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Fish collagen peptide GPLGPR as a potent natural DPP-IV inhibitor against type 2 diabetes

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ABSTRACT: Peptides derived from animal proteins have attracted increasing attention as natural inhibitors of dipeptidyl peptidase-IV(DPP-IV) for the management of type 2 diabetes mellitus (T2DM). Here, we compared the anti-diabetic potential of fish collagen peptides (FCP), casein peptides (CP), and ovalbumin peptides (OP). Among them, FCP showed the strongest hypoglycemic effect, significantly improving insulin sensitivity and tissue damage in the liver and pancreas of type 2 diabetic mice. LC–MS characterization and molecular docking identified GPLGPR as the most active peptide within FCP. GPLGPR bound tightly to the DPP-IV catalytic pocket through hydrogen bonding with Glu205 and Glu206 and hydrophobic interactions with Phe357, Tyr547, and Trp629, consistent with a competitive inhibition mode. *In vivo*, GPLGPR suppressed DPP-IV activity, elevated serum GLP-1 levels, and reduced hepatic gluconeogenic gene expression, thereby improving glucose metabolism and alleviating diabetic symptoms such as weight loss, polydipsia, and tissue injury. Collectively, these findings identify GPLGPR as a potent natural DPP-IV inhibitory peptide with strong potential for dietary intervention in T2DM.

Keywords: Fish collagen hydrolysate; Diabetes; DPP-IV inhibitory peptide; Molecular docking.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by impaired insulin resistance and/or insulin secretion, resulting in persistent hyperglycemia. Prolonged elevation of blood glucose can lead to progressive damage to multiple organs and tissues, including the vasculature, peripheral nerves, retina, kidneys, and lower extremities(Zheng, Ley, & Hu, 2018). Epidemiological data show a marked increase in the prevalence of T2DM from the 1990s to the mid-2000s, largely attributed to rising obesity rates, sedentary lifestyles, and population aging(Reed, Bain, & Kanamarlapudi, 2021). According to the International Diabetes Federation, approximately 537 million adults were living with diabetes in 2021, and this number is projected to exceed 700 million by 2045 (Lara-Castor, O'Hearn, Cudhea, Miller, Shi, Zhang, et al., 2025; Roden & Shulman, 2019). Given its high morbidity, extensive complications, and substantial impact on quality of life and life expectancy, the escalating global burden of T2DM highlights the urgent need for effective therapeutic agents with minimal adverse effects. Currently available pharmacological treatments, including sulfonylureas and biguanides, are often limited by side effects such as hypoglycemia, weight gain,

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gastrointestinal disturbances, edema, and hepatotoxicity(Corathers, Peavie, & Salehi, 2013; Filippatos, Panagiotopoulou, & Elisaf, 2014; Inzucchi, Bergenstal, Buse, Diamant, Ferrannini, Nauck, et al., 2014; Palmer, Mavridis, Nicolucci, Johnson, Tonelli, Craig, et al., 2016), underscoring the importance of developing safer and more sustainable intervention strategies.

Incretins are a class of peptide hormones secreted by the intestine in response to nutrient ingestion, with glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) being the most prominent members(Pol, Puhlmann, & Mars, 2022). Following a postprandial rise in blood glucose, GLP-1 and GIP stimulate pancreatic β -cells to enhance insulin secretion, thereby promoting glucose utilization and lowering blood glucose levels(Baggio & Drucker, 2007). In addition, they suppress glucagon release from pancreatic α -cells, preventing excessive hyperglycemia. However, native incretins are highly unstable *in vivo* and are rapidly degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV), resulting in a short half-life(Dinsmore, Liu, Miao, Ünsal, Sürmeli, Beinborn, et al., 2024; Sicinski, Montanari, Raman, Doyle, Harwood, Song, et al., 2021; Sicinski, Sürmeli, Du, Raman, Montanari, Lee, et al., 2024). Consequently, numerous antidiabetic drugs have been developed as DPP-IV inhibitors, including sitagliptin, vildagliptin, saxagliptin, alogliptin, and linagliptin. While these agents prolong incretin activity and improve glycemic control, their clinical use may be associated with hepatic and renal toxicity, gastrointestinal discomfort (e.g., abdominal pain, nausea, diarrhea), and hypersensitivity reactions(Saini, Sharma, & Khan, 2023).

In recent years, food-derived peptides have received increasing attention for their potential to regulate hyperglycemia, modulate immune responses, and exert multiple physiological benefits. Acting as signaling molecules, these short amino acid sequences can interact with cellular receptors or transporters to initiate specific intracellular cascades, thereby exhibiting high target specificity and desirable pharmacological profiles(Chelliah, Wei, Daliri, Elahi, Yeon, Tyagi, et al., 2021; Ulug, Jahandideh, & Wu, 2021). Dietary proteins from milk, eggs, fish, and livestock meat represent abundant and natural sources of bioactive peptides(Fan, Liu, Du, Zeng, Wu, Pan, et al., 2024). Among them, fish collagen, milk casein, and egg ovalbumin possess distinct structural and functional properties, which may contribute to metabolic regulation, including glycemic control. Bioactive peptides derived from these proteins have demonstrated a wide range of biological functions, such as antioxidant, antihypertensive, and anti-diabetic activities. In the context of T2DM, fish-derived peptides have been extensively studied for their ability to inhibit DPP-IV, thereby prolonging incretin action and improving glucose homeostasis. For example, peptides isolated from sardine or Atlantic salmon exhibit strong DPP-IV inhibitory activity and enhance GLP-1 secretion, resulting in improved insulin sensitivity (Harnedy, Parthasarathy, McLaughlin, O'Keeffe, Allsopp, McSorley, et al., 2018; Rivero-Pino, Espejo-Carpio, & Guadix, 2020). Similarly, casein-derived peptides display DPP-IV inhibition and glucose-lowering potential(S. Wang, Zhou, Qian, Yang, Zheng, & Zhao, 2025). In addition, egg white-derived peptides have been reported to reduce blood glucose levels in high-fat diet and low-dose streptozotocin-induced T2DM mice by modulating hepatic gluconeogenic signaling and improving metabolic profiles(Cao, Chen, Lu, Yu, Xia, Wang, et al., 2024). These findings suggest that biopeptides from fish, milk,

and eggs may target multiple metabolic pathways relevant to T2DM management, supporting their potential as functional food ingredients or nutraceuticals.

Although numerous studies have demonstrated the potential of fish, milk, and egg derived bioactive peptides for glycemic regulation, direct comparative analyses among these peptide sources and the identification of specific short peptide sequences responsible for their effects remain limited. In this work, peptide preparations from FCP, CP, and OP were first evaluated for their antihyperglycemic efficacy using a HFD and STZ-induced T2DM mouse model to determine the most effective source. The most active preparation was subsequently subjected to peptide sequencing via LC–MS/MS, and the resulting peptide profiles were analyzed *in silico* to predict candidates with DPP-IV inhibitory potential. Molecular docking was then performed to assess binding affinity and interaction patterns with the DPP-IV active site, followed by *in vitro* enzymatic assays and *in vivo* validation. This stepwise approach enables the direct linkage of peptide source, sequence, and mechanism, and provides a solid basis for developing safe and effective functional foods or nutraceuticals for the prevention and management of T2DM.

2. Material and method

2.1 Materials and reagents

Tilapia scales, milk, and antibiotic-free eggs used to extract FCP, CNP and OVP were purchased locally in Beijing, China. Chemical reagents included Metformin and Isoflurane from Aladdin (Shanghai, China), and Streptozotocin from Solarbio (Beijing, China). Assay kits for biochemical parameters (TC, TG, AST, ALT, LDL-C, HDL-C) were purchased from Nanjing Jiancheng Technology Co., Ltd. (Nanjing, China). RIPA Lysis Buffer and an Enhanced Chemiluminescence kit were obtained from Beyotime (Shanghai, China). A mouse insulin ELISA kit was supplied by MeiMian (Yancheng, China). Staining kits for H&E and Oil Red O were from G-Clone (Beijing, China), and high-fat diet feed was from Huafukang (Beijing, China). Reagents for qRT-PCR, including primers, RNA extraction reagent, and cDNA synthesis kits, were sourced from Qingke (Beijing, China) and Servicebio (Wuhan, China). Primary antibodies for ACLY, SREBP1, and β -actin were from Proteintech (Wuhan, China); p70 S6 Kinase and Phospho-p70 S6 Kinase from Cell Signaling Technology (Boston, USA); and PPAR α from Affinity (Jiangsu, China). Secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were provided by SouthernBiotech (Birmingham, USA).

2.2 Protein peptides preparation

2.2.1 Extraction of fish collagen peptides

The extraction method was based on previous studies and was adjusted according to this experiment (Gaikwad & Kim, 2024). Tilapia scales were cleaned, immersed in 0.5 M NaOH for 24 h (solution renewed every 6 h) to remove fat and non-collagenous proteins, rinsed to neutrality, and decalcified in 0.5 M HCl for 24 h with the same renewal schedule. After washing to neutrality and drying, acid-soluble collagen (ASC) was obtained by extraction in 0.5 M acetic acid (1:40 w/v) at 4 °C for 48–72 h, followed by filtration, centrifugation ($10,000 \times g$, 20 min, 4 °C), salting-out with NaCl (final concentration 2.6 M), precipitation,

dialysis (0.1 M acetic acid then deionized water), and freeze-drying. Pepsin-soluble collagen (PSC) was extracted similarly, except that pepsin (enzyme-substrate ratio 1:50 w/w) was added during acetic acid extraction. Collagen was hydrolyzed with neutral protease (Alcalase) at pH 8.0, 50 °C for 4 h, inactivated at 90 °C for 15 min, centrifuged, and freeze-dried to obtain collagen peptides.

2.2.2 Preparation of ovalbumin peptides

The extraction method was based on previous studies and was adjusted according to this experiment (Croguennec, Nau, Pezennec, & Brule, 2000; Maggonage, Manjula, Abeyrathne, Shu, Huang, & Ahn, 2025). Whole egg white proteins were diluted to 5% (w/v) with deionized water, adjusted to pH 2.0, and hydrolyzed with pepsin (1:500 w/w) at 37 °C for 4 h. The pH was then adjusted to 7.5 and trypsin (1:500 w/w) was added for a further 4 h hydrolysis at 37 °C. Enzymes were inactivated by heating to 90 °C for 15 min. The hydrolysate was centrifuged to remove insoluble material and fractionated by ultrafiltration (molecular weight cut-off 2 kDa). The permeate was freeze-dried to obtain ovalbumin peptides.

2.2.3 Preparation of casein peptides

The extraction method was based on previous studies and was adjusted according to this experiment (Bonnaillie, Qi, Wickham, & Tomasula, 2014; Malik, De, Singh, Galanakis, Alamri, & Yadav, 2022). Fresh skimmed milk was mixed with 0.02 M CaCl₂, adjusted to pH 4.6, and incubated at 40 °C to precipitate casein. The precipitate was collected by centrifugation, washed three times with deionized water, and dissolved in 0.1 M NaOH (pH 7.0). The casein solution was adjusted to pH 8.0, hydrolyzed with trypsin (1:100 w/w) at 37 °C for 4 h, and enzymes were inactivated at 90 °C for 15 min. After centrifugation, the supernatant was ultrafiltered (2 kDa cut-off), and the permeate was freeze-dried to obtain casein peptides.

