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Insights into rapid screening and molecular mechanism of novel *Rosa roxburghii* seeds pancreatic lipase/cholesterol esterase inhibitory peptides: a combined *in silico* and *in vitro* perspective

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ABSTRACT

Rosa roxburghii seeds are by-products of *R. roxburghii* processing, and their protein hydrolysates (RTSPHs) were found to possess a variety of biological activities. This study aimed to rapidly identify pancreatic lipase (PL) and cholesterol esterase (CE) inhibitory peptides in RTSPHs and to elucidate their molecular mechanisms by combining peptidomics and virtual screening. The simulated intestinal environment worsened the peptide's inhibition of PL but catalyzed the inhibition of CE. The fraction less than 3 kDa in RTSPHs was found to have the highest PL/CE inhibitory activity, among which 17 promising inhibitory peptides were identified and screened by peptidomics and virtual screening. LFCMH, RIPAGSPF, and YFRPR showed good inhibitory abilities against both PL and CE. Molecular docking showed that peptides inhibited PL and CE mainly by hydrogen bonding and hydrophobic interactions with residues in the active site and surface loop. Inhibition kinetic revealed that the peptides were competitive and mixed-type inhibitors of PL/CE. Further, the three peptides, LFCMH, RIPAGSPF, and YFRPR, could effectively inhibit 3T3-L1 preadipocytes differentiation, increase high-density lipoprotein cholesterol content and decrease low-density lipoprotein cholesterol content. This study suggests that combining peptidomics with virtual screening is an effective strategy for rapid screening of PL/CE inhibitory peptides.

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1. Introduction

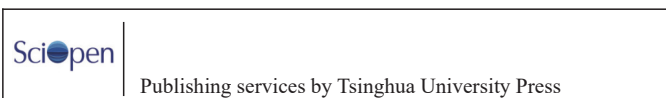
According to the 2023 edition of the World Obesity Atlas, more than 4 billion people worldwide will be obese or overweight by 2035, accounting for 51% of the global population. Obesity is a significant risk factor for cardiovascular disease, diabetes, and other chronic

diseases^[1]. Obesity is caused by excessive food intake or changes in the body's metabolism, which makes the body accumulate too much fat. The digestion and absorption of dietary lipids in the human body require the participation of two key enzymes, pancreatic lipase (PL) and cholesterol esterase (CE)^[2]. Therefore, inhibiting the activity of these two key enzymes can slow down the intake of lipids to achieve the goal of anti-obesity. The only over-the-counter anti-obesity drug currently in clinical use, orlistat, is designed to alleviate obesity by inhibiting PL. However, orlistat may produce several side effects such as nausea, diarrhea, and indigestion during the weight loss^[3]. Recent studies have shown that short fragments of bioactive peptides from

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plants can inhibit lipid digestive enzymes^[2,4-5]. Although bioactive peptides have problems with oral stability and bioavailability compared with chemically synthesized drugs, they still have the incomparable superiority of chemically synthesized drugs from the perspective of safety and applicability.

Several proteins of plant origin and their hydrolysates have been found to inhibit PL and CE activity. The oat peptide SPFWNINAH can inhibit PL activity through the interaction of residue Asn7 with the catalytic triplet of PL^[6]. Sea buckthorn seed peptides can prevent PL from binding to the substrate^[7]. Purifying protein hydrolysates from beer lees by multistage isolation yielded three PL and CE inhibitory peptides^[8]. In a word, plant proteins are promising sources of PL and CE inhibitory peptides. In the aforementioned studies, the activity of PL and CE inhibitory peptides is generally tested in a simplified static buffer system. However, the digestion of lipids in the human gastrointestinal tract is an extremely complex dynamic process. Lipid emulsion mixes with bile and pancreatic juice in the intestine, forming a bilayer of emulsified particles covered by bile salts and other substances^[9]. Herrera et al.^[10] found that the inhibitory activity of quinoa and fenugreek extracts on PL was significantly reduced in the simulated intestinal environment. Zhang et al.^[11] also concluded that PL needs to function in a stable dispersed emulsion. Therefore, further in-depth studies on the PL and CE inhibitory activity of anti-hyperlipidemic peptides based on the simulated gastrointestinal tract are necessary.

