

Bioactive Peptides from Blackberry (*Rubus* L.) Seed Proteins: Enzymatic Production, Fractionation, and Multi-Functional Properties

Shaoyi Wang

Institute of Botany, Jiangsu Province and Chinese Academy of Sciences

Fengyi Zhao

zhaofengyi92@163.com

Institute of Botany, Jiangsu Province and Chinese Academy of Sciences

Hongxia Liu

Institute of Botany, Jiangsu Province and Chinese Academy of Sciences

Huifang Zhao

Institute of Botany, Jiangsu Province and Chinese Academy of Sciences

Wenlong Wu

Institute of Botany, Jiangsu Province and Chinese Academy of Sciences

Yaqiong Wu

Institute of Botany, Jiangsu Province and Chinese Academy of Sciences

Jinshu Tian

Nanjing Tian Guo Agricultural Science and Technology Co., Ltd.

Weilin Li

Nanjing Forestry University

Research Article

Keywords: blackberry seeds, bioactive peptides, enzymatic hydrolysis, ultrafiltration, antioxidant activity, anticancer activity

Posted Date: June 18th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9753294/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Blackberry (*Rubus* L.) seeds are an underutilized byproduct of fruit processing, containing substantial protein (20–25% dry weight) with potential for generating bioactive peptides. This study aimed to optimize enzymatic hydrolysis conditions for blackberry seed protein isolate (BSPI) and evaluate the bioactivities of the resulting peptide fractions. Response surface methodology identified alkaline protease as the optimal enzyme, achieving a 22.98% degree of hydrolysis under optimized conditions (2.5% substrate, 16,500 U/g enzyme, pH 9, 3 h). The hydrolysate was fractionated by ultrafiltration into four molecular weight fractions (< 3, 3–5, 5–10, > 10 kDa). The < 3 kDa fraction exhibited the strongest antioxidant activity (DPPH IC₅₀: 15.38 µg/mL), significant α-glucosidase (50.99%) and α-amylase (63.87%) inhibition, and potent pancreatic lipase inhibition (62.55%). Notably, this fraction demonstrated exceptional cytotoxicity against HepG2 cells (IC₅₀: 0.12 µg/mL) with low toxicity toward normal HUVEC cells (IC₅₀: 288.24 µg/mL). In vivo studies in a xenograft mouse model confirmed significant tumor growth inhibition (48.95%) without observable organ toxicity. Blackberry seed-derived peptides, particularly the < 3 kDa fraction, possess multi-functional bioactive properties including antioxidant, hypoglycemic, hypolipidemic, and anticancer activities. These findings support the potential application of blackberry seed peptides as functional food ingredients or nutraceuticals, contributing to the valorization of agricultural byproducts.

1. Introduction

Plant-derived proteins represent a critical resource for sustainable biomaterials, extending beyond traditional food applications into pharmaceutical and industrial sectors [1, 2]. While seeds from conventional crops like legumes and cereals have been extensively studied, numerous underutilized species offer untapped potential for novel bioactive compounds [3]. Blackberry (*Rubus* L.) seeds, a major byproduct of juice and wine processing, account for 20–30% of fruit pomace and contain approximately 20–25% crude protein on a dry weight basis, yet remain largely underutilized [4].

Previous research on blackberry seeds primarily focused on polysaccharides exhibiting anticoagulant activity and phenolic compounds with anti-inflammatory effects [5, 6]. However, the protein fraction, which constitutes a major component of the seed, has received limited attention [7]. Unlike conventional seed proteins, blackberry seed peptides represent a novel valorization pathway for agro-industrial waste. Importantly, the bioactivities reported for crude extracts cannot be solely attributed to proteins or peptides without dedicated isolation and characterization, such as blood lipid reduction [8, 9]. Our study specifically targets the protein fraction, isolating and hydrolyzing it to generate bioactive peptides, thereby clearly distinguishing our work from prior studies and addressing a significant knowledge gap.

This study addresses key gaps relevant to food and agricultural industries: (1) Waste valorization: utilizing discarded blackberry seeds as a non-food biomass feedstock [4]. (2) process optimization—developing an enzymatic-ultrafiltration biorefinery approach for peptide fractionation [10]. (3)

multifunctional characterization—evaluating antioxidant, hypoglycemic, hypolipidemic, and anticancer activities to support the development of functional food ingredients or nutraceuticals [11, 12].

We establish that optimized blackberry seed peptides exhibit exceptional *in vitro* and *in vivo* bioactivities meriting further development as therapeutic agents or functional biomaterials [13, 14], aligning with industrial crop valorization objectives [15]. This work provides the first comprehensive evidence for transforming horticultural waste into high-value peptide products with food and nutraceutical potential [16].

2. Material and methods

2.1. Material and chemicals

Blackberry seeds were obtained from Baima Experimental Station (Institute of Botany, Jiangsu Province and Chinese Academy of Sciences). Alkaline protease (from *Bacillus licheniformis*, activity ≥ 160 U/mg), α -glucosidase (EC 3.2.1.20), α -amylase (EC 3.2.1.1), porcine pancreatic lipase (Type II), cholesterol esterase, and ultrafiltration membranes (3, 5, 10 kDa MWCO) were sourced from Nanjing Keybionet Biotechnology Co. Ltd. (China). Cell lines (HepG2, MCF-7, Hela, A549, HUVEC) were acquired from the National Collection of Authenticated Cell Cultures. All other chemicals were analytical grade.

2.2. Protein Isolation and Hydrolysis

Blackberry seed protein isolate (BSPI) was prepared according to our optimized method.⁴ Defatted seed powder (1:19 w/v in H₂O) was extracted at 50°C (pH 10, 78 min), centrifuged (6,000 ×g, 20 min), and precipitated at pH 4.5. The isolate was freeze-dried and stored at – 20°C.

Enzymatic hydrolysis was performed using alkaline protease (selected based on preliminary screening due to its high efficiency in hydrolyzing glutelin-rich proteins, which are predominant in blackberry seeds). Response surface methodology (RSM) optimized hydrolysis conditions: A. substrate concentration (1–3%), B. enzyme dose (14,000–18,000 U/g), C. time (2–4 h), D. pH (9–10) (Table 1). Hydrolysates were inactivated (100°C, 10 min), centrifuged (6,000 ×g, 10 min), and supernatants lyophilized.

Table 1
Response surface test factors and levels

Factors	Levels		
	-1	0	1
A	1	2	3
B	14000	16000	18000
C	2	3	4
D	9	9.5	10

2.3. Peptide Fractionation

BSPI hydrolysate was fractionated using Amicon® stirred ultrafiltration (Millipore) with 3, 5, and 10 kDa membranes [17]. Fractions were collected as: F1: <3 kDa; F2: 3–5 kDa; F3: 5–10 kDa; F4: >10 kDa. Lyophilized fractions were stored at – 80°C.

2.4 Bioactivity Assays

2.4.1 Antioxidant activity

Total antioxidant capacity was determined using a T-AOC (A015-2-1) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In similar, DPPH scavenging: A153-1-1 kit. Superoxide anion (O_2^-) scavenging: A052-1-1 kit. Hydroxyl radical ($\cdot OH$) scavenging: A018-1 kit. Results expressed as IC_{50} or Trolox equivalents (TE).

2.4.2 Antihyperglycemic activity

α -Glucosidase inhibition: pNPG substrate method [18]. 500 μL of the sample and 500 μL of α -glucosidase solution (6 mol/L) were added, then incubated in water bath at 37 °C for 10 minutes, added 500 μL of pNPG (2.5 mmol/L) solution and further incubated for 40 minutes. 2.0 mL of Na_2CO_3 (0.2 mol/L) was immediately added to terminate the reaction, and were measured the absorbance at 405 nm. The inhibition rate was calculated as follows: $Y = ((A_0 - (A_1 - A_2)) / A_0) \times 100$.

Where: Y is the α -glucosidase inhibition rate (%); A_0 is the absorbance of the control group; A_1 is the absorbance of the sample group; A_2 is the absorbance of the background group.

α -Amylase inhibition: Bernfeld method [19]. In this experiment, phosphoric acid buffer (0.2 mol/L, pH 6.9) was used to prepare the reagents. A 1% starch solution was prepared and boiled for 10 minutes while stirring with a glass rod, then cooled to the final volume of 1%. A 1 U/mg α -amylase solution was prepared with the same solvent and refrigerated at 4 °C. 0.25 mL of enzyme solution was taken and immersed in water bath at 37 °C for 1 minute, 0.25 mL of samples of different concentrations were added, and incubated at 37 °C for 4 minutes. Then 0.5 mL of starch solution was added and mixed well,

and incubated at 37 °C for 4 minutes. 1 mL of DNS reagent was added and boiled in a water bath for 8 minutes. After cooling to room temperature, 10 mL of distilled water was added and the absorbance of the mixed solution was measured at a wavelength of 540 nm. The mixed solution without α-amylase was used as a blank control to calculate the inhibition rate of α-amylase.

$$\text{Inhibition rate (\%)} = ((A-B)/A) \times 100$$

Where: A is the activity of α-amylase with inhibitor (U/mg); B is the enzymatic activity of α-amylase without inhibitor (U/mg).

2.4.3 Anticancer activity

In vitro cytotoxicity. The methyl thiazolyl tetrazolium (MTT) method was used to study the inhibitory effects of four protein peptide components on human cancer cells, including HeLa, HepG2, MCF-7, A549, and HUVEC cells. The inhibition rate of the samples on the five types of cells was calculated with IC₅₀ values [20, 21].

