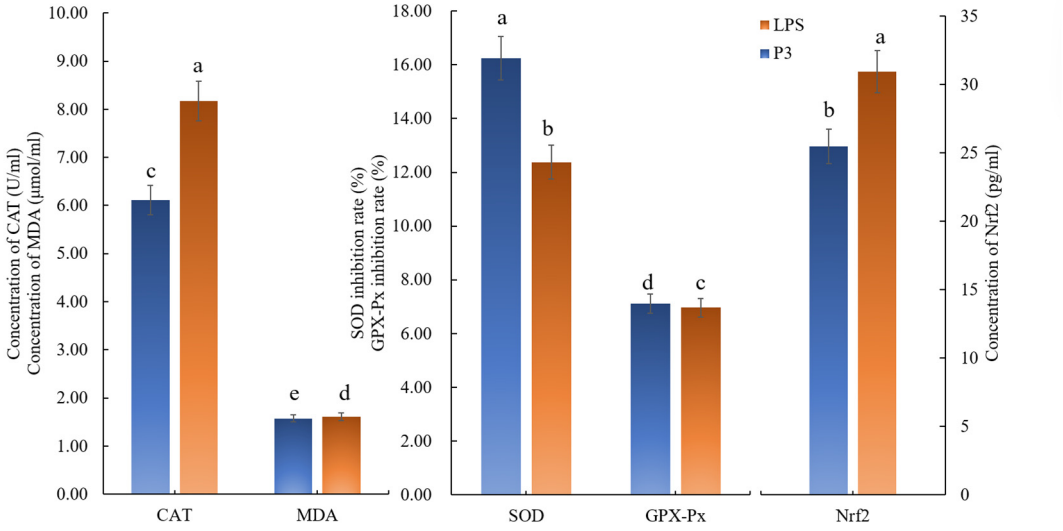


Figure 5 Caco-2 cell survival rate and the effect of giant salamander peptide P3 on intracellular Keap1-Nrf2 signaling pathway

3.4.2.2 Caco-2 cell antioxidant experiment

After cells are oxidatively damaged, many oxidative products are produced. At the same time, the intracellular antioxidant enzyme content will also change. After Caco-2 cells were treated with 8 μmol/mL H_2O_2 for 30 min, the intracellular contents of MDA, SOD, Nrf2, CAT and GPX-Px were measured.

In Figure 5b, after salamander peptide P3 was added to the culture medium, the contents of MDA, ROS and CAT in cells changed. MDA and CAT content decreased while SOD content increased. SOD disproportionated superoxide anions into hydrogen peroxide and oxygen. CAT enzymatically decomposes H_2O_2 into H_2O and O_2. The content of GPX-Px increased, and GPX-Px can reduce the accumulation of lipid peroxides in cells. Due to the reduction in intracellular peroxide, the content of MDA is also reduced. Using Dulbecco's modified eagle medium added P3 to culture cells improves cell survival rate and intracellular antioxidant enzyme content, and reduces the accumulation of peroxides. We also detected the content of Nrf2. As an important transcription factor in the Keap1-Nrf2 pathway, increased levels of Nrf2 contribute to the expression of antioxidant enzymes.

3.5 RAW264.7 cell anti-inflammatory experiment

3.5.1 Construction of RAW264.7 anti-inflammatory cell model

We constructed the RAW264.7 cell anti-inflammatory model by adding LPS to culture cells in DMEM for 25 h. LPS induced cells to produce symptoms of inflammation. After 25 h of culture in DMEM containing 1 mg/mL LPS, in Figure 6a, the cell survival rate reached about 50% (49.40%). The levels of NO, interleukin, NF-κB and TNF-α in cells that produce inflammation all change.