Traditional methods for isolating bioactive peptides from food proteins require enzymatic digestion followed by separation and purification using ultrafiltration, gel permeation chromatography, and reverse HPLC, etc.^[12]. These methods are time-consuming and expensive, limiting subsequent research on activity identification and molecular mechanisms. The new emergence of simulated hydrolysis *in silico* in recent years has the advantages of high efficiency and cheapness^[3,13]. However, virtual hydrolysis ignores the complexity of the actual conditions, and the results can not exactly match the actual situation^[14]. The combination of peptidomics and virtual screening techniques can rapidly screen bioactive peptides while ensuring realism. For example, Hou et al.^[15] combined peptidomics and molecular docking to identify a variety of potential antimicrobial peptides from *Zanthoxylum bungeanum* Maxim seeds. Similarly, Wei et al.^[16] used peptidomics to identify 430 peptides from fermented rubing cheese. Further, 16 antioxidant peptides, ACE inhibitory peptides, and α -glucosidase inhibitory peptides were successfully screened by molecular docking. In conclusion, the combination of peptidomics and virtual screening will become the most promising means to obtain active peptides quickly and accurately. Considering the complexity of the intestinal environment, the validity of using a combination of peptidomics and virtual screening to screen for PL and CE inhibitory peptides needs to be further confirmed.

Rosa roxburghii, a deciduous shrub of the Rosaceae family, is widely distributed in China. The fruits of *R. roxburghii* are rich in vitamin C, flavonoids (especially rutin), and superoxide dismutase (SOD), with detoxification, sedation, and other medicinal value and health care functions^[17]. *R. roxburghii* seeds are a waste product from the processing of *R. roxburghii* and can account for up to one-third of the weight of *R. roxburghii*. The high protein content of *R. roxburghii* seeds (7.85%) has the potential for the preparation of bioactive peptides, but has not attracted attention before. Our preliminary experiment confirmed that the alcalase and papain hydrolysate of

R. roxburghii seeds possessed strong PL and CE inhibitory activity, attributed to the bioactive peptides it contained. Several studies have identified the potential of plant seed protein hydrolysates to inhibit PL and CE. However, further sequence identification, activity verification, and molecular binding mechanisms of peptides inhibiting PL and CE in protein hydrolysates have not been fully confirmed. Therefore, investigating the potential roles of bioactive peptides from *R. roxburghii* seed in inhibiting lipid digestive enzyme is crucial. This study first explored the inhibitory effects of *R. roxburghii* seed protein hydrolysates (RTSPHs) on PL and CE under traditional conditions and simulated intestinal environments to provide a more realistic reflection of the activity. Subsequently, a combination of peptidomics and virtual screening methods was employed to identify PL and CE inhibitory peptides in RTSPHs. Finally, the screened peptides were synthesized to validate their activity, explore the molecular mechanism, and confirm their role in regulating lipid metabolism at the cellular level. This research will provide a better understanding of using RTSPHs as a potential PL and CE inhibitor and functional ingredient in managing the diet for obesity.

2. Materials and methods

2.1 Materials

R. roxburghii seeds were obtained from Xin Yang Agricultural Technology Co., Ltd. (Bijie, China). PL (≥ 3 U/mg) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and CE (70.2 U/mg) was purchased from Solarbio Life Sciences (Beijing, China). All of the other chemicals were given in detail in the supplementary materials.

2.2 RTSPHs preparation

The *R. roxburghii* seed protein was prepared using the standard alkaline dissolution and acid precipitation combined with microwave treatment as previously described^[18]. The optimal hydrolysis conditions for papain and alcalase were given in detail in the supplementary materials.

RTSPHs were separated into three fractions of different molecular weights (> 10 , $3-10$, < 3 kDa) using ultrafiltration^[19]. Briefly, 1 g of lyophilized RTSPHs were dissolved in 100 mL of water and filtered through a $0.45\ \mu\text{m}$ filter membrane. It was then loaded into an ultrafiltration device (Merck Millipore, Germany) and separated under a pressure of 40 psi through filter membranes with different cutoff molecular weights. Then the filtrate was lyophilized and stored at $-20\ ^\circ\text{C}$ for further analysis.

2.3 PL and CE inhibition assay

2.3.1 General

Both PL and CE inhibition assays were performed by incubation of the substrate, enzyme and RTSPHs at $37\ ^\circ\text{C}$, under two different conditions according to methods by Herrera et al.^[10], Alnuaimi et al.^[20] and Zhang et al.^[21] with some modifications: A) traditional method, that is using Tris-HCl buffer (0.1 mol/L, pH 7.5) as buffer; and B) simulation of intestinal conditions, that is shaking incubation using a digestion buffer (0.312 g lecithin, 0.78 g bile salts, 0.056 g CaCl_2 ,

0.88 g NaCl, and 100 mL Trizma maleate buffer (0.1 mol/L, pH 7.5)). This mixture was homogenized in a homogenizer (T18 Ultra-Turrax, IKA, Germany) for 2 min at 10 000 r/min.