2.3 Degree of hydrolysis analysis

The degree of hydrolysis (DH) of fish collagen, casein, and ovalbumin hydrolysates was determined using the o-phthaldialdehyde (OPA) colorimetric method. The OPA reagent was freshly prepared by dissolving 50 mg of OPA in 1 mL of anhydrous ethanol, followed by the addition of 50 mL of 100 mM borate buffer (pH 9.5) and 100 µL of β-mercaptoethanol. Subsequently, 250 µL of the OPA reagent was mixed with 50 µL of an appropriately diluted hydrolysate or L-arginine standard solution (0–0.8 mM). The mixture was incubated at room temperature for 5 min in the dark, and the absorbance was measured at 340 nm using a microplate reader (BioTek Synergy H1, USA). The concentration of free amino groups was determined from the L-arginine standard curve and expressed as mmol per gram of protein. The degree of hydrolysis (DH) was calculated according to the following equation:

$$DH(\%) = (AN_t - AN_0) / (h_{tot}) \times 100\%$$

where AN_t and AN₀ represent the free amino group contents (mmol/g protein) of hydrolyzed and unhydrolyzed samples, respectively, and h_{tot} denotes the total number of peptide bonds per gram of protein. The values of h_{tot} were assumed to be 8.6 mmol/g for fish collagen (Chen, Yu, Guan, & Cao, 2025), 8.0 mmol/g for casein (J. Wang, Bai, Ma, & Ma, 2017), and the total number of peptide bonds per gram protein

(h_{tot}) of ovalbumin was calculated from its amino acid composition and molecular weight, yielding a value of $8.66 \text{ mmol} \cdot \text{g}^{-1}$, which was used for DH determination.

2.4 Soluble peptide content analysis

The soluble peptide content of protein hydrolysates was determined using the Folin–Ciocalteu colorimetric method. Briefly, the hydrolysate was mixed with an equal volume of 10% (w/v) trichloroacetic acid and allowed to stand at room temperature for 30 min, followed by centrifugation at 8000 r/min for 10 min. The supernatant was collected and diluted 20-fold with distilled water. Then, 1 mL of the diluted sample was mixed with 5 mL of alkaline copper reagent (containing 2% Na_2CO_3 , 0.1 mol/L NaOH, 1% sodium potassium tartrate, and 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and incubated for 10 min at room temperature. Subsequently, 0.5 mL of 1 mol/L Folin–Ciocalteu reagent was added, mixed thoroughly, and allowed to react for 30 min at room temperature. The absorbance was measured at 500 nm using a spectrophotometer, with distilled water as the blank. A standard curve was constructed using bovine serum albumin (BSA), and the soluble peptide content was expressed as mg BSA equivalents per mL of hydrolysate.

2.5 Animal models and experimental design

Male C57BL/6J mice (SPF grade, 6 weeks old, $20 \pm 2 \text{ g}$) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were housed at $22 \pm 2 \text{ }^\circ\text{C}$ with 50–60% humidity under a 12 h light/dark cycle, with ad libitum access to water and standard chow for one week prior to experiments. All procedures were approved by the Laboratory Animal Ethical Review Committee of China Agricultural University (Approval No. AW41015207-5-01) and conducted in accordance with the ARRIVE guidelines and the U.K. Animals (Scientific Procedures) Act, 1986.

The study comprised two experimental stages.

Stage 1: Screening of protein peptides with antidiabetic activity

Mice were randomly assigned to a standard chow diet (11.4% kcal fat, 21.3% protein, 67.3% carbohydrate; SPF-F01, Sipeifu, China) or a high-fat diet (HFD; 60% kcal fat, 20% protein, 20% carbohydrate; H10060, HFKbio, China). Control mice received chow and sodium citrate buffer (pH 4.5) via intraperitoneal injection. After 6 weeks of HFD feeding, diabetes was induced by intraperitoneal streptozotocin (STZ; 50 mg/kg in sodium citrate buffer, pH 4.5; Solarbio, China) once daily for 3 days following an overnight fast. This HFD/STZ protocol is widely used to mimic the metabolic characteristics of human type 2 diabetes, as HFD induces obesity and insulin resistance while STZ causes partial pancreatic β -cell dysfunction (Nath, Ghosh, & Choudhury, 2017). Three days after the final injection, mice with fasting blood glucose (FBG) $\geq 11.1 \text{ mmol/L}$ were considered diabetic and randomly allocated to:

DM group: ultrapure water (100 $\mu\text{L/day}$); MET group: metformin (70 mg/kg); FCP group: fish collagen peptides (200 mg/kg); CP group: casein peptides (200 mg/kg); OP group: ovalbumin peptides (200 mg/kg). Treatments were administered daily by gavage for 5 weeks. Food/water intake was recorded daily. FBG, glucose tolerance (GTT), and insulin tolerance (ITT) were assessed.

Stage 2: Identification of active peptides with DPP-IV inhibitory activity

Diabetic mice were generated as in Stage 1 and treated with: DM group: ultrapure water (100 μ L/day); SIT group: sitagliptin (70 mg/kg); GPLGPR group: GPLGPR (200 mg/kg); SPGSWF group: SPGSWF (200 mg/kg). All treatments were given by gavage once daily.

At the end of each stage, mice were fasted for 12 h, anesthetized, and blood was collected from the orbital sinus into heparinized tubes. Serum was obtained by clotting at room temperature for 2 h and centrifugation ($1,000 \times g$, 10 min, 4 °C), then stored at -80 °C. Liver, pancreas, and colon tissues were collected for histopathological, biochemical, and molecular analyses, either fixed in 4% paraformaldehyde or snap-frozen in liquid nitrogen.

2.6 Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

After five weeks of peptide treatment, glucose tolerance and insulin sensitivity were evaluated using GTT and ITT, respectively. For the GTT, mice were fasted for 16 h with free access to water, followed by intraperitoneal injection of glucose (2 g/kg body weight, dissolved in sterile saline). For the ITT, mice were fasted for 4 h and then injected intraperitoneally with human recombinant insulin (0.75 U/kg body weight, Novo Nordisk, Denmark) prepared in sterile saline. Blood glucose concentrations were determined from tail vein blood samples collected at 0, 30, 60, 90, and 120 min after glucose or insulin administration using a handheld glucose meter (Sinocare GA-3, China). The interval between the two tests was at least 72 h to avoid metabolic interference. Glucose tolerance and insulin sensitivity were quantified by calculating the area under the blood glucose–time curve (AUC) using the trapezoidal method.

2.7 Serum Biochemical Analysis

Serum insulin levels were measured using a mouse insulin ELISA kit (Meilian, USA) following the manufacturer's instructions. Briefly, 10 μ L of serum was added to a 96-well microplate pre-coated with an anti-insulin antibody, followed by incubation with a biotinylated detection antibody and streptavidin–horseradish peroxidase (HRP) conjugate. After 60 min incubation at 37 °C and three washes with PBST (0.05% Tween-20 in PBS), tetramethylbenzidine (TMB) substrate was added. The reaction was terminated with 1 M sulfuric acid, and absorbance was measured at 450 nm using a microplate reader (BioTek, USA). Insulin concentrations were calculated from a standard curve generated using known insulin standards.

Serum lipid profiles, including triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), were determined using commercial enzymatic kits (Nanjing Jiancheng Bioengineering Institute, China). Serum samples (2.5–10 μ L) were mixed with kit-specific reagents and incubated at 37 °C for 10–30 min depending on the analyte. Absorbance was measured at 500 nm, and concentrations were calculated from standard curves.

Activities of alanine transaminase (ALT) and aspartate aminotransferase (AST) were assessed using commercial colorimetric kits (Nanjing Jiancheng Bioengineering Institute, China). Serum samples (10 μ L) were incubated with reaction buffers containing specific substrates and coenzymes at 37 °C for 30 min. The reactions generated color changes based on NADH oxidation or pyruvate formation, and absorbance was

measured at 510 nm. Enzyme activities were calculated using calibration curves according to the manufacturer's instructions.

2.8 Liver and Pancreatic Histology

For hematoxylin and eosin (H&E) staining, liver and pancreatic tissues were dissected and immediately fixed in 4% paraformaldehyde (Biosharp, China) at 4 °C for 12 h. Fixed tissues were washed with PBS, dehydrated through a graded ethanol series (70-100%), cleared in xylene, and embedded in paraffin at 65 °C. Paraffin blocks were sectioned at 5 µm using a rotary microtome (Leica RM2235, Germany), mounted on glass slides, deparaffinized in xylene, and rehydrated through descending ethanol concentrations (100-70%). Sections were stained with hematoxylin for 2 min, rinsed in running tap water, counterstained with eosin for 30 s, dehydrated, cleared in xylene, and mounted with resin-based medium. Histopathological changes, including hepatocyte structure, inflammatory infiltration, and islet morphology, were examined under a bright-field microscope (Leica DM2500, Germany) at 20 × magnification.

For Oil Red O staining, liver tissues were fixed in 4% paraformaldehyde at 4 °C for 12 h and cryoprotected in 30% sucrose at 4 °C for 24 h. Samples were embedded in OCT compound and stored at -80 °C. Frozen sections (5 µm) were cut using a cryostat microtome (Leica CM1950, Germany), mounted on slides, air-dried for 10 min, and briefly rinsed in PBS. Sections were pretreated with 60% isopropanol for 30 s, stained with filtered Oil Red O working solution for 10–15 min at room temperature, rinsed with 60% isopropanol, and washed with distilled water. Nuclei were counterstained with hematoxylin for 1 min, followed by washing and mounting with an aqueous medium. Stained sections were observed under a bright-field microscope (Leica DM2500, Germany), and representative images were captured. Lipid accumulation was quantified using ImageJ software (NIH, USA).

For Periodic Acid–Schiff (PAS) staining, liver tissue sections were deparaffinized and rehydrated through a graded ethanol series to water. Sections were then treated with periodic acid solution for 10 min, followed by rinsing under running tap water for 5 min. After carefully removing excess moisture, sections were incubated with Schiff's reagent for 15 min and subsequently washed under running water for 10 min. Nuclei were counterstained with hematoxylin for 1 min, rinsed with distilled water, and blued for 5 min. Finally, sections were dehydrated, cleared with xylene, mounted with neutral resin, and examined under a light microscope.

2.9 Identification, screening, synthesis of active peptides

2.9.1 Identification of active peptides

The active peptides were identified with LC-MS/MS. Firstly, peptides were pretreated prior to analysis. Approximately 5.2 mg of sample was dissolved in 300 µL of deionized water and centrifuged at 12,000 rpm for 10 min at 4 °C using a 10 kDa molecular weight cut-off ultrafiltration tube. The filtrate, containing peptides <10 kDa, was collected and quantified using a NanoDrop spectrophotometer. For subsequent processing, 100 µg of peptide solution was reduced with dithiothreitol (DTT, final concentration 10 mmol/L)

and alkylated with iodoacetamide (IAM, final concentration 20 mmol/L). The reaction mixture was reconstituted in 0.1% trifluoroacetic acid (TFA), desalted using a C18 column, and dried under vacuum.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed using a Reprosil-Pur 120 C18-AQ precolumn and analytical column. Mobile phase A consisted of 0.1% formic acid (FA) in water, and mobile phase B consisted of 0.1% FA in 80% acetonitrile (ACN). A 66-min gradient elution program was applied. Mass spectrometric acquisition was carried out in data-dependent mode with a primary MS resolution of 70,000 and secondary MS resolution of 17,500, with the Top20 most intense precursor ions selected for fragmentation.