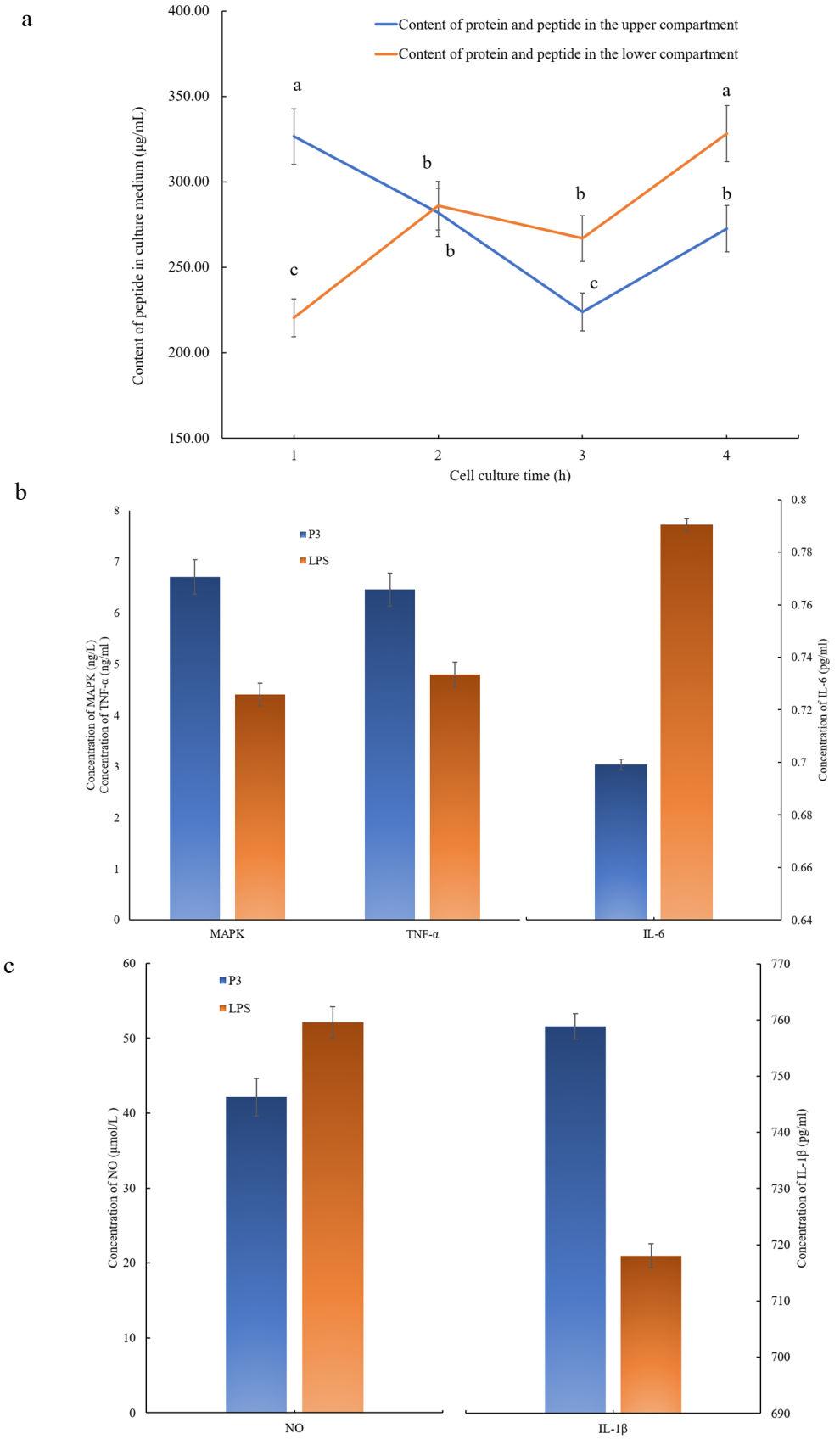


Figure 6 RAW264.7 cell survival rate and the effect of giant salamander peptide P3 on intracellular MAPK/NF-κB signaling pathway

3.5.2 Salamander peptide P3 affects the MAPK/NF-κB signaling pathway

As a ligand, LPS can activate Toll-like receptors (TLRs), which then attracts MyD88 and activates the NF-κB signaling pathway [26]. Finally, it translocated the p65-p50 dimer to the nucleus and binds to DNA sites

to regulate downstream gene expression [27]. Previous studies have shown that LPS-induced inflammation can cause changes in the cellular NF-κB signaling pathway. After LPS comes into contact with the cell wall, it binds to receptor proteins. The receptor protein activates IκB kinase (IKK). IKK phosphorylates the serine at the regulatory site of the IκB subunit of the intracellular NF-κB·IκB complex, leading to ubiquitination and modification of the IκB subunit, which is then degraded by proteases and releases the NF-κB dimer [28]. Free NF-κB will enter the nucleus and bind to genes with NF-κB binding sites, initiating the transcription process. The expression of NF-κB leads to the production of IL-1β, THF-α and IL-6 [29]. LPS stimulates RAW264.7 cells to promote the transcription and expression of nuclear factor NF-κB. The expression of NF-κB leads to the production of IL-1β, THF-α and IL-6. We detected IL-1β, THF-α, and IL-6 in LPS-stimulated RAW264.7 cells. From Figure 6b and 6c we can see cells cultured with NS-P3 released less IL-6, THF-α and MAPK. Compared with the blank group (LPS group), the concentration of inflammatory factors in the experimental group (NS-P3 group) was lower. These three cytokines bind to different anti-inflammatory target organs. IL-6 and THF-α can influence other immune cells to trigger an immune response. We had the same results for IL-6 and THF-α in the NF-κB signaling pathway. At the same time, IL-1β is an important cytokine that produces inflammation. However, exposure of cells to IL-1β at lower concentrations than below may lead to sub-health and other chronic diseases. Excess IL-1 can also promote intracellular NO production. We also measured intracellular NO concentration. In Figure 6d, even though the IL-1β concentration increased, the NO concentration did not increase. NO and IL-1β are important indicators of inflammation in cells, and they also affect the conduction of other signal pathways. Food-borne anti-inflammatory peptides mainly work by regulating the release of inflammatory cytokines, oxidative stress response, inflammation-related signaling pathways and the expression of transcription factors [30].