2.3.2 *In vitro* PL and CE inhibition activity and inhibition mode

PL and CE inhibition by RTSPHs were conducted according to methods by Jafar et al.^[5] and Mudgil et al.^[22] with some modifications. Experimental conditions were given in detail in the supplementary material.

The inhibition modes of peptides were examined via Lineweaver-Burk double-reciprocal plots by measuring the reaction rates at different substrate concentrations (1–8 mmol/L) without inhibitors or in the presence of peptides (0.5–1 mg/mL).

2.4 Peptide identification and sequencing by LC-MS/MS analysis

The peptide composition of RTSPHs was analyzed by LC-MS/MS. LC-MS/MS analysis was performed by a Q Exactive plus mass spectrometer (Thermo Scientific, USA) and connected to an Easy nLC1200 system (Thermo Scientific, USA)^[23]. Experimental conditions were given in detail in the supplementary material.

2.5 Virtual screening and molecular docking

The potential biological activity of the identified peptides was scored using PeptideRanker (<http://distilldeep.ucd.ie/PeptideRanker/>)^[24] and those scoring above 0.5 were used as an alternative for further screening. All screened peptides were used for further studies.

AutoDock Vina software was used to screen the promising peptides^[25–26]. The structures of the peptides were built using RPBS online server (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>)^[27]. ILPB and 1AQL were selected as the target protein crystal structure due to they have been used in PL and CE inhibitor studies elsewhere^[2,28]. After removing extraneous small molecules using PyMOL 2.1 software, the protein molecule was imported into AutoDock Tools-1.5.6 software to remove water molecules, add hydrogen atoms, and set the atom type. The peptides were molecularly docked to the enzyme using AutoDock Vina. The center positions of the Grid Box used in the screening process was determined by the position of the active site of the enzyme (PL: Ser152-His263-Asp176; CE: Ser194-Asp320-His435) as follows: ILPB: $x = 8.024$, $y = 23.538$, $z = 55.706$; 1AQL: $x = 34.757$, $y = 66.474$, $z = 23.515$, and the length, width and height were set to $30 \text{ \AA} \times 30 \text{ \AA} \times 30 \text{ \AA}$ ^[28].

Molecular docking was performed by AutoDock Vina to investigate the probable interactions between peptides and PL/CE. The screened peptides were used as small molecule ligands, and the two proteins ILPB and 1AQL were used as receptors. The mode of interactions between the compound and the target protein were analyzed to obtain the compound-protein residue interactions, such as the resulting hydrogen bonding interactions, π - π interactions, hydrophobic interactions, etc. The 3D and 2D docking images were obtained from PyMOL 2.1 and LigPlot, respectively.

2.6 Solid-phase peptides synthesis

Peptides were synthesized by Nanjing PeptideValley Biotechnology Co., Ltd. (Nanjing, China).

2.7 In silico peptide analysis

The physico-chemical properties of selected peptides with potent PL and CE inhibitory activities were calculated using the online available tool PepDraw (<http://www.tulane.edu/~biochem/WW/PepDraw/>)^[29].

2.8 Intracellular lipid-lowering activity of peptides analysis

2.8.1 Parallel artificial membrane permeability analysis (PAMPA)

The PAMPA method was used to determine the passive permeability of peptides in artificial membranes according to the methods by Navarro del Hierro et al.^[30].

2.8.2 Cell culture and differentiation

3T3-L1 preadipocytes were cultured in a high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and placed in a 5% CO₂, 37 °C cell incubator. 3T3-L1 preadipocytes were inoculated in 6-well plates (1×10^5 per well) and cultured until the cells produced contact inhibition. They were then cultured in a primary differentiation medium (0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 μ mol/L dexamethasone and 10 μ g/mL insulin) for 3 days and in secondary differentiation medium (10 μ g/mL insulin) for 4 days. The test group was cultured with the corresponding medium containing different concentrations of peptides. Differentiation was completed until 80% of the cells differentiated into mature adipocytes.