Raw data were processed using the PEAKS de novo sequencing algorithm. Search parameters were set as follows: fixed modification, carbamidomethylation of cysteine; variable modifications, methionine oxidation and N-terminal acetylation; enzyme specificity, non-specific. Mass tolerances were set to 20 ppm for precursor ions and 0.02 Da for fragmentations.

2.9.2 Screening of active peptides

Following the establishment of a peptide library using LC–MS, peptides with de novo sequencing scores greater than 0.8 were retained for further analysis. The bioactivity potential of these peptides was subsequently predicted using PeptideRanker (<http://distilldeep.ucd.ie/PeptideRanker/>), and sequences with a score >0.5 were selected. To ensure safety and novelty, peptides were further screened using ToxinPred (<https://webs.iitd.edu.in/raghava/toxinpred/index.html>) for toxicity assessment, AllerTop 2.0 (https://www.ddg-pharmfac.net/allertop_test/) for allergenicity prediction, and the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/biopep/start_biopep.php) to exclude previously reported bioactive peptides. Candidate peptides were then subjected to molecular docking against DPP-IV (PDB ID: 1WCY) to evaluate binding affinity, and the top five peptides with the lowest binding energies were selected. These sequences were chemically synthesized and tested for in vitro DPP-IV inhibitory activity.

2.9.3 Synthesis of active peptides

The active peptides (GPLGPR and SPGSWF) were chemically synthesized and purchased from Beijing Zhongke Yaguang Biotechnology Co., Ltd. The purity of the peptides was $\geq 95\%$, as verified by the supplier using analytical HPLC and high-resolution mass spectrometry (HRMS).

2.10 DPP-IV inhibitory activity assay

The DPP-IV inhibitory activity of peptides was determined using a chromogenic substrate assay. In a 96-well microplate, 25 μL of DPP-IV solution (0.01 U/mL in 100 mM Tris–HCl buffer, pH 8.0) was incubated with 100 μL of peptide samples at various concentrations or buffer (control) at 37 °C for 10 min. The reaction was initiated by adding 50 μL of Gly–Pro–p-nitroanilide (1.5 mM in Tris–HCl buffer), and the release of p-nitroaniline was continuously monitored at 405 nm every 2 min for 2 h using a microplate reader. All experiments were performed in triplicate. The inhibitory rate of DPP-IV was calculated as:

$$\text{Inhibition (\%)} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{Control}}}\right) \times 100$$

where A(sample) and A(control) denote the absorbance of the sample and control groups, respectively. The half-maximal inhibitory concentration (IC_{50}) values were calculated by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

2.11 *In vitro simulated gastrointestinal digestion*

Simulated gastrointestinal digestion was conducted according to a previously reported method with slight modifications (Zhang, He, Liu, Chen, Sun, Wu, et al., 2019). A 2% (w/v) peptide suspension was stirred for 1 h at room temperature and subsequently heated at 95 °C for 10 min to denature residual proteins and enhance enzymatic susceptibility. After cooling to 37 °C, the pH was adjusted to 2.0 with 1 mol/L HCl to simulate gastric conditions. Pepsin was added at an enzyme-to-substrate ratio of 4% (w/w), and the mixture was incubated at 37 °C for 2 h under continuous stirring.

Following gastric digestion, the pH was adjusted to 7.5 with 1 mol/L NaOH to initiate the intestinal phase. Pancreatin was added at an enzyme-to-substrate ratio of 4% (w/w), and the reaction proceeded at 37 °C for another 2 h with constant stirring. Aliquots were collected at 30 min intervals during both phases to monitor the digestion process. The enzymatic reaction was terminated by heating at 95 °C for 15 min. The digests were centrifuged at $10,000 \times g$ for 10 min at 4 °C, and the supernatants were collected and stored at -20 °C for subsequent determination of DPP-IV inhibitory activity.

2.12 *Enzyme kinetic analysis of peptides*

The enzyme kinetics of peptide inhibition against DPP-IV were analyzed using the Lineweaver–Burk double-reciprocal plot method to investigate the inhibition mechanism. The enzyme concentration was maintained at 0.01 U/mL, while peptide concentrations (0, 80, 250/300, and 500 μ M) and substrate (Gly–Pro–pNA) concentrations (0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 mM) were varied. Peptides were preincubated with the enzyme in 50 mM Tris–HCl buffer (pH 8.0) at 37 °C for 10 min before substrate addition. The initial reaction velocity (V_0) was calculated from the change in absorbance at 405 nm during the first 30 min of reaction. Lineweaver–Burk plots ($1/V_0$ vs. $1/[S]$) were constructed for each peptide concentration, and the inhibition type was determined based on the intersection pattern of the regression lines.

2.13 *Selectivity of peptides toward DPP-IV*

To evaluate the selectivity of fish collagen–derived peptides toward DPP-IV, their inhibitory activities against DPP8 and DPP9 were also determined. Enzyme inhibition assays were performed in 96-well plates at 37 °C using 50 mM Tris–HCl buffer (pH 8.0). Each reaction mixture (175 μ L) contained 1 mM Gly–Pro–pNA as the chromogenic substrate for DPP-IV, and 0.2 mM Ala–Pro–pNA for DPP8 and DPP9, with a final enzyme concentration of 0.01 U/mL. Peptide samples at various concentrations were preincubated with the enzyme for 10 min prior to substrate addition. The release of p-nitroaniline (pNA) was continuously monitored at 405 nm using a microplate reader. Residual enzyme activity (%) was calculated relative to the control (without inhibitor), and IC_{50} values were determined by nonlinear regression analysis using GraphPad

Prism 9. Selectivity indices were expressed as $IC_{50}(\text{DPP8})/IC_{50}(\text{DPP-IV})$ and $IC_{50}(\text{DPP9})/IC_{50}(\text{DPP-IV})$, where higher ratios indicate stronger selectivity of the peptide toward DPP-IV over DPP8 or DPP9.

2.14 Real-Time Quantitative PCR

Total RNA was extracted from liver tissues using TRIzol reagent (Qingke Biotechnology, Beijing, China) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 1 μg RNA using the SewScript RT II First Strand cDNA Synthesis Kit (Servicebio, Wuhan, China). Quantitative PCR was performed using $2 \times$ Universal Blue SYBR Green qPCR Master Mix (Servicebio, Wuhan, China) on a real-time PCR system. Gene expression levels were normalized to β -actin, and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are listed in Table S1 and were synthesized by Qingke Biotechnology (Beijing, China).

2.15 Statistical Analysis

All data are presented as mean $\pm$ SEM. Statistical analyses were performed using Student's t-test and one-way ANOVA with GraphPad Prism 9.0 (GraphPad Software, USA) and IBM SPSS 26.0. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Fish collagen peptides demonstrate superior anti-diabetic activity

To comparatively evaluate the anti-diabetic efficacy of different dietary peptides, we first determined the degree of hydrolysis and soluble peptide content of three animal-derived peptide preparations. All samples exhibited a hydrolysis degree exceeding 60% and a soluble peptide content above 80% (Fig. 1A, B), indicating efficient enzymatic digestion and good solubility. Subsequently, their anti-diabetic effects were assessed in a T2DM model, as illustrated in Fig. 1C. As shown in Fig. 1D-F, diabetic mice exhibited hallmark symptoms of hyperglycemia, including progressive body weight loss, polydipsia and polyphagia. Treatment of FCP effectively mitigated these phenotypes, with improvements comparable to those observed in the metformin-treated group. In contrast, CP and OP peptides failed to elicit significant changes relative to untreated T2DM mice. GTT and ITT analyses further demonstrated that FCP treatment markedly improved glucose tolerance and insulin sensitivity (Fig. 1G–J). To substantiate these findings, fasting blood glucose (FBG) and fasting serum insulin concentrations were determined, and the homeostatic model assessment of insulin resistance (HOMA-IR) index was calculated. FCP significantly reduced both fasting blood glucose and HOMA-IR values, indicating a robust improvement in systemic insulin sensitivity (Fig. 1K–M). Furthermore, PAS staining of liver sections revealed a marked depletion of glycogen stores in T2DM mice (Fig. 1N), indicative of impaired hepatic glucose utilization and glycogen synthesis, reflecting disrupted glucose metabolism. Notably, treatment with FCP or metformin substantially restored hepatic glycogen content, suggesting a reversal of these metabolic abnormalities.