3.6 NS-peptide resists gastric digestion

In the ABTS free radical scavenging experiment, DPPH free radical scavenging experiment and iron ion reduction experiment of giant salamander peptide P3, in Figure 7, the antioxidant performance of P3 decreased rapidly after gastric digestion, while the antioxidant performance of P3 increased significantly after gastrointestinal digestion. It may be that gastric acid and pepsin destroy the integrity and spatial structure of P3, and P3 becomes a shorter peptide with enhanced hydrophobicity. P3 becomes short peptides and amino acids after digestion in the gastrointestinal tract. Although P3 also has antioxidant properties, it is also quickly absorbed by the intestine [31]. The antioxidant property of NS-P3 solution reached the highest level 15 min after gastric digestion, but the antioxidant property of NS-P3 was much smaller than that of P3 at this time. After 45 min of gastric digestion, the antioxidant properties of NS-P3 decreased slightly. But at this time, 90% of the liquid has entered the intestines [32].

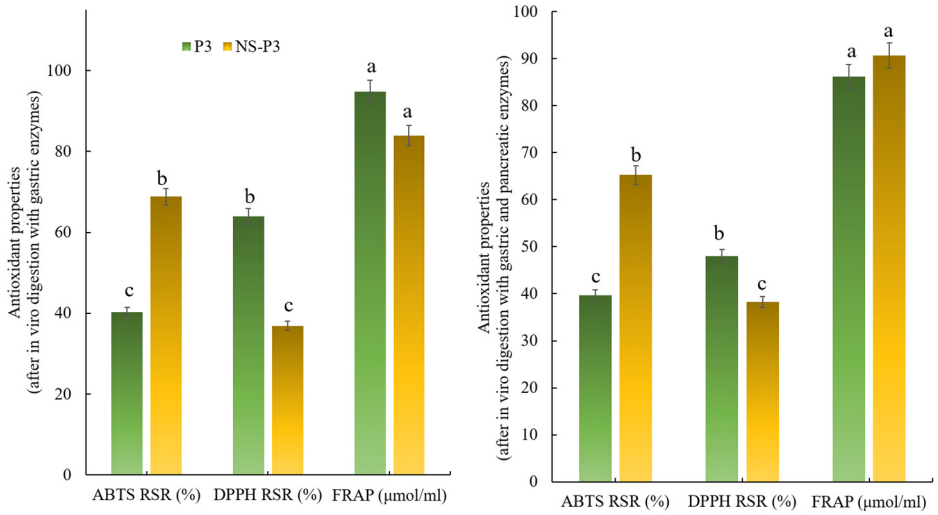


Figure 7 Antioxidant properties of giant salamander peptide P3 and NS-P3 in *in vitro* digestion

We tested the ABTS free radical scavenging rate, DPPH free radical scavenging rate, FRAP free radical scavenging rate, cellular antioxidant properties, and cellular anti-inflammatory properties of NS-P3 before and after *in vitro* digestion. As can be seen from Figure 7, NS improved the antioxidant properties of P3 in *in vitro* digestion and did not affect the anti-inflammatory properties of P3. P3 embedded in NS is also digested normally by the intestine.

4. Conclusion

Giant salamander is an emerging food ingredient that is also very rare in China, where it originated. But with the development of freshwater aquaculture, the nutritional value of giant salamanders has also been studied. Giant salamander meat is a source of high-quality protein and has the potential to prepare functional peptides. We obtained giant salamander protein hydrolyzate through enzymatic hydrolysis of giant salamander meat. Giant salamander proteolytic solution has strong antioxidant properties, ABTS free radical scavenging rate (79.72%), DPPH free radical scavenging rate (98.26%) and iron ion reducing capacity (0.0801 μmol/mL). We separated the giant salamander proteolytic solution through G-15 dextran gel. Giant salamander proteolytic solution is divided into four groups (P1-P4), among which P3 has the strongest antioxidant property. Oxidative reactions often occur simultaneously with inflammatory reactions. We also examined the inhibitory effect of P3 on inflammatory responses. We found that P3 can act in the MAPK/NF-κB signaling pathway. P3 can reduce the production of inflammatory factors TH-α, IL-6, IL-8 and NO, and reduce cellular inflammatory response. P3 can also increase the production of IL-1β and IL-2 and promote cell resistance to LPS. As a proteolytic solution, P3 is first digested and degraded by pepsin and gastric acid in the stomach after entering the digestive system. But stomach acid and pepsin can affect the antioxidant properties of P3. We make NS from rice starch through anti-solvent extraction method. In the particle size analyzer, the diameter of NS is 300-500 nm. NS can embed P3 to form a complex. The effects of gastric enzymes and gastric acid on the antioxidant properties of NS P3 complex were reduced. In Caco-2 cell antioxidant experiments, NS P3 complex can reduce the production of peroxide, increase the expression of antioxidant enzymes and reduce the damage of hydrogen peroxide to cells by affecting nuclear factor Nrf2. In

the anti-inflammatory experiment on RAW264.7 cells, NS did not affect the anti-inflammatory properties of P3. NS P3 complex can be used as a potential material to alleviate oxidative damage and inflammatory damage. The combination of NS and P3 is also an effective method to prepare highly functional active substances.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Reference

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