2.8.3 Cell viability assay

3T3L1 cells were inoculated in 96-well plates (10^4 per well). After 24 h of incubation, the medium was discarded and medium containing different concentrations of peptides was added. After 24 h of incubation, the medium was discarded, 10% CCK-8 medium was added, and the absorbance was detected at 450 nm after 2 h of incubation. Cell viability was calculated by the following formula:

$$\text{Cell viability (\%)} = \frac{A_t}{A_c} \times 100$$

Where A_t was the absorbance value of the test group, and A_c was the absorbance value of the control group.

2.8.4 High-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) assay

After completion of cell differentiation, the cells were digested with trypsin and the medium was centrifuged to collect the cell precipitate. Cells were washed with PBS and broken by cell lysis buffer. Finally, HDL-C and LDL-C contents were measured according to the kit instructions.

2.9 Statistical analysis

Statistics differences were evaluated in SPSS 20 software (IBM, Chicago, IL, USA) by a one-way analysis of variance using Duncan's post hoc test. Differences were considered to be significant when the $P < 0.05$. Each experiment was repeated three times.

3. Results and discussion

3.1 Degree of hydrolysis (DH) and PL/CE inhibitory activity of RTSPHs

As shown in Fig. 1A, the DH of *R. roxburghii* Tratt seed protein gradually increased with the hydrolysis time. The DH of the alcalase-treated group could reach 17% within 0.5 h, then slowly increased and finally reached about 25%. The DH in the papain-treated group was much lower, reaching about 7% after 3 h of hydrolysis. These differences in DH values were mainly due to differences in enzyme reaction rates, specificity, and substrate affinity^[18]. Alcalase preferentially cleaves on the carboxyl side of hydrophobic amino acid residues, while papain primarily hydrolyzes peptide bonds of adjacent amino acids such as lysine, arginine and phenylalanine^[31].

The PL inhibition activity of RTSPHs is shown in Figs. 1B-C, and the PL inhibition activity of alcalase hydrolysates is slightly higher than that of papain hydrolysates, both under traditional condition and in the intestinal environment. This is mainly due to the higher DH leading to more short peptide production and thus higher biological activity^[32]. The IC_{50} for PL inhibition in the papain-treated group under traditional condition was (4.888 ± 0.244) mg/mL, while the value for the alcalase treated group was (4.256 ± 0.374) mg/mL. Baba et al.^[33] hydrolyzed camel whey protein and found the PL IC_{50} of its hydrolysate to be 0.465–7.653 mg/mL, and the IC_{50} of amaranth protein hydrolysate ranged from 0.38–7.34 mg/mL^[4]. However, the abovementioned

studies on PL inhibitory peptides were mainly conducted in a simplified static system environment without considering the actual situation and dynamics of lipid digestion in the gastrointestinal tract (GIT). In this study, the inhibition of PL by peptides was examined using an environment simulating intestinal digestion, and it was observed that the inhibitory activity of both protease hydrolysates on PL was reduced to some extent in the simulated intestinal environment. The IC_{50} for PL inhibition in the papain-treated group under simulated intestinal environment was (13.636 ± 2.368) mg/mL, while the value for the alcalase treated group was (6.035 ± 1.417) mg/mL. The reasons were complex, as the changes of the lipase activity, the interaction among the reaction components, and the peptide composition all may affect the results. Herrera et al.^[10] attributed the decrease in PL inhibitory activity in the simulated intestinal for the ethanolic extracts of fenugreek and quinoa seeds to the degradation of bioactive compounds. In addition, PL can interact with surfactants, bile salts, and lecithin in the simulated intestinal environment, allowing PL to remain adsorbed on the surface of emulsified lipids and making it easier to interact with the substrates, and thus enhancing the activity of PL and led to a more difficult inhibition of PL activity by the peptide^[34].

Figs. 1D-E demonstrates the inhibitory activity of the two protein hydrolysates against CE. As same as the PL inhibitory activity, the alcalase-treated group inhibited CE more than the papain-treated group. The IC_{50} for CE inhibition in the papain-treated group under traditional condition was (36.798 ± 2.955) mg/mL, while the value for the alcalase treated group was (23.853 ± 2.722) mg/mL. However, unlike PL, the inhibitory activity of both hydrolysates against CE was stronger in the simulated intestinal environment than in the conventional conditions. The IC_{50} for CE inhibition in the papain-treated group under simulated intestinal environment was (7.571 ± 0.783) mg/mL, while the value for the alcalase treated group was (5.031 ± 0.707) mg/mL. This is mainly due to the difference in enzyme structure. Bile salts will bind two different binding sites present in CE, which enhances