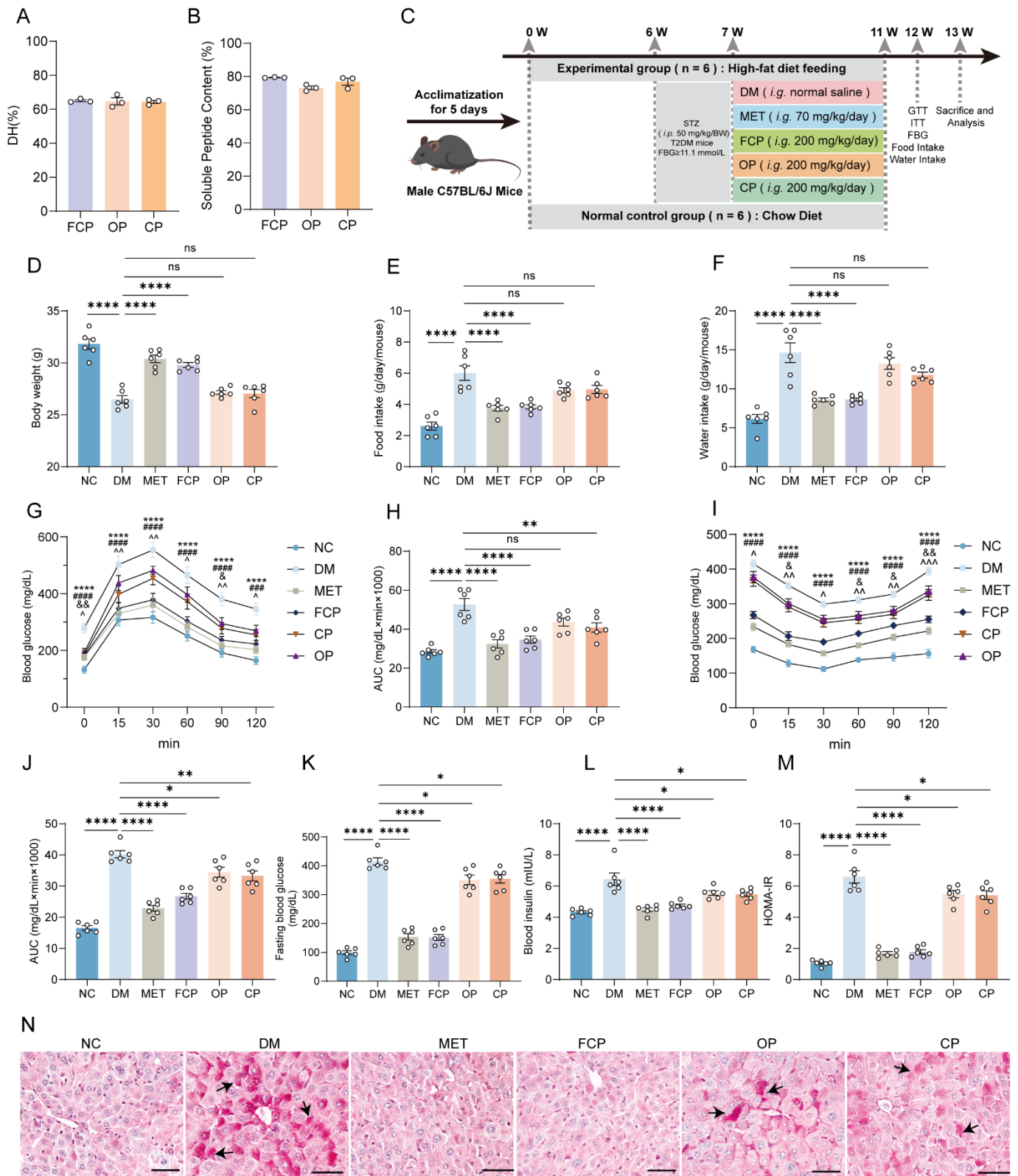


Fig. 1. FCP significantly improve glucose homeostasis and insulin sensitivity in diabetic mice. (A) Degree of hydrolysis (DH); (B) Soluble peptide content of hydrolysates derived from fish collagen, ovalbumin, and casein; (C) Schematic diagram of animal experiment; (D) Final body weight of mice after intervention; (E, F) Daily food and water intake after intervention. (G, H) Glucose tolerance test (GTT) and the corresponding area under the curve (AUC). (I, J) Insulin tolerance test (ITT) and AUC. (K) Fasting blood glucose levels at the end of treatment. (L) Serum insulin concentrations. (M) Homeostasis model assessment of insulin resistance (HOMA-IR) calculated from fasting blood glucose and serum insulin. (N) Periodic acid–Schiff (PAS) staining of liver sections. Scale bar: 50 μm. Data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. *, Met vs. DM; #, FCP vs. DM; ^, OP vs. DM; \$, CP vs. DM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.2 FCP markedly attenuate histopathological damage in T2DM mice

Diabetes is frequently associated with multi-organ injury, particularly affecting the pancreas and liver. As shown in Fig. 2A, pancreatic sections from T2DM mice exhibited pronounced morphological abnormalities, including poorly demarcated islet boundaries, disorganized cellular architecture, and vacuolization within the islet core. Treatment with FCP or metformin markedly preserved islet integrity, characterized by well-defined borders and a more orderly cellular arrangement. In contrast, CP and OP produced only minimal histological improvements.

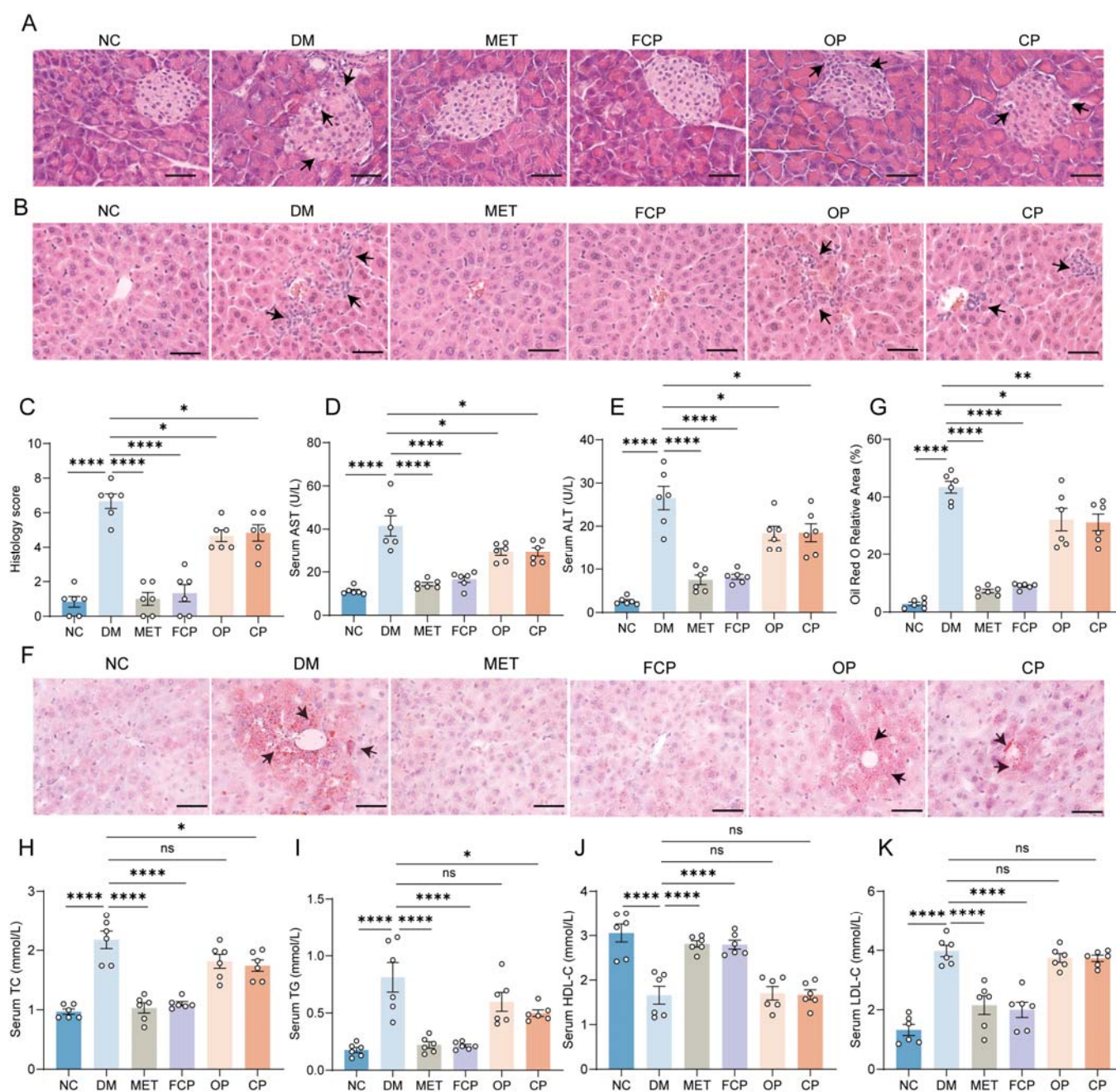


Fig. 2. FCP attenuate histopathological damage and improve metabolic profiles. (A) Hematoxylin and eosin (H&E) staining of pancreatic tissue. Scale bar: 50 μ m; (B) H&E staining of liver tissue; (C) Histopathological scoring of liver sections; (D) Serum AST levels; (E) Serum ALT levels; (F) Oil Red O staining of liver sections; (G) Quantification of Oil Red O positive area; (H) Serum TC levels; (I) Serum TG levels; (J) Serum high-density HDL-C levels; (K) Serum LDL-C levels. Scale bar: 50 μ m. All data are presented as mean $\pm$ SEM. * P < 0.05, ** P < 0.01, **** P < 0.0001.

H&E staining of liver sections, together with quantitative histopathological scoring (Fig. 2B, C), revealed that T2DM mice developed extensive inflammatory infiltration and lipid deposition. FCP treatment significantly attenuated these pathological changes, outperforming both CP and OP. To further assess hepatic protection, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured (Fig. 2D, E). Consistent with histological observations, FCP markedly reduced AST and ALT levels, indicating a pronounced improvement in liver function.

3.3 FCP markedly improve lipid homeostasis in T2DM mice

To specifically assess hepatic lipid deposition, Oil Red O staining was performed (Fig. 2F, G). Diabetic mice exhibited large, densely packed lipid droplets distributed throughout the hepatocytes, indicating lipid accumulation. Administration of FCP or metformin markedly reduced both the number and size of lipid droplets, suggesting a strong protective effect against hepatic steatosis. In contrast, CP and OP treatments resulted in only partial reductions in lipid accumulation.

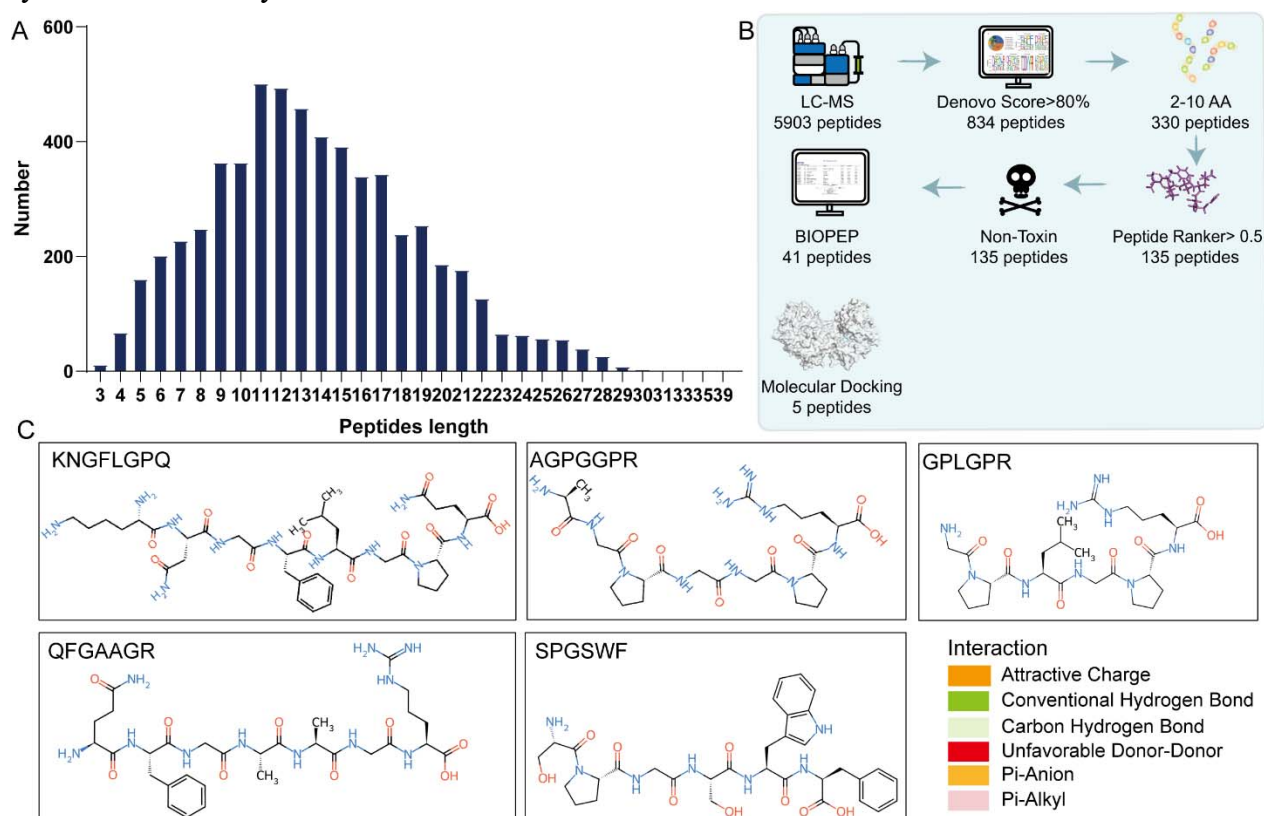
Given that dyslipidemia in T2DM affects both hepatic and systemic metabolism, we next measured serum lipid profiles (Fig. 2H–K). Fish collagen peptide treatment significantly lowered total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C), while concomitantly increasing high-density lipoprotein cholesterol (HDL-C). These findings indicate that FCP not only protect against hepatic lipid deposition but also restore systemic lipid homeostasis, which may contribute to their overall anti-diabetic efficacy.