In vivo antitumor efficacy. This study was contracted to Nanjing KeyGEN BioTECH Co., Ltd. (China). Female BALB/c nude mice (age: 5–6 weeks) were utilized. HeLa cells were harvested. Control Group: Intravenous injections of PBS. Treatment Group: Intravenous injections of < 3 kDa protein peptide component (10 mg/kg body weight; 100 µL/10 g body weight). Treatments were administered once daily. Tumor volumes and body weights were measured every 3 days for 21 days. Tumor volume (V) was calculated as: $V (\text{mm}^3) = (\text{Length} \times \text{Width}^2) / 2$. Tumor growth inhibition rate (TGI%) was calculated as: $\text{Inhibition efficiency (\%)} = (1 - \text{The weight of the experimental group} / \text{The weight of the control group}) \times 100\%$ [20, 21].

2.4.4 Hypolipidemic Activity

Bile acid binding

Sodium taurocholate binding assay [22]. The cholate binding rate was determined by adding 1 mL of the sample solution into a centrifuge tube, followed by 1 mL of 0.01 mol/L HCl solution to simulate the environment of the human stomach. The mixture was incubated in a constant temperature water bath at 37 °C for 1 hour. The pH of the solution was then adjusted to 6.3 using 0.1 mol/L NaOH solution. Subsequently, 5 mL of 1 mmol/L taurine cholic acid solution was added, and for the blank tube, 5 mL of phosphate buffer (0.1 mol/L, pH 6.3) was added. After 1 hour of ultrasound in a constant temperature water bath at 37 °C, the mixture was centrifuged at 6000 r/min for 25 minutes. 2.5 mL of the supernatant was taken, 7.5 mL of 60% sulfuric acid solution was added and incubated at 70 °C for 30 minutes, then cooled to room temperature. The absorbance was measured at 387 nm. The test was repeated three times, and the cholate binding rate was calculated using the following formula

$$\text{Cholate binding rate (\%)} = (C_0 - C_1) / C_0 \times 100$$

Where: C_0 is the concentration of cholate in the blank (without peptide), $\mu\text{mol/mL}$; C_1 is the concentration of cholate in the supernatant after adding the peptide, $\mu\text{mol/mL}$.

Pancreatic lipase inhibition

p-Nitrophenyl laurate method [23]. Type II porcine pancreatic lipase was prepared into a 5 mg/mL solution with distilled water. A reaction substrate, p-nitrophenyllaurate (pNPG), was dissolved in an aqueous solution containing 1% Triton X-100 and 0.05 mol/L sodium acetate to prepare a 0.1% (w/v) pNPG solution. The lipase inhibition rate was calculated, and the IC_{50} value was determined using the following formula

$$\text{Pancreatic lipase inhibition rate (\%)} = (1 - (A_1 - A_2) / A_0) \times 100$$

Where: A_1 is the absorbance of the sample supernatant; A_2 is the absorbance of the sample control supernatant; A_0 is the absorbance of the blank supernatant.

Cholesterol esterase inhibition

p-Nitrophenyl butyrate method [24]. The inhibitory effect of protein peptides on cholesterol esterase was determined using porcine pancreatic cholesterol esterase prepared with distilled water at a concentration of 4.2 $\mu\text{g/mL}$. All reactions were performed in sodium phosphate buffers containing NaCl (0.1 mol/L), p-nitrophenylbutyrate (PNPB) (0.2 mmol/L), and sodium taurocholate (STC) (5.16 mmol/L), with a buffer concentration of 0.1 mol/L and a pH of 7.0. The absorbance was measured at 405 nm. The blank control tube replaced the enzyme solution and sample solution with primary water, while the background control tube replaced the enzyme solution with distilled water. Cholesterol esterase inhibition rate (%) was calculated using the following formula

$$\text{Cholesterol esterase inhibition rate (\%)} = (1 - (C - D) / (A - B)) \times 100$$

Where: A is the absorbance of the blank tube; B is the absorbance of the blank focus; C is the absorbance of the sample tube; D is the absorbance of the background

2.5 Statistical analysis

Data expressed as mean $\pm$ SD ($n = 3$). One-way ANOVA (SPSS 16.0) with Tukey's test ($p < 0.05$). Response surface analysis used Design-Expert® v8.0.5. A Box-Behnken Design (BBD), a widely used RSM design for optimizing process parameters, was employed with four independent variables (Factors A-D) at three levels each (-1, 0, +1 as defined in Table 1). This design allowed for efficient fitting of a quadratic model to predict the hydrolysis degree (Y).

3. Results and discussion

3.1. Preparation of protein hydrolysate isolated from blackberry seed

3.1.1 Determination of hydrolysis degree

The degree of hydrolysis (DH) is a measure of the ability of a protein to hydrolyze into a polypeptide molecule. The chain length of the polypeptide chain and the types of terminal amino acids significantly affect its biological activity. The higher the degree of protein hydrolysis, the higher the degree of hydrolysis of the polypeptide chain, resulting in a greater variety of terminal amino acids [25]. In this experiment, neutral protease, alkaline protease, acid protease and papain were used to hydrolyze blackberry seed protein under the optimum conditions for each enzyme. The degree of hydrolysis was shown in Fig. 1. The highest degree of hydrolysis was achieved with alkaline protease at 10.95%, followed by acid protease at 9.61%, while neutral protease exhibited the lowest degree of hydrolysis at 2.57%. Liu also concluded that the highest degree of hydrolysis with alkaline protease was 17.86%, which corresponded to its highest antioxidant capabilities [26]. The higher the degree of hydrolysis, the greater the amount of peptides produced, which can indicate a higher potential for biological activity. The high degree of hydrolysis observed with alkaline protease and acid protease may be due to the abundance of enzyme-specific cleavage sites. After comprehensive consideration, alkaline protease was selected as the enzyme for the preparation of blackberry seed protein peptide. This represents a 2.1-fold improvement over conventional hydrolysis, attributed to enhanced cleavage of glutelin-rich blackberry proteins (> 65% gluten content) [4].

3.1.2. Single factor test design

The results of four factors were below: Substrate concentration (Fig. 2a): Hydrolysis degree plateaued beyond 2 g/100 mL, indicating saturation kinetics. Therefore, the optimal was 2 g/100 mL. Enzyme dosage (Fig. 2b): Hydrolysis peaked at 16,000 U/g (enhanced 58% compared with 10,000 U/g), declining at higher doses due to enzyme aggregation. Time (Fig. 2c): Maximal DH (22.98%) achieved at 3 h – prolonged hydrolysis caused peptide reaggregation. pH (Fig. 2d): Alkaline conditions (pH 9) neutralized proton release during proteolysis, enhancing solubility and enzyme activity by 32% under pH 7.5 [4, 10].

3.1.3 Response surface test design

The Box-Behnken design was used for multiple regression fitting analysis (Table S1 and S2). The quadratic multiple regression equation predicting the degree of hydrolysis (Y) for protein concentration (A), enzyme addition amount (B), time (C), and pH (D) was $Y = 22.58 + 2.3A + 0.52B + 0.56C + 0.53D + 0.59AB + 0.45AC + 0.094AD - 0.54BC + 0.092BD + 0.2CD - 1.62A^2 - 1.27B^2 - 1.21C^2 - 1.11D^2$.

The ANOVA for the quadratic model (Table S2) indicated that the model was highly significant ($P < 0.001$) with a non-significant lack of fit ($P > 0.05$), confirming the model's adequacy. The high coefficient of determination ($R^2 = 0.9770$) and adjusted ($R^2_{Adj} = 0.9540$) demonstrate an excellent fit of the model to

the experimental data. Analysis of variance revealed that the linear terms (A, B, C, D), all quadratic terms (A^2, B^2, C^2, D^2), and the interaction terms AB and AC had significant effects on the degree of hydrolysis ($P < 0.05$). The order of factor influence, determined by F -values, was $A > C > D > B$.

Within the test range, the optimal hydrolysis conditions were determined according to the mathematical model. Optimal conditions (2.5% substrate, 16,500 U/g enzyme, pH 9, 3 h) yielded 22.98% DH – aligning with the predicted 23.44% (error < 2%). This validates the model's precision for industrial-scale peptide production.

3.2 Biological activity

3.2.1 Antioxidant activity

The ultrafiltration-derived peptide fractions exhibited significant antioxidant potential, with pronounced molecular-weight dependence (Fig. 4). To determine the total antioxidant capacity and the scavenging abilities of Vitamin C (V_C) against DPPH radicals, ABTS cation radicals, and hydroxyl radicals, thereby evaluating its *in vitro* antioxidant activity and establishing it as a standard for assessing the antioxidant capacity of other samples.

The ABTS⁺ free radical scavenging ability is a measure of the total antioxidant capacity of a substance. Under the action of an oxidizing agent, ABTS⁺ is oxidized to a stable blue-green ABTS⁺. Oxidative stress is known to be a major cause of many different diseases, including cancer, diabetes, obesity, and heart disease. The reason behind oxidative stress is the unbalanced production and insufficient removal of intracellular reactive oxygen species (ROS). During normal cell metabolism, ROS is produced to play physiological roles such as intercellular signal transduction. The total antioxidant activity of different molecular weight protein peptides can be determined. As shown in Fig. 4a, F1 (< 3 kDa) exhibited the best total antioxidant capacity, up to 2.70 mol/g, followed by F4 (> 10 kDa), compared with V_C (only 3.8 mmol/g).

The free radical scavenging activity of DPPH is used to detect the ability of a substance to provide electrons or hydrogen, thereby transforming the free radical into a more stable substance [27]. It has been widely used to test the ability of natural compounds or extracts of plant and animal origin to act as free radical scavengers or hydrogen donors [28]. For context, the IC_{50} value of the common antioxidant V_C measured under the same conditions was 12.5 μ g/mL, indicating that the F1 fraction possesses remarkably strong DPPH radical scavenging activity, comparable to this potent standard.