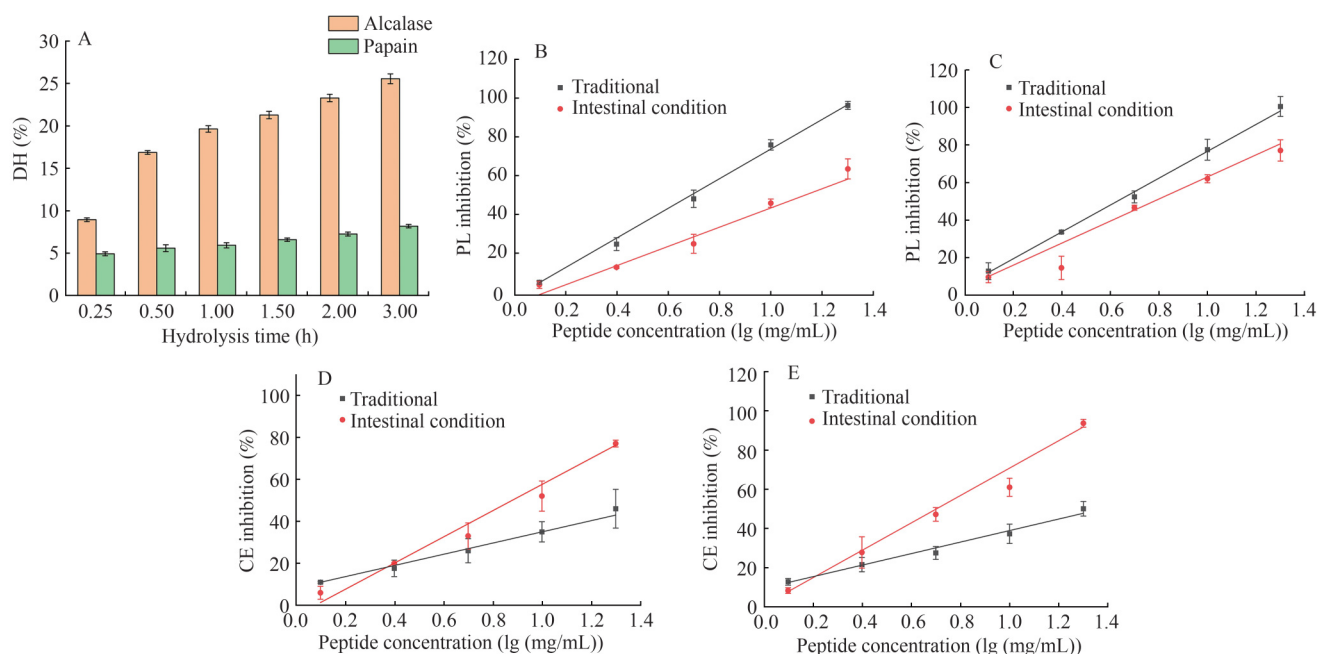


Fig. 1 (A) DH of RTSPHs obtained by alcalase and papain, respectively. (B-C) PL inhibition effect of crude RTSPHs under conventional and simulated intestinal conditions. (B) Papain treated group; (C) Alcalase treated group. (D-E) CE inhibition effect of crude RTSPHs under conventional and simulated intestinal conditions. (D) Papain treated group; (E) Alcalase treated group. Data were represented as means $\pm$ SEM.

the hydrophobicity of binding sites of CE^[35]. As a result, those peptides that are more hydrophobic than *p*-nitrophenyl butyrate become more readily bound to the active site, thereby limiting the binding of the substrate to the active site. Herrera et al.^[10] also found that the catalytic activity of amylase became stronger in the simulated intestinal environment. Since the simulated intestinal environment is more in line with the actual scenario, all subsequent assays for the inhibitory activity of peptides were suggested performed using the simulated intestinal environment method.