3.4 Identification of bioactive peptides within FCP

Given the pronounced anti-diabetic efficacy of FCP, we next aimed to identify the specific short peptide sequences responsible for this activity. Previous studies have demonstrated that fish collagen derived peptides can inhibit DPP-IV activity (Rivero-Pino, Espejo-Carpio, & Guadix, 2020), thereby enhancing endogenous GLP-1 secretion and improving glucose homeostasis. Consistent with these findings, FCP administration markedly increased serum GLP-1 levels and reduced DPP-IV activity (Fig. S1A, B), indicating that FCP may exert its hypoglycemic effects through DPP-IV inhibition. To further characterize the active components, the FCP mixture was analyzed by LC–MS to determine its composition and sequences. The analysis revealed that the peptides were predominantly 5–22 amino acids in length (Approximately 93%), providing a basis for subsequent identification of bioactive sequences (Fig. 3A). Subsequently, all identified peptides were subjected to *in silico* screening based on their physicochemical properties, the presence of known DPP-IV inhibitory motifs, and predicted bioactivity scores (Fig. 3B). A total of 41 peptides were obtained from the BIOPEP database and were subjected to molecular docking to evaluate their potential DPP-IV inhibitory activity. Among these, five peptides KNGFLGPQ, AGPGGPR, GPLGPR, QFGAAGR, and SPGSWF exhibited the most favorable docking scores and interaction profiles with DPP-IV (Table. S2). Their chemical structures are illustrated in Fig. 3C. As shown in the docking model, all five peptides were stably accommodated within the catalytic pocket of DPP-IV, occupying the region surrounding the key residues Ser630, Tyr547, Tyr666, Glu205, and Glu206, which are known to be essential for substrate binding and

catalytic activity (Fig. 3D). Specifically, KNGFLGPQ was deeply embedded in the catalytic pocket and formed prominent hydrogen bonds with SER630, GLU205, GLU206, and ARG125, together with hydrophobic and π - π interactions involving PHE357, TYR547, and TYR666, which firmly anchored the peptide within the groove. AGPGGPR adopted an extended configuration, forming aromatic and hydrophobic contacts with TRP629 and TYR547, as well as polar interactions with GLN553 and LYS554, while its C-terminal region interacted electrostatically with ASP708, suggesting a combination of charge complementarity and aromatic stabilization. GPLGPR exhibited a compact orientation stabilized by hydrogen bonding with ARG125, GLU205, and GLU206, and hydrophobic stacking with PHE357, TYR547, and TRP629, indicating a dual polar-hydrophobic binding mode. QFGAAGR formed multiple hydrogen bonds with GLU205, GLU206, and ARG125, along with additional hydrophobic interactions involving PHE357 and TYR547; an auxiliary contact with ARG358 was also observed, extending the peptide toward the rim of the binding cleft. SPGSWF adopted a relatively open conformation, characterized by strong aromatic and hydrophobic contacts with PHE357, TRP629, and TYR547, complemented by hydrogen bonds to GLU205 and SER630 and peripheral interactions with ARG358.

Collectively, all candidate peptides exhibited a conserved interaction pattern, comprising polar anchoring to catalytic residues (GLU205/GLU206, ARG125, SER630) and hydrophobic/ π - π stabilization by aromatic residues (PHE357, TYR547, TRP629). This dual interaction mode positioned the peptides within the substrate-binding cleft of DPP-IV, suggesting that they may competitively interfere with substrate access and thereby contribute to enzymatic inhibition.



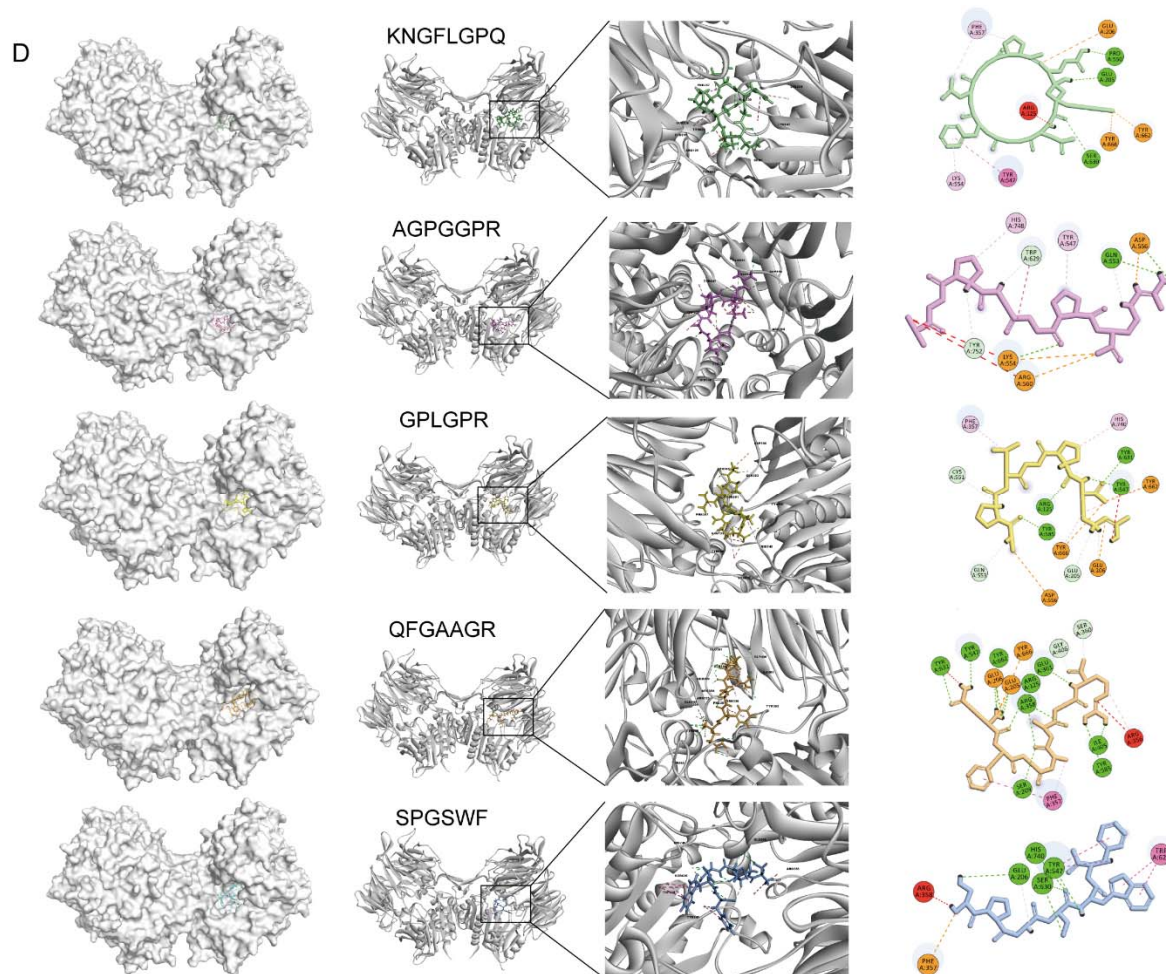


Fig. 3. Identification and in silico screening of bioactive peptides from FCP. (A) Length distribution of peptides derived from FCP; (B) Schematic flowchart illustrating the computational screening strategy; (C) 1D structures and amino acid sequences of the five candidate peptides identified through in silico screening; (D) Molecular docking interactions of the selected peptides with DPP-IV.

3.5 DPP-IV inhibitory effects in vitro

The top candidates obtained from in silico molecular docking including KNGFLGPQ, AGPGGPR, GPLGPR, QFGAAGR, and SPGSWF were chemically synthesized and evaluated for their inhibitory potency against recombinant human DPP-IV *in vitro*. As illustrated in Figure 4A, each peptide displayed a distinct molecular ion peak corresponding to its theoretical molecular weight, with the predominant signals typically observed as $[M+H]^+$ and $[M+2H]^{2+}$ ions. The close agreement between experimental and calculated m/z values verified the accurate synthesis of the peptides without detectable impurities or fragmentation (Fig. 4A). Among the tested peptides, GPLGPR and SPGSWF exhibited the strongest DPP-IV inhibitory activity, with IC_{50} values of 184.7 μ M and 180.8 μ M, respectively, representing the lowest values among the five candidates (Fig. 4B).

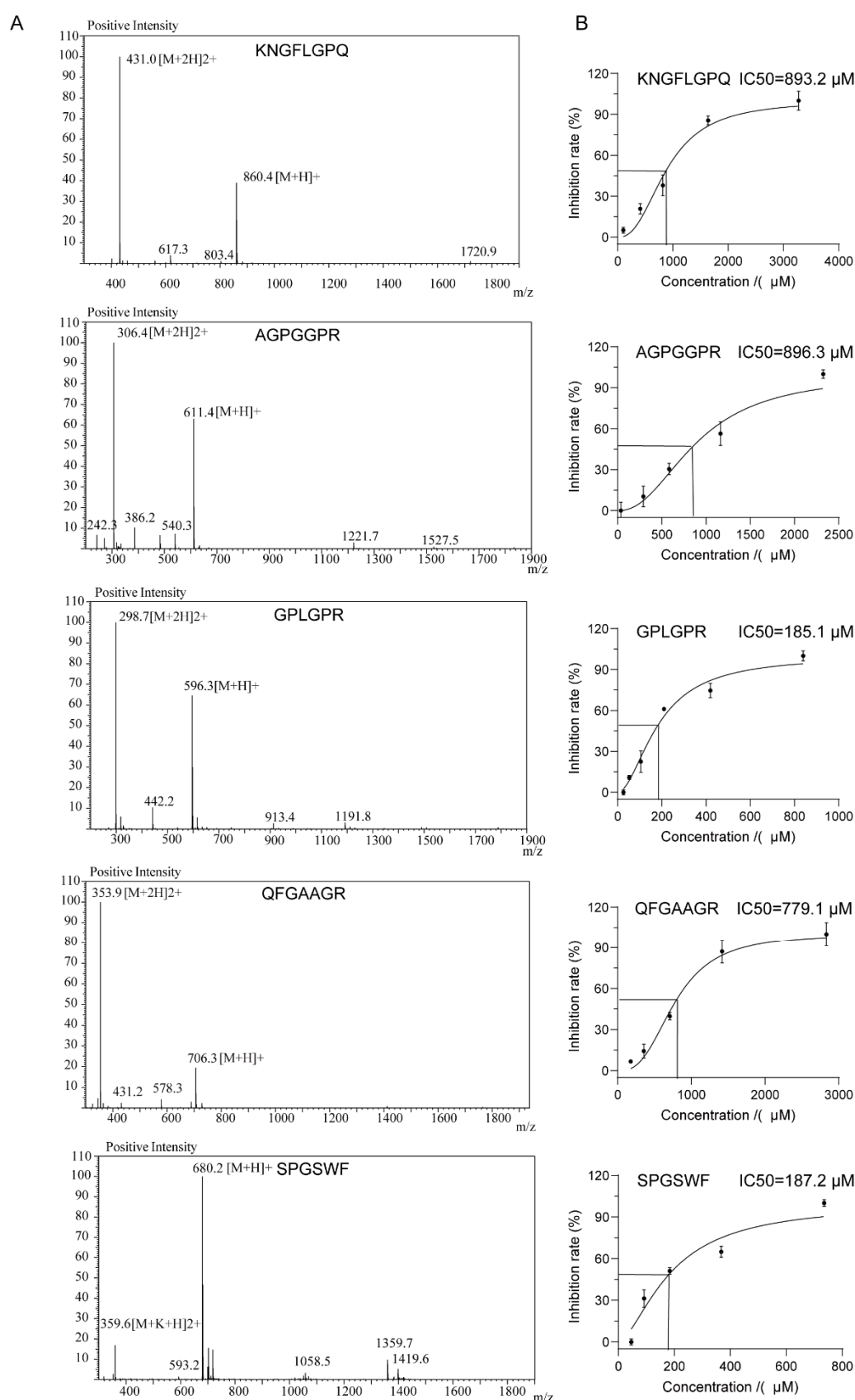


Fig. 4. Verification of peptide synthesis and evaluation of DPP-IV inhibitory activity *in vitro*. (A) Mass spectrometric validation of synthesized peptide candidates; (B) Dose-response inhibition curve and IC₅₀ determination.