In Fig. 4b, with the increase in the concentration of the peptide component solution, the ability of DPPH to scavenge free radicals also increased. All the peptides reached their maximum DPPH free radical scavenging activity at 0.5 mg/mL. At a concentration of 5 mg/mL, F1 achieved nearly 100% DPPH free radical scavenging rate. The overall free radical clearance rate follows the order: $F1 > F4 > F2 > F3$. At 0.0625 mg/mL, the free radical scavenging rates of F2 (3–5 kDa) and F3 (5–10 kDa) were very close.

Table 2
IC₅₀ value of DPPH free radical scavenging capacity

Protein peptides	F1	F2	F3	F4
IC ₅₀ (μg/mL)	15.38	25.93	41.39	17.96

In Table 2, the semi-inhibitory concentrations (IC₅₀) for F1, F2, F3, and F4 were 15.38 μg/mL, 25.93 μg/mL, 41.39 μg/mL, and 17.96 μg/mL, respectively. F1 had the smallest IC₅₀ value, indicating that its inhibitory effect was the best. The IC₅₀ value of F3 was the highest, indicating that its inhibitory effect was relatively poor. These results suggest that the < 3 kDa and > 10 kDa protein peptide components of blackberry seed protein peptides may contain a large number of hydrogen donors, which can react with free radicals to produce more stable products and terminate the free radical chain reaction. Small molecular weight peptides < 3 kDa showed the best DPPH radical scavenging rate, which may be due to their higher content of hydrophobic amino acids that can bind to peroxy DPPH, or the fact that small molecular weight peptides are more soluble and have more binding sites [29, 30].

Oxidative stress is known to be a major cause of many different diseases, including cancer, diabetes, obesity, and heart disease [31]. The reason behind oxidative stress is the unbalanced production and insufficient removal of intracellular reactive oxygen species (ROS). ROS are produced in the normal process of cell metabolism to play physiological roles such as intercellular signal transduction [32]. Superoxide anions promote oxidation and further react with hydroxyl radicals, resulting in damage to organelles. Superoxide anions are produced during normal cellular activities, but under disease conditions, they are overproduced, indirectly leading to lipid oxidation and cell damage. Therefore, the development of oxidative stress-related diseases can be reduced by eliminating superoxide anions.

As shown in Fig. 4c, in terms of superoxide anion resistance, the smaller the molecular weight, the better the superoxide anion resistance, especially when the molecular weight is < 10 kDa. The antioxidant capacity of F3 was the lowest (52.12 ± 1.25 U/g), while that of F1 was the highest (59.30 ± 2.15 U/g). To contextualize this activity, the reference antioxidant V_C possesses a potent superoxide anion scavenging capacity of approximately 4000 U/g under analogous assay conditions. The results indicated that smaller molecular weight blackberry seed protein peptides were more suitable as antioxidants.

Hydroxyl radical inhibition ability is also used in antioxidant evaluation. Using multiple antioxidant evaluation systems allows for a more comprehensive assessment of the antioxidant ability of protein peptide components. Hydroxyl radicals are highly reactive and can interact with almost all cell macromolecules they contact, causing severe damage to cells and eventually leading to various chronic diseases. Thus, hydroxyl radical inhibition is also very important. From Fig. 4d, the inhibitory capacity of hydroxyl radicals is similar with that of superoxide anions. When the molecular weight is > 10 kDa, the inhibitory capacity gradually increases with the decrease in molecular weight. The inhibitory ability of F1 was the strongest, reaching 48.56 U/mg, while the other three protein peptide components also exhibited good inhibitory abilities. The potency of the classic antioxidant V_C is approximately 1.8 U/mg

under comparable assay conditions. This comparison highlights the remarkably strong hydroxyl radical scavenging capability of the F1 fraction.

The superior antioxidant capacity of the < 3 kDa fraction (F1) aligns with the well-established structure-activity relationship of bioactive peptides, wherein lower molecular weight often correlates with enhanced ability to donate electrons or hydrogen atoms [33]. The DPPH scavenging IC_{50} of F1 (15.38 $\mu\text{g/mL}$), while lower than that of ascorbic acid (2.5 $\mu\text{g/mL}$, this study), is notably superior to many other plant-derived peptides. For instance, peptide fractions from rapeseed and soybean protein hydrolysates have been reported with DPPH IC_{50} values of 85.2 $\mu\text{g/mL}$ and 106.5 $\mu\text{g/mL}$, respectively [34, 35]. This suggests that the blackberry seed peptides, particularly the F1 fraction, possess a potent radical scavenging capability. The high antioxidant potency may be attributed to the abundance of hydrophobic amino acids in the gluten-rich blackberry seed protein [4], which can enhance interactions with radical species and contribute to the observed strong ABTS⁺ and hydroxyl radical scavenging activities.

3.2.2 Antihyperglycemic activity

Diabetes is a very old disease in human civilization. In recent years, various effective treatments have been successfully applied, but many side effects of drug treatments can endanger the health of patients [36]. Protein hydrolysates from different edible seeds have anti-diabetic activity and have the potential to be used as alternative therapies. Studies of hypoglycemia have focused on the inhibition of α -glucosidase and α -amylase, as these enzymes are the main digestive enzymes involved in daily carbohydrate metabolism [37].

All protein peptide components were prepared as 0.5 mg/mL protein peptide solutions for determination. As shown in Fig. 5a, F1 exhibited the best α -glucosidase inhibition effect, with an inhibition rate of 50.99%. The α -glucosidase inhibition rate decreased with the increase in molecular weight of protein peptide components. F3 had an inhibition rate of only 29.99%, while the inhibition rate of F4 increased to 37.38%. Among the four components, only F1 had an inhibition rate exceeding 50%. Therefore, F1 has better potential to reduce blood glucose *in vitro*, making it worthy of further study.

α -Amylase is one of the main enzymes involved in the digestion of dietary starch. The oligosaccharides released by its activity can be further decomposed into glucose, which is rapidly absorbed by the body. Therefore, the inhibition of α -amylase activity is considered one of the effective strategies to control diabetes [38].

From Fig. 5b, different protein peptide components exhibited an increasing inhibition rate of α -amylase activity with increasing concentration. Among them, F1 showed the best α -amylase inhibitory activity, with an inhibition rate of up to $63.87 \pm 0.95\%$ at 5 mg/mL, and consistently exhibited the highest inhibitory rate across different concentrations. It was followed by F4. The α -amylase inhibitory activities of F2 and F3 were relatively weak and very similar, with inhibition rates of $42.38 \pm 1.11\%$ and $41.95 \pm 1.25\%$ at 5 mg/mL, respectively.

Our finding that the < 3 kDa fraction (F1) exhibited the strongest inhibition against α-glucosidase and α-amylase is particularly intriguing. Olagunju and coworkers found that all peptides could inhibit α-amylase and α-glucosidase activities, with the 3–5 kDa and > 10 kDa peptides having the most pronounced effects, which differed from the present study. This discrepancy might be due to the division of < 3 kDa peptides into two fragments, < 1 kDa and 1–3 kDa components. Both hydrolyzed Mexican black bean and Brazilian cacao bean peptide components have shown good hypoglycemic effects [39, 40]. This discrepancy underscores the source-dependent functionality of bioactive peptides. The unique amino acid sequence and composition derived from blackberry seed gluten, which comprises over 65% of the seed protein, may yield specific low-molecular-weight peptides that act as potent enzyme inhibitors. This appears to contradict a study by Olagunju et al. on pigeon pea protein, where the 3–5 kDa and > 10 kDa fractions showed the most pronounced effects. This hypothesis is supported by Jorge et al. [41], who found that glutenin hydrolysates from amaranth seeds possessed superior hypoglycemic activity. The high glutelin content in blackberry seeds may thus be a critical factor defining the efficacy of its small peptide fractions, offering a novel and targeted material for managing postprandial blood glucose levels.

3.2.3 Anticancer activity

In vitro cytotoxicity. The toxic activity of four protein peptide components (F1-F4) and the positive control drug 5-fluorouracil (5-Fu) against four cancer cells (Hela, HepG2, MCF-7, A549), and normal human umbilical vein endothelial cells (HUVEC) was studied using the MTT thiazolium blue colorimetric assay. Since the 1980s, 5-fluorouracil (5-Fu) has been a common main drug for the treatment of cancer, and it is clinically used in the treatment of colon cancer, stomach cancer, breast cancer, ovarian cancer, liver cancer, and other cancers. However, relevant studies have pointed out that 5-Fu can also cause damage to normal cells while eliminating cancer cells [42].

Table 3
Cytotoxicity of four protein peptides against axenic cancer cells and normal cells

IC ₅₀ ± SD / µg/mL					
Sample	Hela	HepG2	MCF-7	A549	HUVEC
5-Fu	7.55 ± 1.25	4.08 ± 0.09	22.17 ± 1.55	19.41 ± 1.83	50.04 ± 6.59
F1	4.32 ± 0.71 ^a	0.12 ± 0.01 ^a	47.32 ± 5.04 ^c	33.92 ± 2.58 ^b	288.24 ± 19.57 ^d
F2	12.42 ± 1.05 ^a	0.21 ± 0.03 ^a	49.40 ± 6.11 ^b	38.81 ± 3.15 ^a	168.80 ± 20.04 ^c
F3	88.59 ± 15.37 ^c	7.04 ± 1.18 ^b	60.35 ± 4.21 ^a	42.62 ± 5.84 ^b	146.87 ± 13.29 ^a
F4	77.37 ± 16.44 ^b	70.52 ± 14.05 ^b	89.04 ± 10.05 ^d	96.17 ± 12.73 ^c	410.24 ± 52.46 ^d
^a p < 0.001, ^b p < 0.01, ^c p < 0.05, ^d = not significant, complexes compared with 5-Fu, respectively. Values with different superscript letters are significantly different (p < 0.05).					