3.2 Separation of PL and CE inhibitory peptides from RTSPHs

In order to further investigate the inhibitory activity of RTSPHs on lipid-digesting enzymes, three fractions of RTSPHs were separated into > 10, 3–10 and < 3 kDa by ultrafiltration membranes with different retention molecular weights. Fractions less than 3 kDa accounted for the vast majority, with 65.4% of fractions less than 3 kDa in the papain-treated group and 73.2% of fractions less than 3 kDa in the alcalase treated group. The inhibitory activities on lipid-digesting enzymes were measured under simulated intestinal conditions and the results are displayed in Figs. 2A–B. All groups showed a trend that the smaller molecular weight resulted in stronger inhibitory activity. Since small peptides are more likely to attach to the catalytic centers of PL and CE, RTSPHs with lower molecular weights have a higher inhibitory capacity against PL and CE^[12]. Awosika et al.^[36] studied the PL inhibitory activity of pea protein hydrolysate and found that the lower molecular weight hydrolysate could inhibit PL better than the other fractions. Therefore, peptide fractions less than 3 kDa in alcalase-treated group and the papain-treated group were selected for the next experiments.

3.3 Identification of the peptides

In contrast to the MS/MS method for identifying peptides after bioassay-based multi-column purification, peptidomics could identify all peptides simultaneously after Nano-LC-MS/MS and PEAKS protein identification software. In present study, peptide sequences with a false discovery rate of < 1% were selected and a total of 1 676 peptides were identified after the removal of duplicate terms. The molecular weight distribution of all peptides is shown in Fig. 2C, ranging from 350.198 3

to 2 862.925 15 Da. Among them, 85.4% of the peptides had molecular weights < 1.6 kDa, and the lower molecular weights may contribute to their effective PL and CE inhibitory properties. These peptides mainly from major storage proteins A0A2P6PV95, A0A2P6PV77, A0A2P6PV78, A0A2P6PV89, and A0A2P6PV90 which are abundant in *R. roxburghii* (Fig. 2D). Table S1 provides the sequence information of all identified peptides. Considering the abundance, peptides containing more than 0.1% were selected for further virtual screening to filter out the exact peptide fragment with the highest inhibitory activity.

3.4 Peptide fragment screening with PL and CE inhibitory activity

The potential bioactivity of peptides containing more than 0.1% of the hydrolysate (291 peptides in total) was first scored using the PeptideRanker. A total of 66 peptides with a score of more than 0.5 were screened. 3D structures of these peptides (323 in total) were generated for the next step of virtual screening using the RPBS online server. PL (PDB ID: 1LPB) and CE (PDB ID: 1AQL) have been widely used for virtual screening of PL and CE inhibitors due to their high precision structures (resolution: 1LPB: 2.46 Å; 1AQL: 2.80 Å)^[37–38]. The peptides were then screened virtually for inhibition of PL and CE activities using AutoDock Vina. Considering the number of peptides, the top 12 peptides with the highest binding energies to PL and CE, respectively, were selected for the next step of analysis. A total of 17 peptides with inhibitory potential were screened (Table 1). Among them, 12 peptides showed binding energies below −7 kcal/mol to PL, with the highest being −7.0 kcal/mol for QGALL and the lowest being −8.6 kcal/mol for RIPAGSPF and SPHWNLN. For CE inhibitory peptides, 12 peptides showed inhibitory potential, with the highest binding energy being −7.6 kcal/mol for LFCMH and the lowest being −9.7 kcal/mol for RPFLY. Since there is no absolute correspondence between binding energy and inhibitory capacity, *in vitro* validation of the inhibitory capacity of these peptides is required^[12]. Seven peptides (LFCHM, LFCMH, QGALL, RGFFY, RIPAGSPF, RPRFY, and YFRPR) showed low binding energies for both PL and CE, suggesting that they may have the potential to inhibit both enzymes. PepSite2 (<http://pepsite2.russelllab.org/>) is a website that can predict the binding sites of peptides to enzymes and has been used to explore the binding sites of peptides that inhibit PL and CE^[4,7,39]. The PepSite2 results (Table 1)