To elucidate the mode of inhibition, enzyme kinetic parameters were determined by Lineweaver–Burk analysis (Fig. 5A). Both GPLGPR and SPGSWF displayed a competitive inhibition pattern, as evidenced by an increase in the apparent Michaelis–Menten constant (K_m) without affecting the maximum reaction velocity

($V_{\max}$). The inhibition constants (K_i) for peptides A and B fell within the low micromolar range, further supporting their high binding specificity toward the DPP-IV catalytic site (Fig. 5A).

Gastrointestinal stability was next evaluated using simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion assays. Both peptides retained approximately 80% of their initial DPP-IV inhibitory activity after 2 h of SGF exposure and over 60% following SIF treatment, with GPLGPR exhibiting higher stability than SPGSWF. These results indicate that GPLGPR and SPGSWF can largely maintain their bioactivity under gastrointestinal conditions (Fig. 5B, C).

Since DPP-IV is part of the dipeptidyl peptidase family, which includes DPP8 and DPP9, off-target inhibition poses safety concerns, particularly cytotoxicity and immune-related adverse effects. We therefore assessed the selectivity of peptides GPLGPR and SPGSWF against recombinant DPP8 and DPP9 enzymes. Both peptides showed markedly higher inhibitory activity toward DPP-IV compared with DPP-8 and DPP-9, with F3 and F5 exhibiting over 80-fold and 60-fold greater potency, respectively, indicating strong target specificity. (Fig. 5D, E). This selectivity profile is particularly favorable for therapeutic development, as it minimizes the risk of side effects commonly associated with non-selective DPP inhibition.

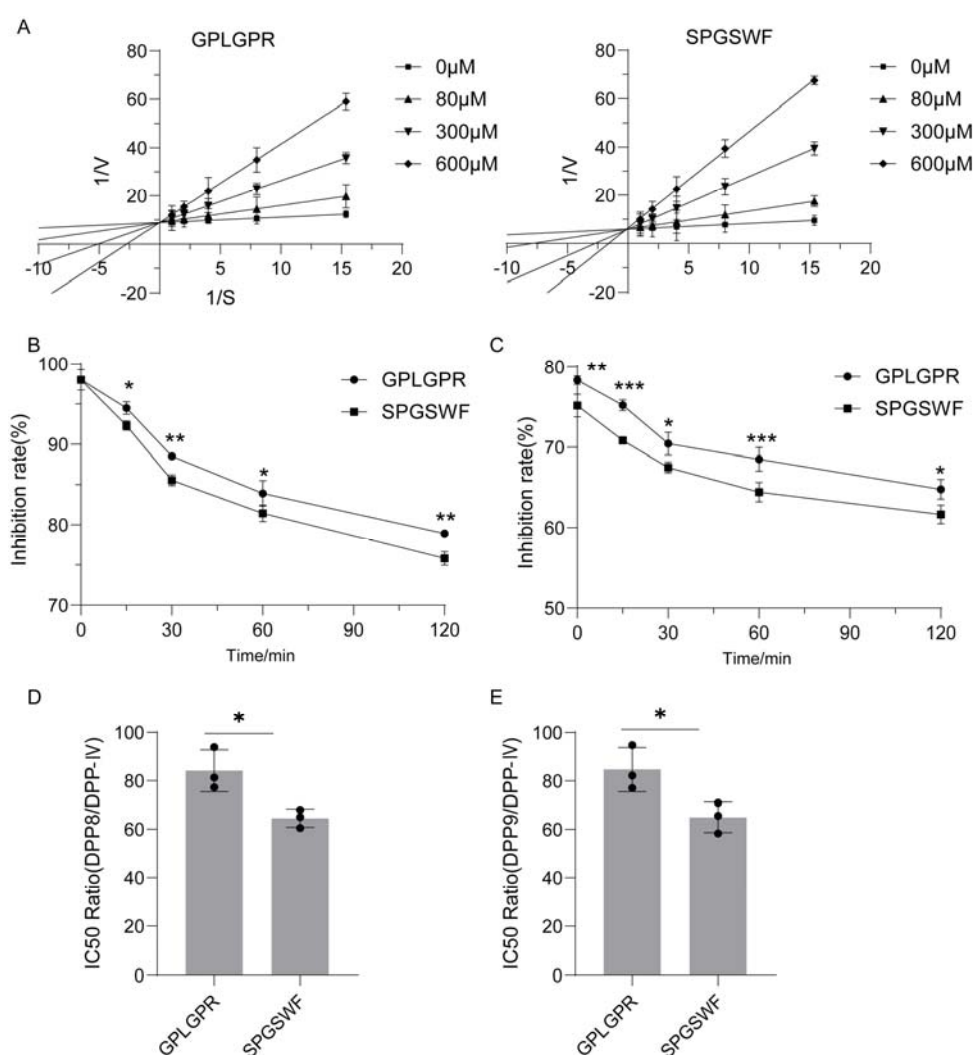


Fig. 5. Evaluation of the stability and safety of the identified DPP-IV inhibitory peptide. (A) Enzyme kinetic analysis showing the Lineweaver–Burk plot, inhibition constant (K_i), and inhibition type; (B) Stability of the peptide in SGF; (C) Stability of the peptide in SIF; (D) Selectivity of the peptide toward DPP-IV over DPP8; (E) Selectivity of the peptide toward DPP-IV over DPP9. All data are expressed as mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

3.6 GPLGPR inhibits DPP-IV activity and enhances GLP-1 stability in vivo

Since GPLGPR and SPGSWF demonstrated strong specificity toward DPP-IV inhibition and favorable gastrointestinal stability *in vitro*, their *in vivo* effects on DPP-IV activity were evaluated in T2DM mice (Fig. 6A). Plasma enzyme activity analysis showed that GPLGPR exhibited inhibitory potency comparable to that of the clinically used inhibitor sitagliptin (Fig. 6B). Consistent with DPP-IV inhibition, GPLGPR treatment markedly increased circulating GLP-1 levels (Fig. 6C). GLP-1 is known to suppress glucagon secretion from pancreatic α -cells in a glucose-dependent manner, thereby reducing hepatic glucose production through glycogenolysis and gluconeogenesis. Accordingly, the expression of key genes involved in these processes, including *Pygl* (glycogenolysis), *Foxo1* (transcriptional regulator), and the gluconeogenic enzymes *Pepck* and *G6pase*, was analyzed. GPLGPR treatment significantly downregulated these genes (Fig. 6D–G), indicating effective suppression of hepatic glycogenolysis and gluconeogenesis. Collectively, these results demonstrate that GPLGPR potently inhibits DPP-IV *in vivo*, leading to elevated GLP-1 levels and reduced hepatic glucose output.