As can be seen from Table 3, the four protein peptide components exhibit the highest inhibitory activity on HepG2 cell proliferation, with IC_{50} values of 0.12, 0.21, 7.04, and 70.52 $\mu\text{g/mL}$, respectively, especially F1 and F2. The inhibitory activity of HepG2 cell proliferation was higher than that of 5-Fu (4.08 $\mu\text{g/mL}$). At the same time, the toxic effects of the four protein peptide components were very low, with their IC_{50} values for HUVEC all higher than 100 $\mu\text{g/mL}$, which was much higher than that of the positive drug 5-Fu, especially F4 ($IC_{50} = 410.24 \mu\text{g/mL}$). For Hela cells, the inhibitory activity of F1 was the highest (4.32 $\mu\text{g/mL}$), even higher than that of the positive control drug 5-Fu (7.55 $\mu\text{g/mL}$). This was followed by F2 (12.42 $\mu\text{g/mL}$), F4 (77.37 $\mu\text{g/mL}$), and F3 (88.59 $\mu\text{g/mL}$) components. For MCF-7 cells and A549 cells, the inhibitory activity of F1 was the highest (47.32 $\mu\text{g/mL}$, 33.92 $\mu\text{g/mL}$), followed by F2 (49.40 $\mu\text{g/mL}$, 38.81 $\mu\text{g/mL}$), F3 (60.35 $\mu\text{g/mL}$, 42.62 $\mu\text{g/mL}$), and F4 (89.04 $\mu\text{g/mL}$, 96.17 $\mu\text{g/mL}$) components. Ding also reached a similar conclusion in a study on the inhibitory activity of almond proteolysis on breast cancer cells, where the peptide component $< 10 \text{ kDa}$ showed the best inhibitory activity. In general, small-molecule peptide components are better able to induce apoptosis of cancer cells.⁴³ The remarkably low IC_{50} of the F1 fraction against HepG2 cells (0.12 $\mu\text{g/mL}$), which is significantly lower than that of the chemotherapeutic drug 5-Fu (4.08 $\mu\text{g/mL}$), highlights its exceptional potency. More importantly, the F1 fraction demonstrated markedly lower cytotoxicity towards normal HUVEC cells ($IC_{50} = 288.24 \mu\text{g/mL}$) compared to 5-Fu ($IC_{50} = 50.04 \mu\text{g/mL}$), indicating a superior selective toxicity against cancer cells and a potentially wider therapeutic window. This high selectivity is a coveted feature in anticancer drug development. Our results are consistent with the trend observed by Ding, where $< 10 \text{ kDa}$ almond protein hydrolysates showed the highest inhibitory activity against breast cancer cells [43].

In vivo antitumor efficacy. Figure 6(a)-(e) demonstrated that administration of the $< 3 \text{ kDa}$ protein peptide component (F1, 10 mg/kg) induced significant suppression of both tumor growth and body weight in tumor-bearing mice compared to the PBS control group. At day 21 post-treatment: PBS control mice exhibited mean HepG2 tumor volumes of 1.196 cm^3 and tumor weights of 1.62 g. The 3 kDa protein peptide component-treated mice displayed markedly reduced mean tumor volumes of 0.570 cm^3 and tumor weights of 0.83 g. The T/C value (tumor/control ratio) for the $< 3 \text{ kDa}$ protein peptide component group was 53.07%, corresponding to a tumor growth inhibition rate (TGIR) of 48.95%. Figure 6(f) revealed no discernible toxicopathological alterations in the heart, liver, spleen, lungs, kidneys, or brain tissues of protein peptide-treated mice compared to controls, indicating negligible systemic toxicity. Analysis of tumor angiogenesis (g) via CD31 immunohistochemistry on day 21 showed a significant decrease in microvessel density within tumors from protein peptide-treated mice relative to PBS controls. This reduction in neovascularization correlates with the observed anticancer efficacy.

In general, these findings establish that the $< 3 \text{ kDa}$ protein peptide component possesses marked *in vivo* anticancer efficacy against HepG2 cells, coupled with an excellent safety profile based on the absence of significant organ toxicity. The *in vivo* efficacy and negligible systemic toxicity of the F1 fraction further corroborate its promise as a safe and effective anticancer agent. The anti-angiogenic effect observed via reduced CD31 expression provides a plausible mechanism for its tumor-suppressive action *in vivo*.

3.2.4 Hypolipidemic activity

Bile acids are the main components of micelles and are released during liver cholesterol oxidation. Molecules with the ability to bind bile acids have dual roles. They can inhibit micelle formation and promote bile acid release by increasing the rate of cholesterol oxidation. Additionally, some molecules can reduce the absorption of dietary cholesterol by inhibiting pancreatic cholesterol esterase, which is involved in the release of free cholesterol from dietary cholesterol esters [44].

As shown in Fig. 7a, the cholate binding rate decreased as the protein peptide concentration decreased. For < 10 KDa peptides, the binding capacity of cholate decreases with the increase of molecular weight, but for F4, the binding capacity increases again and is higher than that of F2 and F3. It can also be seen from Table 4 that F1 showed the best inhibitory effect, with an IC₅₀ of 9.43 mg/mL. The second most effective component was F4, with an IC₅₀ of 12.67 mg/mL. F3 showed the worst inhibitory effect, with an IC₅₀ of 29.7 mg/mL.

Table 4

IC₅₀ value of sodium taurine cholate binding rate of different protein peptide components

Protein peptide	< 3 kDa	3–5 kDa	5–10 kDa	> 10 kDa
IC ₅₀ (mg/mL)	9.43	15.93	29.07	12.67

From Fig. 7b, the inhibitory activity of pancreatic lipase increased with the concentration of protein peptides from different components, showing a positive correlation. F1 exhibited the best inhibitory effect on pancreatic lipase. When the protein concentration was 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL, the inhibition rate gradually increased to 11.25%, 21.52%, 36.22%, 47.24%, and 62.55%, respectively. At different concentrations, the inhibition rates of F1 were the highest, and the other three protein peptide components showed similar inhibition rates. At 10 mg/mL, the inhibition rates followed the order: F1 > F2 > F4 > F3.

A 5 mg/mL solution of different protein peptide components can inhibit cholesterol lipase. The inhibitory effect of F1 on cholesterol lipase was the best, with an inhibition rate of 65.05%. The inhibition rate of F4 was 58.91%. The inhibition rates of F3 and F2 components were 52.23% and 51.22%, respectively. The inhibition rate of all four protein peptide components on cholesterol lipase exceeded 50%, indicating a good inhibitory effect on cholesterol lipase (Fig. 7c).

The potent hypolipidemic activity of the F1 fraction, particularly its inhibition of pancreatic lipase (62.55%), positions it as a strong candidate for managing obesity and hyperlipidemia. This efficacy is comparable to, and in some cases surpasses, that of well-studied soy protein hydrolysates. For example, Zhong et al. [45] identified a soy peptide with a pancreatic lipase inhibition IC₅₀ of approximately 0.5 mg/mL, whereas our F1 fraction achieved a similar level of inhibition at tested concentrations. The simultaneous high binding capacity to bile acids and inhibition of cholesterol esterase suggest a multi-targeted mechanism of action for the F1 fraction. This multifaceted hypolipidemic effect may be linked to the high proportion of hydrophobic and acidic amino acids in the blackberry seed protein hydrolysate,

as these residues are known to facilitate interactions with lipids, bile acids, and the active sites of digestive enzymes [44].

4. Conclusions

This study demonstrates an integrated biorefinery approach for converting blackberry seeds—an underutilized agricultural byproduct—into high-value bioactive peptides. Through optimized enzymatic hydrolysis and ultrafiltration fractionation, the <3 kDa peptide fraction was identified as a promising multifunctional ingredient with potent antioxidant, hypoglycemic, hypolipidemic, and anticancer activities. The exceptional *in vitro* and *in vivo* efficacy, combined with low toxicity toward normal cells, highlights its potential for functional food and nutraceutical applications. These findings support the sustainable valorization of fruit processing residues, contributing to a circular bioeconomy.

Declarations

Ethics statement

The animal study was reviewed and approved by the ethics committee of Nanjing Advanced Academy of Life and Health (Nanjing Keygen Biotech. Co. Ltd.). Approval code: NAALH-W-2503002; approval date: 03/03/2025.

Funding

We are very grateful for the financial support from National Natural Science Foundation of China (32400310), Central Guidance Funds for Local Science and Technology Development in 2024 (Batch 3) (BE2024010) and Jiangsu Province Key Project of R&D Plan (Modern Agriculture) (BE2020344).