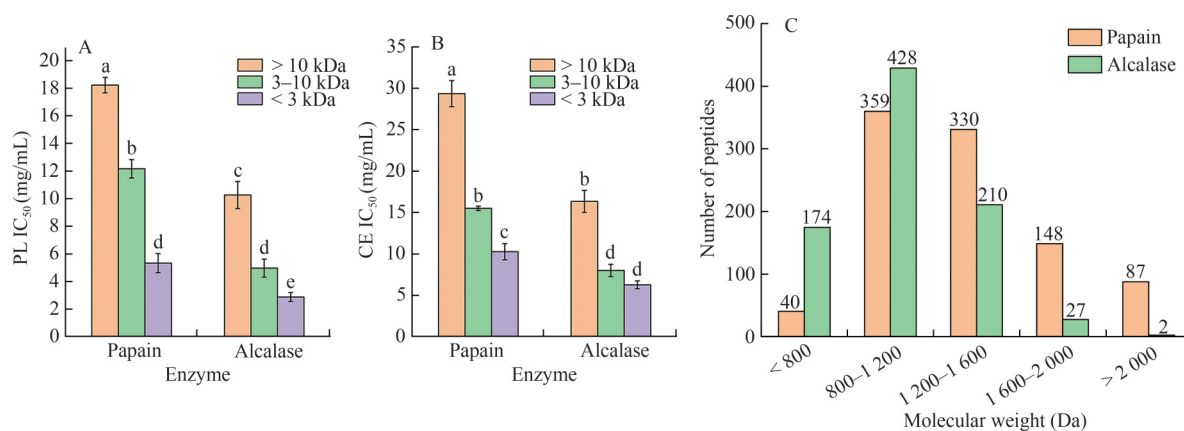


Fig. 2 (A) PL and (B) CE inhibition effect of different RTSPHs fractions. (C) Number of peptides with different molecular weights identified from < 3 kDa RTSPHs fractions. (D) Peptide mapping of A0A2P6PV95 that released most peptides. Data were represented as means ± SEM. Different lowercase letters indicate significant differences between groups ($P < 0.05$).