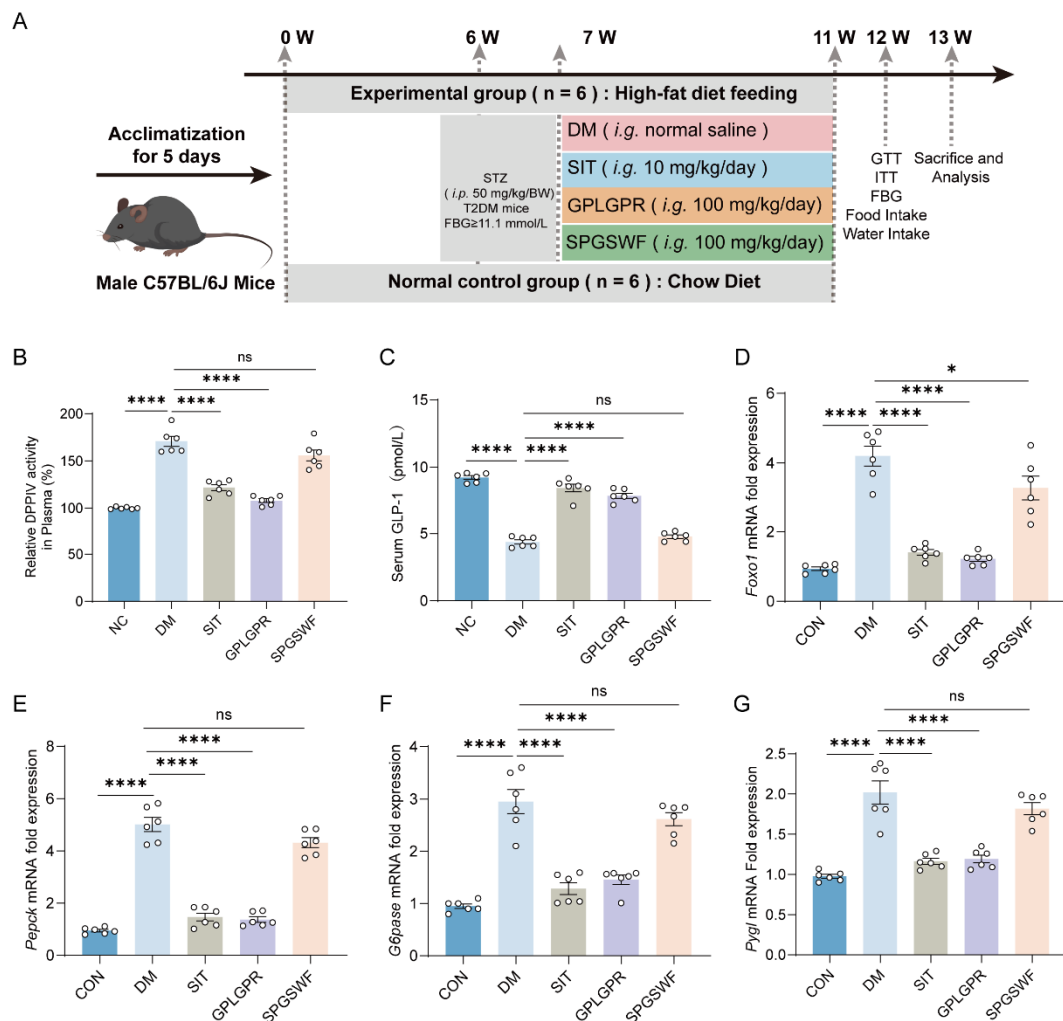
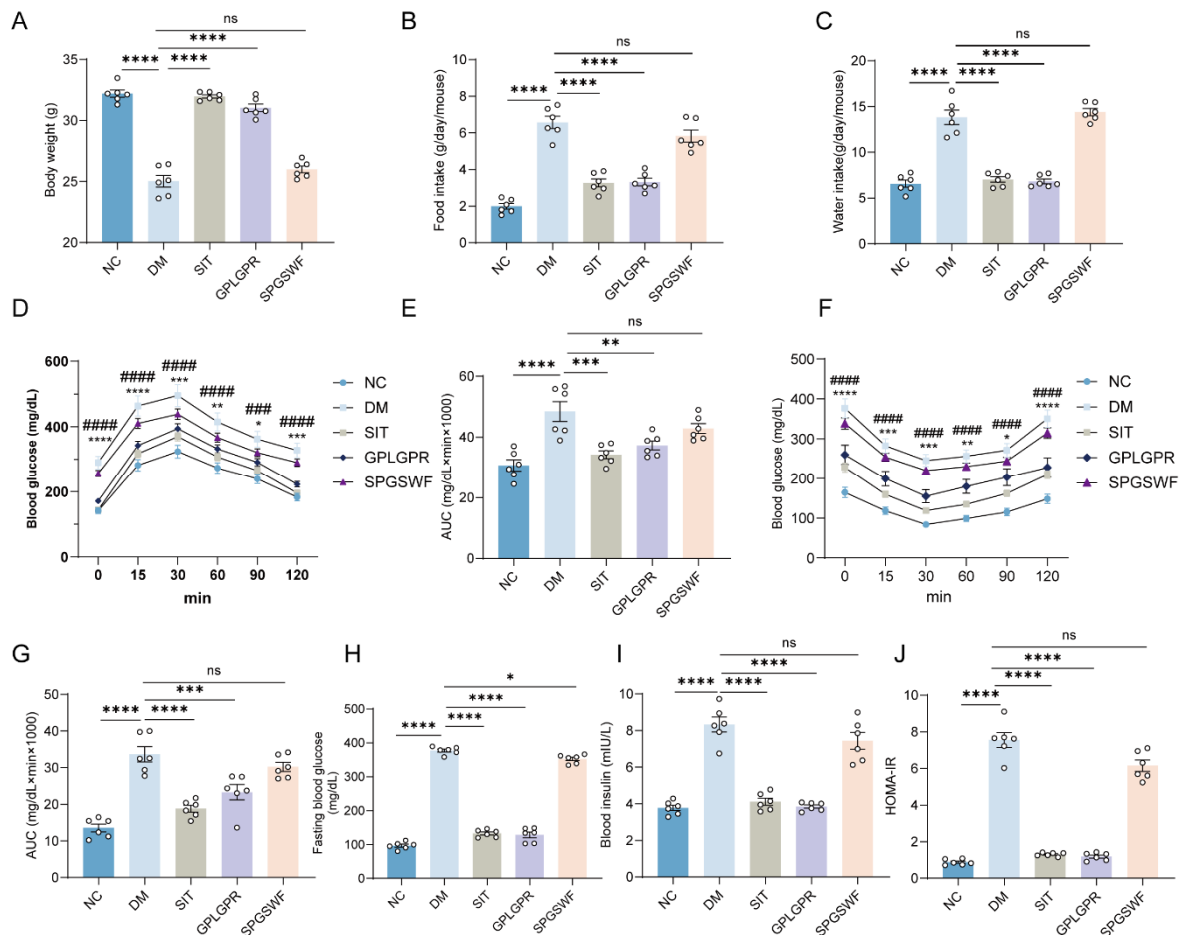


Fig. 6. *In vivo* DPP-IV inhibition, GLP-1 stabilization, and downstream signaling modulation. (A) Schematic diagram of animal experiments; (B) Inhibitory effects of peptides on intestinal DPP-IV activity in T2DM mice; (C) Plasma concentrations of active GLP-1 following peptide administration; Effects of peptides on gluconeogenesis-related genes: (D) Hepatic *Foxo1* mRNA expression, (E) Hepatic *Pepck* mRNA expression, and (F) Hepatic *G6pase* mRNA expression. Effect of peptides on the glycogenolysis-related gene: (G) Hepatic *Pygl* mRNA expression. All data are presented as mean $\pm$ SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

3.7 GPLGPR improves glucose homeostasis and attenuates tissue injury in T2DM mice

Building on the observed *in vivo* DPP-IV inhibition and the consequent elevation of GLP-1, we next evaluated whether these mechanistic effects translated into improvements in systemic diabetic phenotypes. As shown in Fig. 7A-C, GPLGPR more effectively ameliorated diabetes-associated weight loss and excessive food and water intake in T2DM mice compared with SPGSWF. In addition, glucose tolerance and insulin sensitivity assessments revealed that GPLGPR significantly decreased blood glucose levels throughout the testing period and accelerated normalization to baseline values, surpassing the effects of sitagliptin (Fig. 7D-G). Consistently, the observed reductions in FBG, serum insulin concentrations, and HOMA-IR provided further evidence that GPLGPR effectively mitigates hyperglycemia and insulin resistance in T2DM mice (Fig. 7I-K). Furthermore, histopathological assessment of pancreatic and hepatic tissues was performed to evaluate the protective effects of the DPP-IV inhibitory peptide. As shown in Fig. 7L-N, GPLGPR markedly ameliorated pancreatic islet injury, characterized by disorganized cellular arrangement, blurred islet boundaries, and vacuolation, as well as hepatic lesions, including inflammatory infiltration. We further investigated its impact on lipid metabolism. Oil Red O staining of the liver (Supplementary Fig. S2.A-B) and serum lipid profile analysis (Supplementary Fig. S2.C-F) demonstrated that GPLGPR effectively reduced hepatic lipid accumulation and improved systemic lipid metabolism.



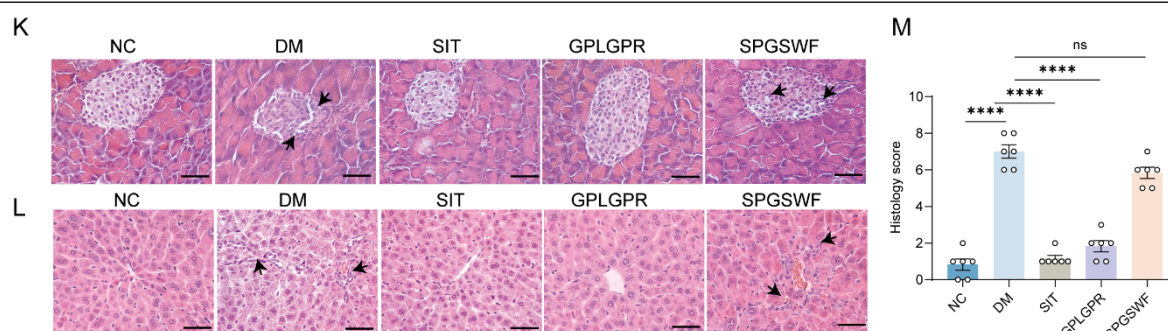


Fig. 7. GPLGPR improves glucose homeostasis and attenuates tissue injury in T2DM mice. (A) Final body weight; (B) Daily food intake after peptides intervention; (C) Daily water intake after peptides intervention; (D) Glucose tolerance test after peptides intervention; (E) AUC of Glucose tolerance test; (F) Insulin tolerance test after peptides intervention; (G) AUC of Insulin tolerance test; (H) Final fasting blood glucose; (I) Serum insulin levels; (J) HOMA-IR calculated from fasting blood glucose and serum insulin. (K) H&E staining of pancreas; (L) H&E staining of liver; (M) Histopathological scoring of liver. Scale bar: 50μm. All data are presented as mean ± SEM. *, SIT vs. DM; #, FCP vs. DM; ^, OP vs. DM; \$, CP vs. DM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4. Discussion

DPP-IV inhibitors, commonly referred to as gliptins, represent a major class of oral hypoglycemic drugs that improve glucose homeostasis by prolonging the half-life of incretin hormones such as GLP-1. By preventing the rapid degradation of GLP-1, DPP-IV inhibitors enhance glucose-dependent insulin secretion, suppress glucagon release, and ultimately contribute to the maintenance of normal blood glucose levels. These agents are generally well tolerated and are associated with a relatively low risk of hypoglycemia compared with other hypoglycemic drugs. However, long-term use of synthetic DPP-IV inhibitors has been linked to adverse effects such as gastrointestinal discomfort, nasopharyngitis, and upper respiratory infections (Saini, Sharma, & Khan, 2023). Consequently, there is growing interest in identifying natural, food-derived DPP-IV inhibitory peptides that can achieve comparable glucose-lowering efficacy while offering improved safety profiles.

In recent years, a number of animal protein hydrolyzed peptides have been reported to have promising anti-diabetic potential. For example, the tripeptide Ile-Pro-Pro (IPP), derived from casein hydrolysate, has been shown to improve glucose metabolism in skeletal muscle cells by activating the AMP-activated protein kinase (AMPK) pathway, promoting glucose uptake independent of the insulin pathway (Iwasa, Takezoe, Kitaura, Sutani, Miyazaki, & Aoi, 2021). For another example, 8 weeks of supplementation with glycomacropeptide (GHP) hydrolysate from bovine casein significantly reduced fasting blood glucose, improved insulin resistance, and promoted skeletal muscle IRS-1/PI3K/Akt signaling and GLUT4 translocation in HFD-STZ induced T2DM. Furthermore, it modulated the gut microbiome, suggesting a multi-target mechanism of action (Yuan, Zhan, Chang, Du, & Mao, 2020). Furthermore, discarded bovine hide collagen has also been used to prepare DPP-IV inhibitory peptides. Studies have repeatedly shown that peptides such as GPVG and FGPGP possess hydrogen bonding and hydrophobic interaction characteristics for binding to DPP-IV (He, Wang, Wang, Luo, Zhao, Han, et al., 2023).

Although these studies have established a mechanistic basis for the use of animal-derived peptides in glucose lowering and improving insulin sensitivity, few studies have systematically compared multiple

animal-derived peptides in the same experimental system. Based on this, this study aimed to provide more direct comparative data for the screening and functional mechanism of animal-derived natural peptides by comparing the anti-diabetic potential of fish collagen peptide (FCP), casein peptide (CP) and ovalbumin peptide (OP). Unlike previous studies that investigated these peptides independently under different experimental conditions, the present work compared the three peptide sources under identical experimental settings, including the same animal model, intervention period, and dosage. In addition, the peptide fractions were standardized to similar low molecular weight ranges, which minimized variability related to peptide size and allowed a more objective comparison of their biological effects. Among these, FCP exhibited the strongest pronounced glucose-lowering effect in T2DM. FCP supplementation effectively alleviated typical diabetic symptoms such as polydipsia, polyphagia, polyuria, hyperglycemia, insulin resistance, and tissue injury.