CRediT authorship contribution statement

Shaoyi Wang: Data Curation, Formal Analysis, Investigation, Writing – Original draft preparation. **Fengyi Zhao:** Conceptualization, Investigation, Writing – Original draft preparation, Writing – Review and Editing, Visualization, Funding Acquisition. **Hongxia Liu:** Investigation, Resources. **Huifang Zhao:** Investigation, Resources. **Wenlong Wu:** Supervision, Resources. **Yaqiong Wu:** Supervision, Writing – Review and Editing. **Jinshu Tian:** Resources, Funding Acquisition. **Weilin Li:** Project Administration, Funding Acquisition.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

References

1. Sá AGA, Moreno YMF, Carciofi BAM (2020) Plant proteins as high-quality nutritional source for human diet. *Trends Food Sci Technol* 97:170–184. <https://doi.org/10.1016/j.tifs.2020.01.011>
2. Ahnen RT, Jonnalagadda SS, Slavin JL (2019) Role of plant protein in nutrition, wellness, and health. *Nutr Rev* 77:735–747. <https://doi.org/10.1093/nutrit/nuz028>
3. Choe U, Li Y, Yu L, Gao B, Wang TTY, Sun J, Chen P, Yu L (2020) Chemical composition of cold-pressed blackberry seed flour extract and its potential health-beneficial properties. *Food Sci Nutr* 8:1215–1225. <https://doi.org/10.1002/fsn3.1410>
4. Wang S, Zhao F, Wu W, Lyu L, Li W (2023) Proteins from blackberry seeds: extraction, Osborne isolate, characteristics, functional properties, and bioactivities. *Int J Mol Sci* 24:15371. <https://doi.org/10.3390/ijms242015371>
5. Wang J, Lian P, Yu Q, Wei J, Kang W (2017) Antithrombotic mechanism of polysaccharides in blackberry (*Rubus* spp.) seeds. *Food Nutr Res* 61:1379862. <https://doi.org/10.1080/16546628.2017.1379862>
6. Yin Z, Zhang W, Feng F, Kang W (2014) Study on effective extracts of *Rubus* spp. seed for reducing blood lipid. *Chin J Exp Tradit Med Form* 20:194–198. (in Chinese)
7. Apostolopoulos V, Bojarska J, Chai T-T, Elnagdy S, Kaczmarek K, Matsoukas J, New R, Parang K, Lopez OP, Parhiz H, Perera CO, Pickholz M, Remko M, Saviano M, Skwarczynski M, Tang Y, Wolf WM, Yoshiya T, Zabrocki J, Zielenkiewicz P, AlKhazindar M, Barriga V, Kelaidonis K, Sarasia EM, Toth I (2021) A global review on short peptides: frontiers and perspectives. *Molecules* 26:430. <https://doi.org/10.3390/molecules26020430>
8. Du Z, Li Y (2022) Review and perspective on bioactive peptides: a roadmap for research, development, and future opportunities. *J Agric Food Res* 9:100353. <https://doi.org/10.1016/j.jafr.2022.100353>
9. Sánchez A, Vázquez A (2017) Bioactive peptides: a review. *Food Qual Saf* 1:29–46. <https://doi.org/10.1093/fqsafe/fyx006>
10. Li C, Yang J, Yao L, Qin F, Hou G, Chen B, Jin L, Deng J, Shen Y (2020) Characterisation, physicochemical and functional properties of protein isolates from *Amygdalus pedunculata* Pall seeds. *Food Chem* 311:125888. <https://doi.org/10.1016/j.foodchem.2019.125888>
11. Leong YK, Chang JS (2024) Proteins and bioactive peptides from algae: insights into antioxidant, anti-hypertensive, anti-diabetic and anticancer activities. *Trends Food Sci Technol* 148:104352. <https://doi.org/10.1016/j.tifs.2024.104352>
12. Akbarian M, Khani A, Eghbapour S, Uversky VN (2022) Bioactive peptides: synthesis, sources, applications, and proposed mechanisms of action. *Int J Mol Sci* 23:1445. <https://doi.org/10.3390/ijms23031445>
13. Wang L, Wang N, Zhang W, Cheng X, Yan Z, Shao G, Wang X, Wang R, Fu C (2022) Therapeutic peptides: current applications and future directions. *Signal Transduct Target Ther* 7:48.

<https://doi.org/10.1038/s41392-022-00904-4>

14. Ulug SK, Jahandideh F, Wu J (2021) Novel technologies for the production of bioactive peptides. *Trends Food Sci Technol* 108:27–39. <https://doi.org/10.1016/j.tifs.2020.12.002>
15. Kussmann M (2022) Prediction, discovery, and characterization of plant- and food-derived health-beneficial bioactive peptides. *Nutrients* 14:4810. <https://doi.org/10.3390/nu14224810>
16. Lau JL, Dunn MK (2018) Therapeutic peptides: historical perspectives, current development trends, and future directions. *Bioorg Med Chem* 26:2700–2707. <https://doi.org/10.1016/j.bmc.2017.06.052>
17. Li C, Ma Y, Li H, Peng G (2017) A convenient method for the determination of molecular weight cut-off of ultrafiltration membranes. *Chin J Chem Eng* 25:62–67.
18. Flores-Jiménez NT, Ulloa JA, Urías-Silvas JE, Ramírez-Ramírez JC, Bautista-Rosales PU, Gutiérrez-Leyva R (2022) Influence of high-intensity ultrasound on physicochemical and functional properties of a guamuchil *Pithecellobium dulce* (Roxb.) seed protein isolate. *Ultrason Sonochem* 84:105976. <https://doi.org/10.1016/j.ultsonch.2022.105976>
19. Fontanari GG, Martins JM, Kobelnik M, Pastre IA, Arêas JAG, Batistuti JP (2012) Thermal studies on protein isolates of white lupin seeds (*Lupinus albus*). *J Therm Anal Calorim* 108:141–148. <https://doi.org/10.1007/s10973-011-1898-6>
20. Xue H, Gu Z, Lin L, Lu W, Yang S, Xu L, Li Z (2025) Synthesis and biological evaluation of C-ring nitro-substituted dehydroabietylamine analogs: in vitro cytotoxicity, DNA affinity, and molecular docking studies. *Bioorg Med Chem* 130:118349. <https://doi.org/10.1016/j.bmc.2025.118349>
21. Zhao F, Du L, Wang J, Liu H, Zhao H, Lyu L, Wang W, Wu W, Li W (2023) Polyphenols from *Prunus mume*: extraction, purification, and anticancer activity. *Food Funct* 14:4380–4391. <https://doi.org/10.1039/D3FO01211E>
22. Li S, Chu S, Lu J, Wang P, Ma M (2018) Molecular and structural properties of three major protein components from almond kernel. *J Food Process Preserv* 42:e13536. <https://doi.org/10.1111/jfpp.13536>
23. Zhang MZ, Zhang LF, Chen FS, Jiang SY (2021) Ultrasound-assisted enzymatic extraction of zein and effects of different extraction methods on its structure and functional properties. *China Oils Fats* 46:26–32. (in Chinese)
24. Wang HD, Zhang HY, Cao J, Li GZ, Yan MM, Zhao DQ (2024) Comparison of protein structure, physicochemical properties, and functional properties of four components in *Schisandra chinensis*. *Food Ferment Ind* 50:67–77. (in Chinese)
25. Beaubier S, Framboisier X, Ioannou I, Galet O, Kapel R (2019) Simultaneous quantification of the degree of hydrolysis, protein conversion rate and mean molar weight of peptides released in the course of enzymatic proteolysis. *J Chromatogr B* 1105:1–9. <https://doi.org/10.1016/j.jchromb.2018.12.005>
26. Liu XY, Zhou YY, Guo LM, Hao GF (2022) Study on the stability of antioxidant peptides from safflower seeds with different molecular weight. *Sci Technol Food Ind* 43:94–102. (in Chinese)

27. Pownall TL, Udenigwe CC, Aluko RE (2010) Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) enzymatic protein hydrolysate fractions. *J Agric Food Chem* 58:4712–4718. <https://doi.org/10.1021/jf904456r>
28. Gilgun-Sherki Y, Eliaz RE, McCann DJ, Loupe PS, Eyal E, Blatt K, Cohen-Barak O, Bassan M (2016) Placebo-controlled evaluation of a bioengineered, cocaine-metabolizing fusion protein, TV-1380 (AlbuBChE), in the treatment of cocaine dependence. *Drug Alcohol Depend* 166:13–20. <https://doi.org/10.1016/j.drugalcdep.2016.05.019>
29. Shekoohi N, Harnedy-Rothwell P, Sharkey S, Lafferty R, Khatib N, O'Harte F, FitzGerald RJ (2024) Purification and characterization of multifunctional peptides with in situ insulinotropic and antioxidative activity from a blue whiting (*Micromesistius poutassou*) protein hydrolysate. *J Funct Foods* 116:106173. <https://doi.org/10.1016/j.jff.2024.106173>
30. Wang B, Li L, Chi C-F, Ma J-H, Luo H-Y, Xu Y (2013) Purification and characterization of a novel antioxidant peptide derived from blue mussel (*Mytilus edulis*) protein hydrolysate. *Food Chem* 138:1713–1719. <https://doi.org/10.1016/j.foodchem.2012.12.002>
31. Martemucci G, Portincasa P, Di Ciaula A, Mariano M, Centonze V, D'Alessandro AG (2022) Oxidative stress, aging, antioxidant supplementation and their impact on human health: an overview. *Mech Ageing Dev* 206:111707. <https://doi.org/10.1016/j.mad.2022.111707>
32. Lennicke C, Cochemé HM (2021) Redox metabolism: ROS as specific molecular regulators of cell signaling and function. *Mol Cell* 81:3691–3707. <https://doi.org/10.1016/j.molcel.2021.08.018>
33. He R, Ju X, Yuan J, Wang L, Girgih AT, Aluko RE (2012) Antioxidant activities of rapeseed peptides produced by solid state fermentation. *Food Res Int* 49:432–438. <https://doi.org/10.1016/j.foodres.2012.08.023>
34. Zhu L, Chen J, Tang X, Xiong YL (2008) Reducing, radical scavenging, and chelation properties of in vitro digests of alcalase-treated zein hydrolysate. *J Agric Food Chem* 56:2714–2721. <https://doi.org/10.1021/jf703697e>
35. Agyei D, Danquah MK (2011) Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. *Biotechnol Adv* 29:272–277. <https://doi.org/10.1016/j.biotechadv.2011.01.001>
36. Xie X, Wu C, Hao Y, Wang T, Yang Y, Cai P, Liu Y, Fu Q (2023) Benefits and risks of drug combination therapy for diabetes mellitus and its complications: a comprehensive review. *Front Endocrinol* 14:1301093. <https://doi.org/10.3389/fendo.2023.1301093>
37. Xu Y, Yang Y, Ma C-M, Bian X, Liu X-F, Wang Y, Zhou Z-K (2023) Characterization of the structure, antioxidant activity and hypoglycemic activity of soy (*Glycine max* L.) protein hydrolysates. *Food Res Int* 173:113473. <https://doi.org/10.1016/j.foodres.2023.113473>
38. Uraipong C, Zhao J (2018) In vitro digestion of rice bran proteins produces peptides with potent inhibitory effects on α -glucosidase and angiotensin I converting enzyme. *J Sci Food Agric* 98:758–766. <https://doi.org/10.1002/jsfa.8523>
39. Maria FQ-S, Ana GS-P, Jeanett G-T, Jose AC-O, Karen VPH, Francisco D-V, Jorge M-S (2018) Phenolic profiles and their contribution to the antioxidant activity of selected chickpea genotypes from

- Mexico and ICRISAT collections. *Plant Foods Hum Nutr* 73:121–127.
<https://doi.org/10.1007/s11130-018-0661-6>
40. Olagunju AI, Omoba OS, Enujiugha VN, Alashi AM, Aluko RE (2021) Thermoase-hydrolyzed pigeon pea protein and its membrane fractions possess in vitro bioactive properties (antioxidative, antihypertensive, and antidiabetic). *J Food Biochem* 45:e13429. <https://doi.org/10.1111/jfbc.13429>
 41. Jorge SS, Raúl RB, Isabel G-L, Edith P-A, Bernardo E-B, César A-P, Sandra AP, Alma LC (2015) Dipeptidyl peptidase IV inhibitory activity of protein hydrolyzates from *Amaranthus hypochondriacus* L. grain and their influence on postprandial glycemia in streptozotocin-induced diabetic mice. *Afr J Tradit Complement Altern Med* 12:90–98. <https://doi.org/10.4314/ajtcam.v12i1.13>
 42. Sethy C, Kundu CN (2021) 5-Fluorouracil (5-FU) resistance and the new strategy to enhance the sensitivity against cancer: implication of DNA repair inhibition. *Biomed Pharmacother* 137:111285. <https://doi.org/10.1016/j.biopha.2021.111285>
 43. Ding MM (2016) Apricot kernel protein enzymatic hydrolysate significantly inhibits proliferation of human breast cancer cell. Master's thesis, Beijing Forestry University, Beijing. (in Chinese)
 44. Prados IM, Plaza M, Marina ML, García MC (2020) Evaluation of the relationship between the peptide profiles and the lipid-lowering properties of olive seed hydrolysates as a tool for tuning hypocholesterolemic functionality. *Food Funct* 11:4973–4981.
<https://doi.org/10.1039/D0FO00576B>
 45. Zhong F, Zhang X, Ma J, Shoemaker CF (2007) Fractionation and identification of a novel hypocholesterolemic peptide derived from soy protein Alcalase hydrolysates. *Food Res Int* 40:756–762. <https://doi.org/10.1016/j.foodres.2007.01.005>

Figures

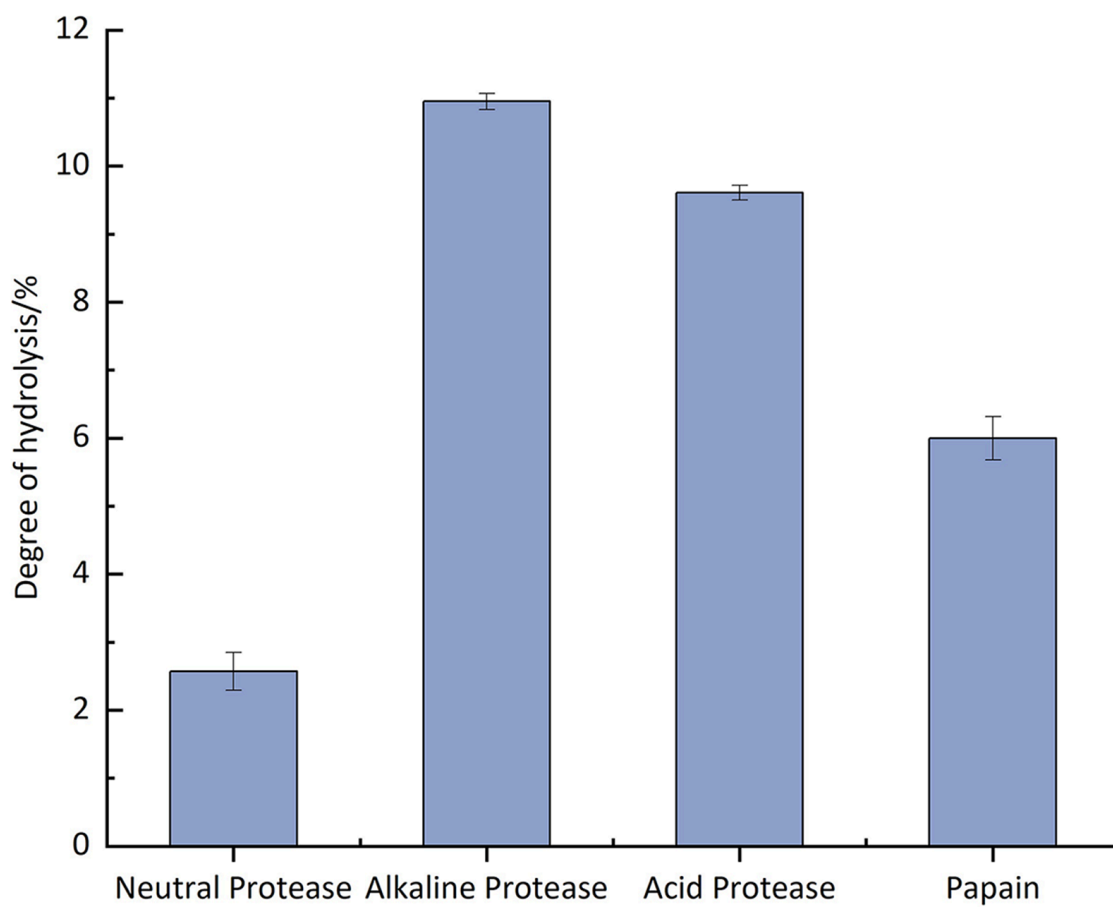


Figure 1

The degree of hydrolysis of different proteases

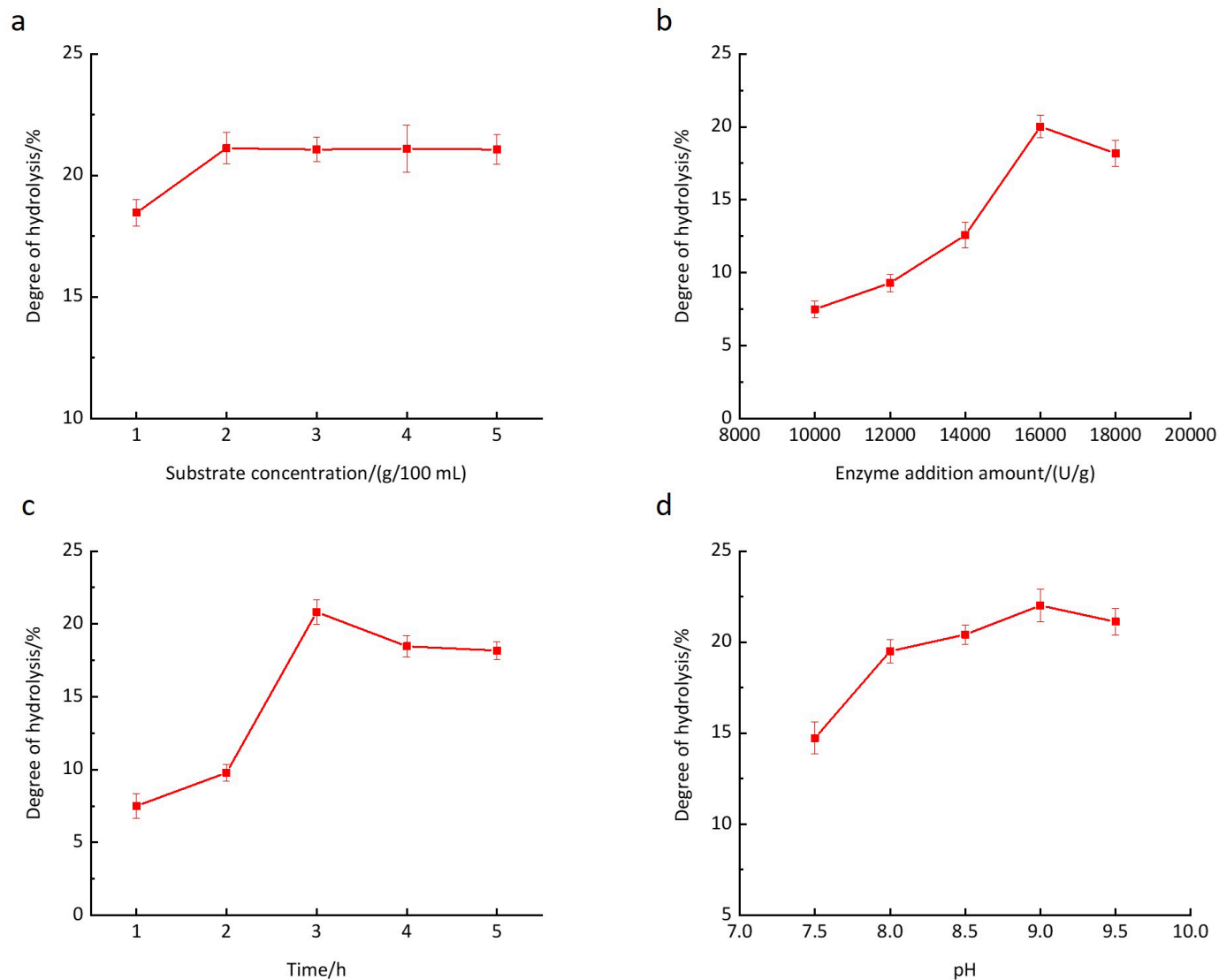


Figure 2

Effect of different factors on degree of hydrolysis: a. substrate concentration; b. enzyme dosage; c. time; d. pH.