To further elucidate the active components responsible for the bioactivity of FCP, we performed LC–MS analysis, revealing that most peptides ranged from 5 to 22 amino acids, accounting for approximately 93% of the total peptide population. Subsequent *de novo* sequencing identified candidate peptides with PeptideRanker scores above 0.5, which were further screened using ToxinPred and AllerTop 2.0 to exclude toxic or allergenic sequences. In addition, the BIOPEP-UWM database was used to ensure novelty by filtering out previously reported bioactive peptides. Molecular docking against DPP-IV (PDB ID: 1WCY) identified five top candidates, including KNGFLGPQ, AGPGGPR, GPLGPR, QFGAAGR, and SPGSWF, based on binding affinity and interaction with the DPP-IV catalytic pocket. DPP-IV contains three major subsites (S1, S2, and S3), which together determine substrate recognition and inhibitor specificity (Meduru, Wang, Tsai, & Chen, 2016). The S1 pocket, characterized by residues Tyr662, Val656, Trp659, Tyr631, Tyr666, and Val711, provides a hydrophobic cavity favoring proline- or alanine-containing substrates. The S2 pocket, dominated by the negatively charged residues Glu205 and Glu206, plays a crucial role in anchoring the N-terminal amino group of the substrate via electrostatic interactions, while the S3 pocket provides an auxiliary binding region that stabilizes the inhibitor and enhances affinity (Arulmozhiraja, Matsuo, Ishitsubo, Okazaki, Shimano, & Tokiwa, 2016; Bayanati, Ismail Mahboubi Rabbani, Sirous Kabiri, Mir, Rezaee, & Tabatabai, 2024).

Among the screened peptides, molecular docking indicated that all five interact within the S1–S2 subpockets through polar anchors and hydrophobic stacking. Notably, GPLGPR and KNGFLGPQ demonstrated the deepest and most stable binding modes, characterized by dual polar–hydrophobic interactions with key residues such as Glu205, Glu206, Tyr547, and Phe357. Although KNGFLGPQ displayed a greater number of polar contacts, its longer sequence and high polarity may result in higher entropic penalties and reduced binding efficiency in aqueous environments. In contrast, GPLGPR exhibited a compact conformation and balanced dual-mode binding, which likely contributes to its superior affinity and inhibitory potency. It should be noted that docking-derived binding energy does not always directly correlate with the experimentally measured inhibitory activity, as enzyme inhibition is also influenced by factors such as binding orientation within the catalytic pocket, steric effects, and peptide conformational flexibility.

Peptides such as AGPGGPR, QFGAAGR, and SPGSWF primarily interacted with peripheral residues (Arg358, Trp629), showing relatively weaker and less stable binding orientations. Such differences in binding orientation and interaction with key catalytic residues may partly explain why some peptides predicted to have favorable docking energies exhibit comparatively lower inhibitory activity in enzymatic assays. In contrast, GPLGPR exhibits superior glucose-lowering activity, likely due to the presence of key residues such as Proline, Leucine, and Arginine. These residues may contribute to enhanced peptide stability in the gastrointestinal tract, DPP-IV inhibitory potency, and potential interactions with metabolic pathways or gut microbiota. These structural insights collectively highlight GPLGPR as the most promising DPP-IV inhibitory candidate among the tested sequences.

Consistent with the docking results, *in vitro* assays confirmed that GPLGPR exhibited the strongest DPP-IV inhibitory activity and superior gastrointestinal stability. Enzyme kinetic analysis revealed that GPLGPR functions as a competitive inhibitor of DPP-IV, directly occupying its catalytic pocket. Molecular docking and dynamic simulation demonstrated that GPLGPR establishes stable hydrogen bonds with Glu205 and Glu206, which are essential residues for substrate recognition, while forming hydrophobic interactions with Phe357, Tyr547, and Trp629 within the S1 and S2 sub-sites. These interactions collectively stabilize the peptide–enzyme complex and prevent the natural substrate from entering the active site. Such a binding mode is consistent with the inhibition pattern observed for classical DPP-IV inhibitors such as sitagliptin and vildagliptin, suggesting that GPLGPR mimics the substrate orientation while competitively blocking catalysis.

Importantly, GPLGPR retained its activity *in vivo*, effectively suppressing plasma DPP-IV activity and elevating circulating GLP-1 levels in T2DM mice. Elevated GLP-1 subsequently inhibited glucagon secretion and reduced hepatic glycogenolysis and gluconeogenesis, as evidenced by the downregulation of *Pygl*, *Foxo1*, *Pepck*, and *G6pase*. These molecular changes translated into marked physiological benefits, including improved glucose tolerance, enhanced insulin sensitivity, and alleviation of diabetic symptoms such as excessive thirst and body weight loss. The consistency between the molecular docking, *in vitro*, and *in vivo* findings provides robust evidence that GPLGPR improves glucose homeostasis primarily through DPP-IV inhibition and GLP-1 stabilization.

Collectively, these results demonstrate that GPLGPR is a potent, selective, and stable DPP-IV inhibitory peptide derived from fish collagen. Its natural origin, competitive inhibition mechanism, and favorable gastrointestinal stability make it a promising candidate for the development of functional foods or nutraceuticals for diabetes prevention and management.

Conclusion

Among the three animal-derived peptides evaluated, FCP exhibited the most pronounced anti-diabetic efficacy, as evidenced by improved glucose tolerance, enhanced insulin sensitivity, and amelioration of hepatic steatosis in T2DM mice. Through peptide profiling and functional validation, GPLGPR was identified from FCP as the most potent bioactive sequence responsible for DPP-IV inhibition. Mechanistic analyses

revealed that GPLGPR acts as a competitive inhibitor of DPP-IV by binding to the catalytic residues Glu205 and Glu206 and forming hydrophobic interactions with Phe357, Tyr547, and Trp629, thereby preventing substrate access and promoting GLP-1 stability. Consequently, GPLGPR administration effectively restored incretin function, suppressed hepatic gluconeogenesis, and mitigated hyperglycemia-associated metabolic disorders. These findings underscore the potential of FCP as a rich source of natural DPP-IV inhibitory peptides, with GPLGPR representing a promising candidate for the dietary management of type 2 diabetes (Fig.8).

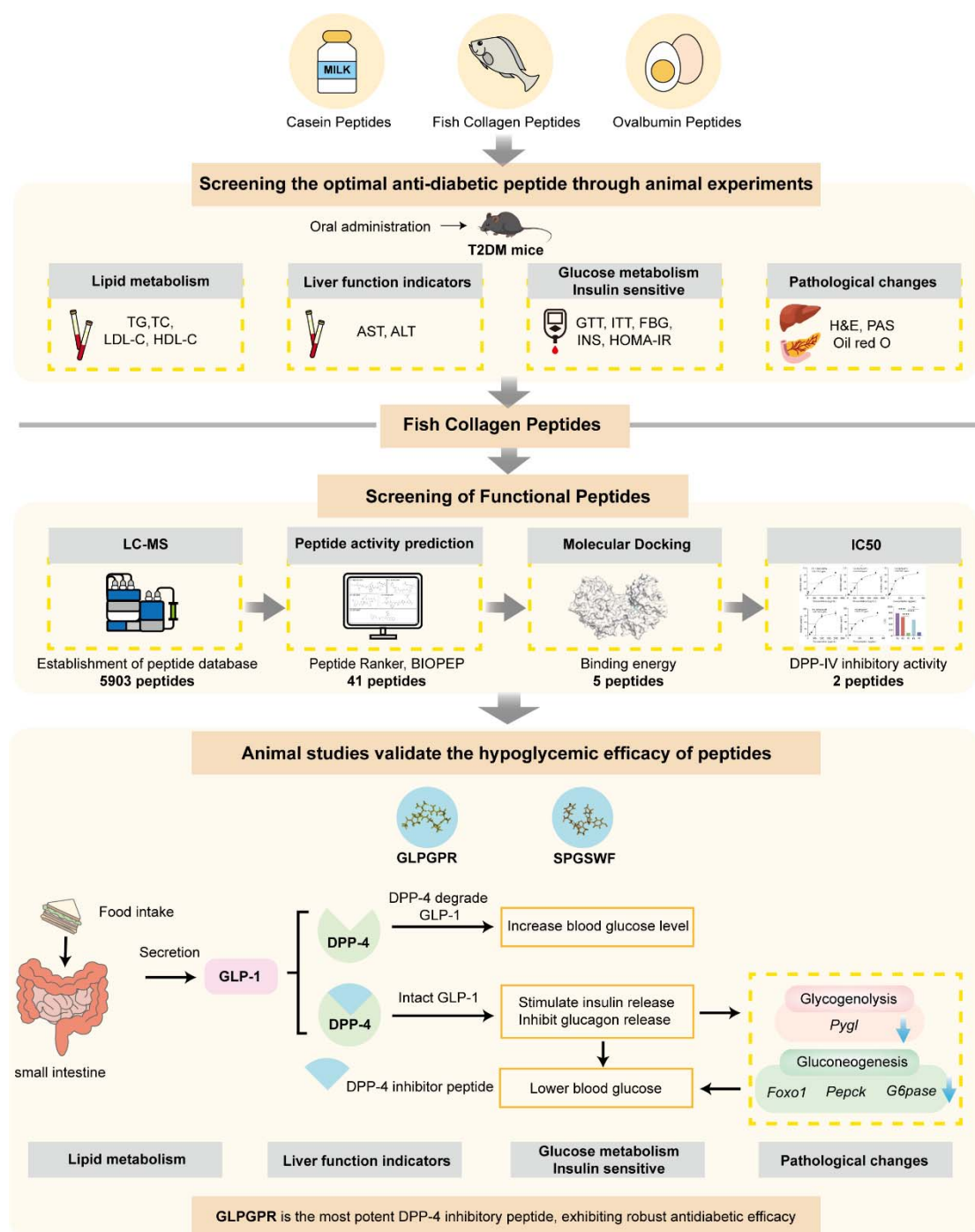


Fig. 8. Systematic screening of animal-derived peptides identifies fish collagen peptide GPLGPR as the most effective DPP-4 inhibitor, enhancing GLP-1 secretion and ameliorating hyperglycemia in T2DM mice.

Nevertheless, several limitations should be acknowledged. The present study was conducted in a murine model, and the long-term bioavailability, metabolic stability, and safety of GPLGPR remain to be fully established in humans. Moreover, while molecular docking provided insights into the binding mechanism, direct structural evidence from crystallography or surface plasmon resonance analysis is still lacking. In addition, the potential influence of GPLGPR on other metabolic pathways, such as insulin signaling and gut microbiota modulation, warrants further exploration. It should also be noted that the GPLGPR used in this study was chemically synthesized due to its low abundance in fish collagen hydrolysates (~0.08%), which limits direct extraction from natural sources and increases production costs. Future research should explore economically feasible strategies to increase peptide yield, such as optimizing enzymatic hydrolysis conditions, employing membrane separation or chromatography for peptide enrichment, or using bioengineering approaches to enhance target peptide production. Overall, future studies integrating multi-omics approaches, chronic intervention models, cost-effective peptide preparation, and clinical validation are needed to clarify the translational potential of fish collagen derived peptides as natural DPP-IV inhibitors.

Ethics Statement

All animal procedures were approved by the Laboratory Animal Ethical Review Committee of China Agricultural University (Approval No. AW41015207-5-01, dated 10/14/2025) and complied with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledge

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