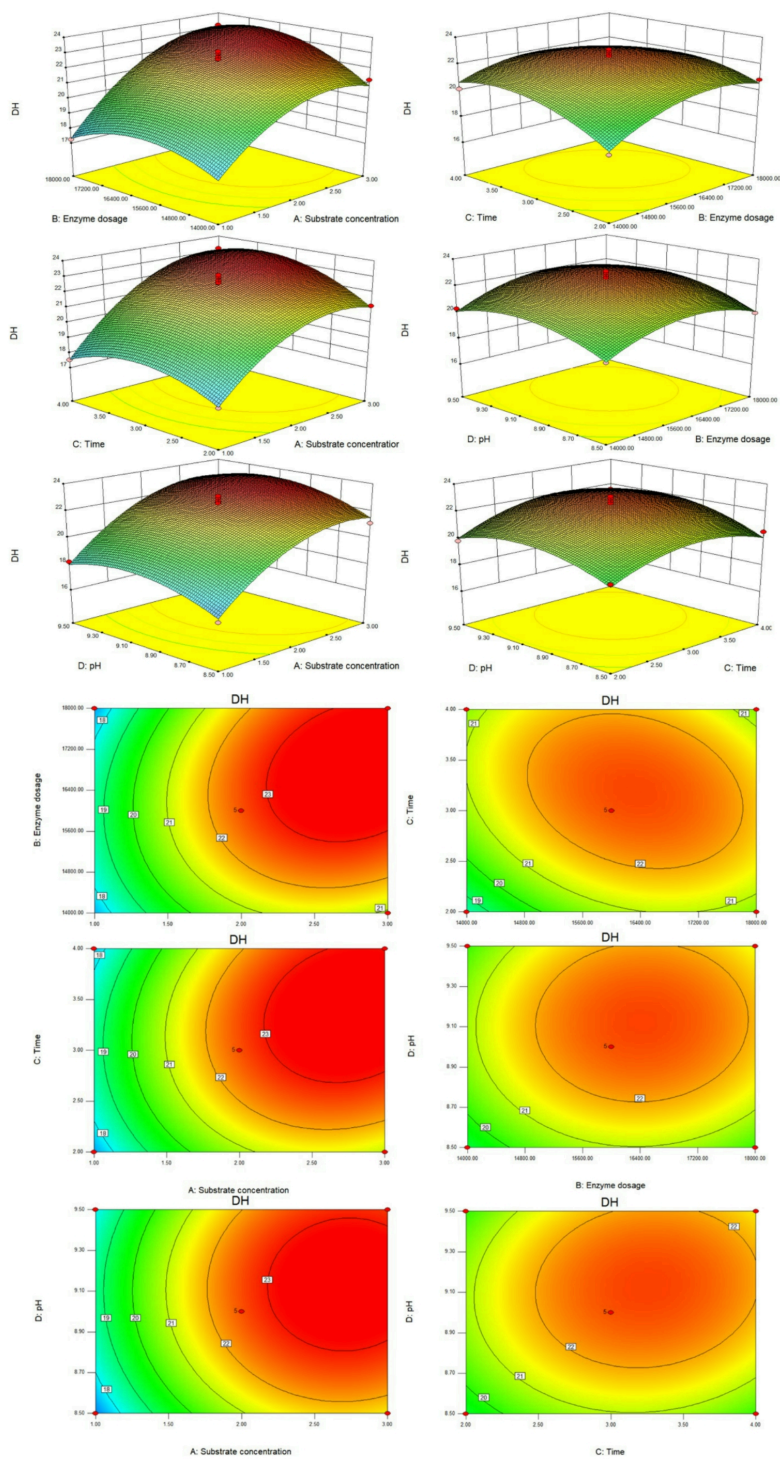


Figure 3

The influence of different conditions on the degree of hydrolysis

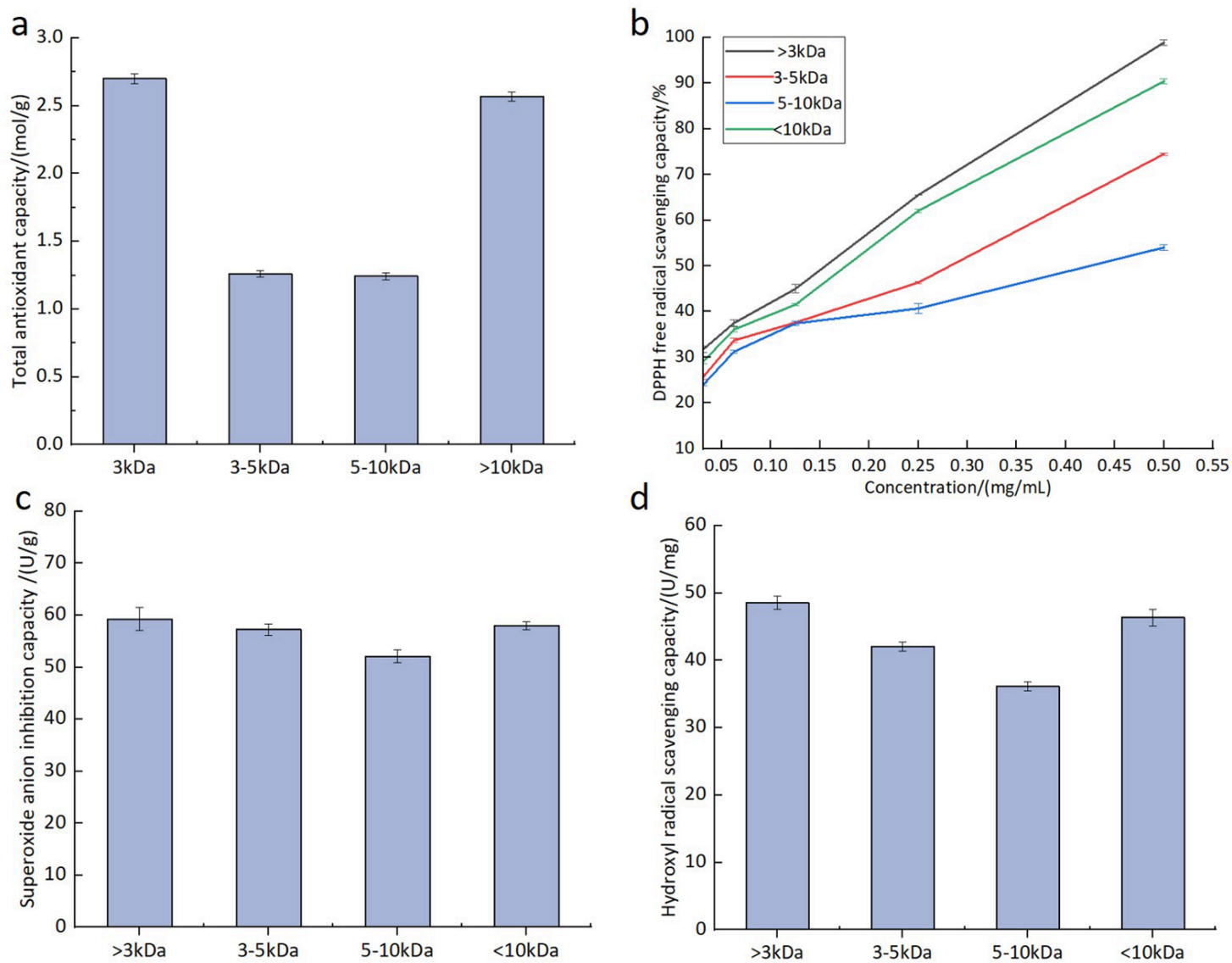


Figure 4

Antioxidant activity of four component protein peptides with different molecular weight: a. T-AOC; b. DPPH; c. O_2^- ; d. $\cdot OH$.

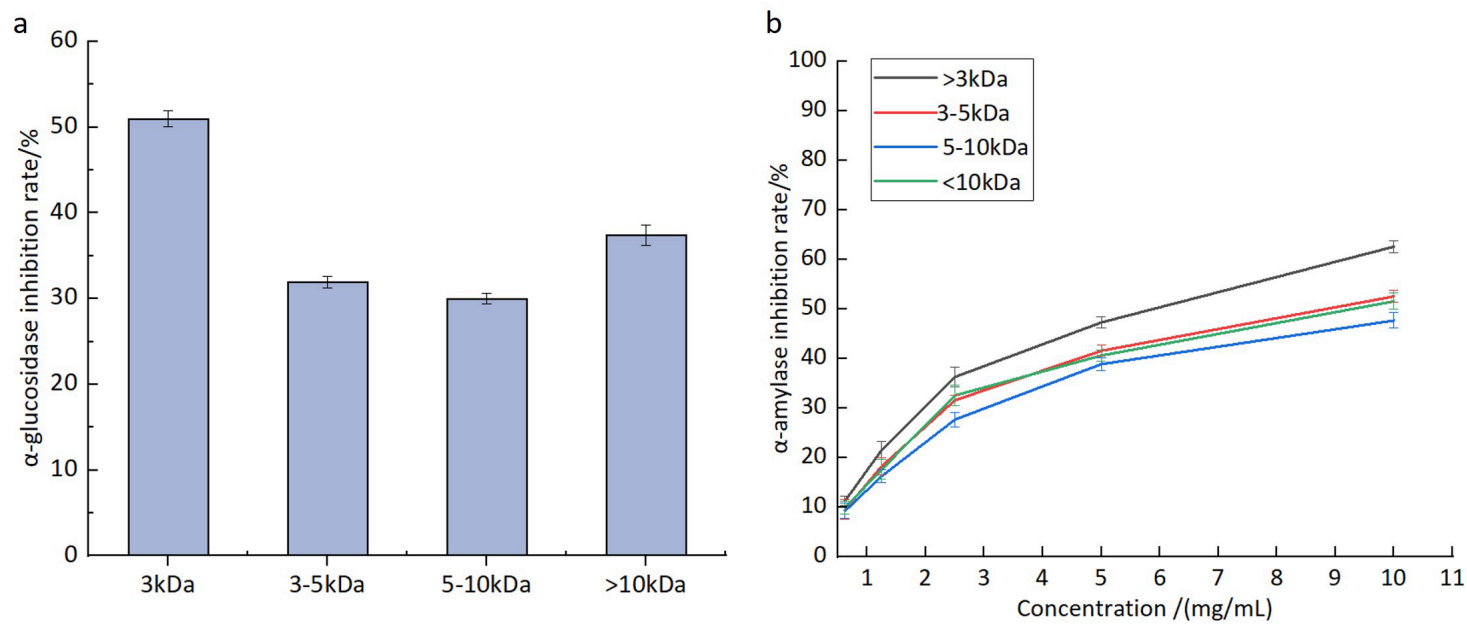


Figure 5

α -glucosidase(a) and α -Amylase (b) inhibition rate of different protein peptides

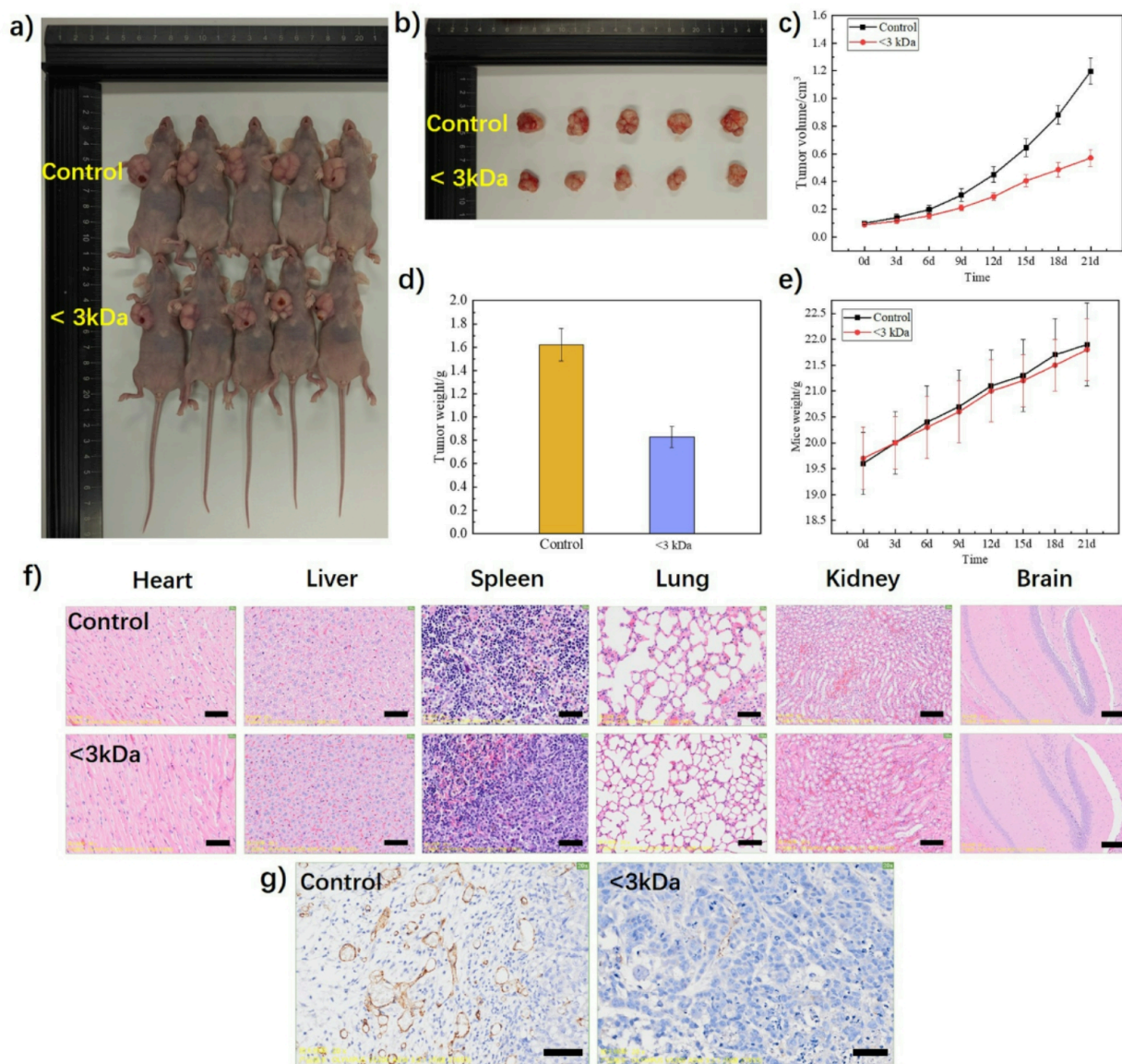


Figure 6

In vivo antitumor efficacy and safety assessment of the <3 kDa protein peptide component in HepG2 xenograft-bearing mice. (a) Representative photographs of mice from PBS control and <3 kDa protein peptide component-treated (Dose: 10 mg/kg) groups at day 21. (b) Comparative tumor volumes at endpoint. (c) Tumor volume progression curves over 21 days. (d) Body weight change profiles during the treatment period. (e) Endpoint tumor weight comparison between treatment groups. (f) Hematoxylin and eosin (H&E)-stained sections of brain, heart, liver, spleen, lung, and kidney tissues. (g) Representative CD31-immunostained tumor sections depicting microvessel density. Scale bar = 20 μm. Error bars represent standard error of the mean (SEM; n = 5 mice per group).

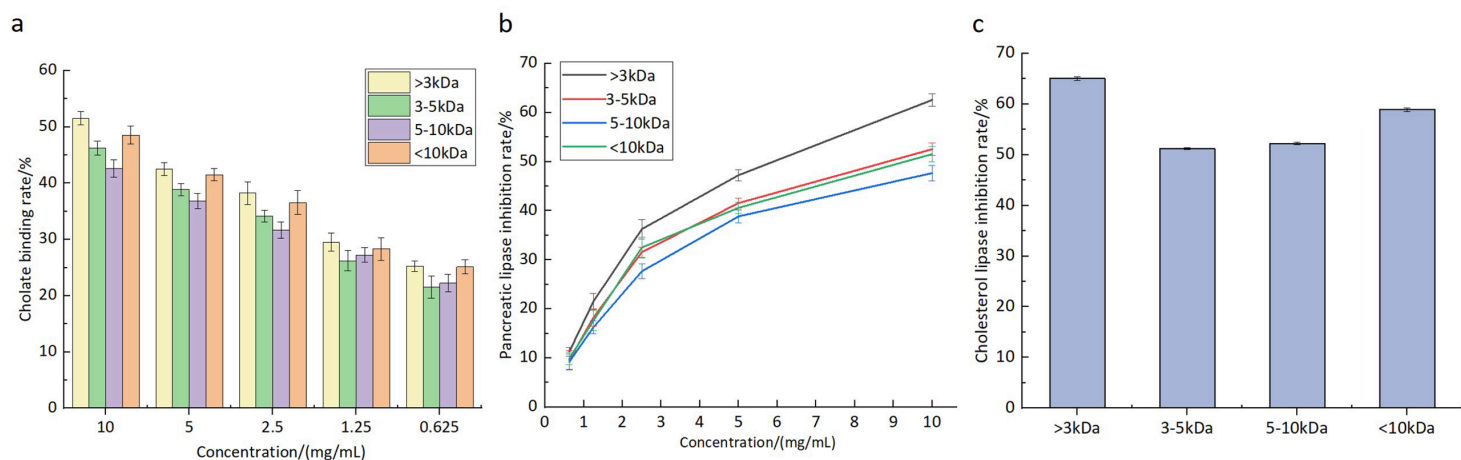


Figure 7

Binding rates with sodium taurine cholate(a), Pancreatic lipase inhibition rate (b), and Cholesterol lipase inhibition rate of four protein peptides

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supportinginformation.docx](#)
- [animaethicsproof.pdf](#)