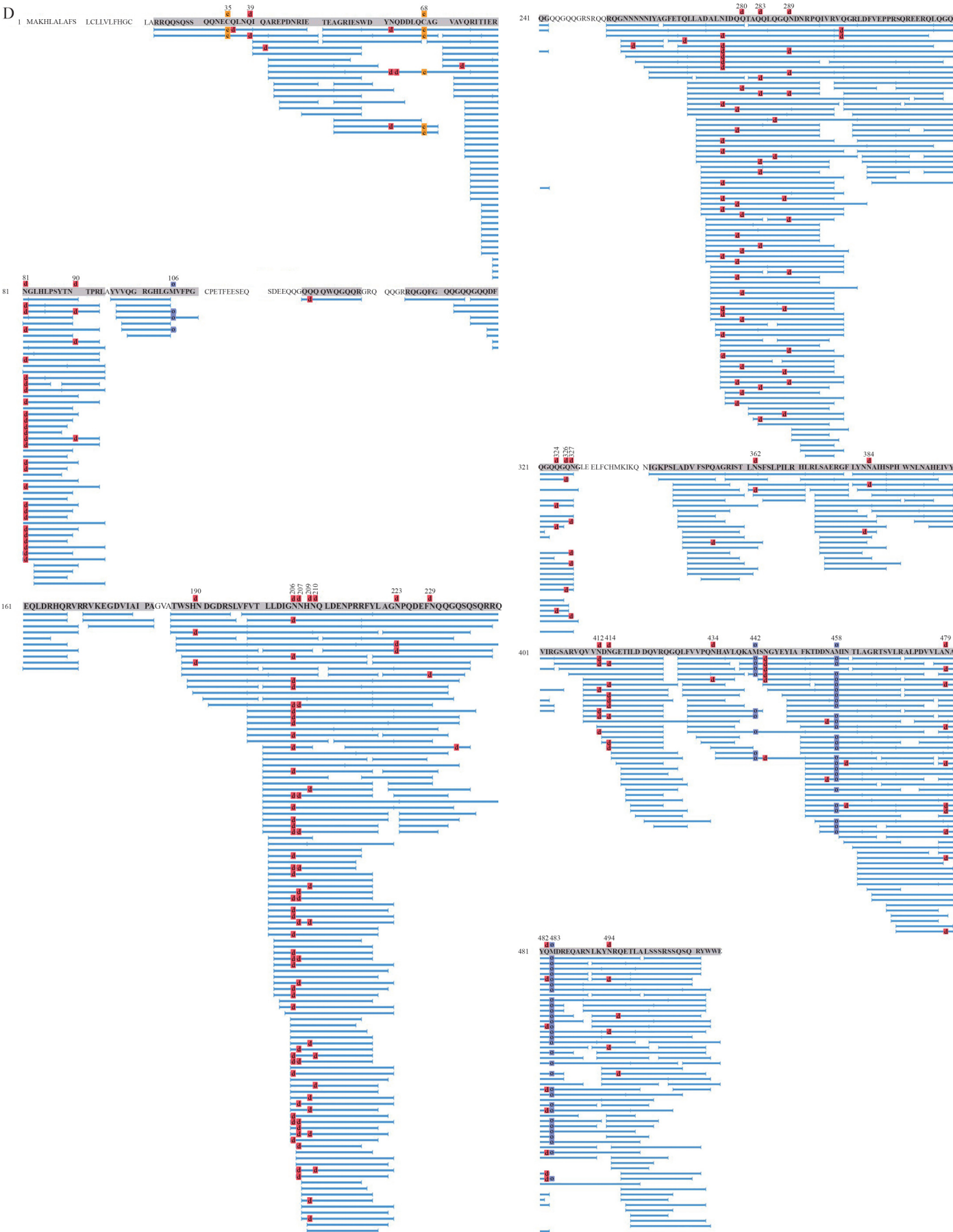


Fig. 2 (Continued)

Table 1

Results of virtual screening for PL and CE inhibitory activity of RTSPHs and physico-chemical property analysis of the selected PL and CE inhibitory peptides.

Peptide sequence	Source	Content/%	PeptideRanker score	Enzyme type	PepSite2 <i>P</i> -value	Vina score (kcal/mol)	Molecular weight (Da)	Isoelectric point	Net charge	Hydrophobicity (kcal/mol)
AKDNVRFPL	AH	0.12	0.611 5	PL	0.022	−8.2	1 058.59	10.14	1	14.22
FIEPPR	PH	0.28	0.735 5	CE	0.030	−8.8	757.411	6.51	0	10.79
HGNQPLVF	PH	0.19	0.702 7	CE	0.099	−8.8	910.465	7.68	0	9.72
LFCHM	AH	0.51	0.918 5	PL	0.009	−7.7	649.271	7.15	0	6.58
				CE	0.032	−7.9				
LFCMH	AH	0.10	0.905 5	PL	0.030	−7.4	649.271	7.15	0	6.58
				CE	0.021	−7.6				
NSFSMPILR	AH	0.34	0.813 5	CE	0.040	−8.1	1 063.550	10.73	1	6.87
QGALL	AH	0.46	0.569 8	PL	0.023	−7.0	500.295	5.44	0	7.82
				CE	0.017	−8.3				
RFYLAG	PH	0.13	0.710 0	PL	0.031	−8.2	725.385	9.93	1	7.69
RGFFY	AH	2.94	0.964 1	PL	0.047	−8.5	688.332	9.64	1	6.73
				CE	0.066	−8.9				
RIPAGSPF	AH	0.11	0.633 8	PL	0.097	−8.6	843.459	10.90	1	9.27
				CE	0.196	−8.1				
RPFLY	AH	3.32	0.899 8	CE	0.017	−9.7	694.379	9.64	1	6.18
RPRFY	PH	0.36	0.906 9	PL	0.098	−8.4	737.396	11.15	2	9.24
				CE	0.029	−9.1				
SMPLIR	AH	0.84	0.729 0	PL	0.020	−7.1	715.404	10.73	1	7.27
SPHWNL	AH	6.32	0.887 3	CE	0.039	−8.7	752.360	7.89	0	8.34
SPHWNLN	AH	3.88	0.832 7	PL	0.194	−8.6	866.402	7.38	0	9.19
SWDHY	AH	0.15	0.711 6	PL	0.191	−7.5	706.270	4.98	−1	11.53
YFRPR	PH	0.17	0.890 3	PL	0.107	−8.1	737.396	11.14	2	9.24
				CE	0.029	−9.3				

show that the vast majority of peptides have a binding *P*-value below 0.05 (highly significant) and all peptides have a *P*-value below 0.2 (moderately significant) with enzymes, further proofing the enzyme inhibitory ability of these peptides.

The physicochemical properties of peptides such as hydrophobicity, fixed point and charge determine the inhibitory properties of bioactive peptides^[40]. The predicted physicochemical properties of the screened peptides are shown in Table 1, and most of the peptides have an isoelectric point greater than 7, indicating the presence of more basic amino acids. Due to the presence of hydrophobic amino acids, all peptides are highly hydrophobic, with hydrophobicity between 6.58 and 14.22 kcal/mol, suggesting that the peptides may have a strong enzyme inhibitory activity^[41].

3.5 *In vitro* PL and CE inhibitory peptide activities

To avoid false positive results, these 17 peptides were prepared by a solid-phase synthesis method and their inhibitory activities were shown in Table 2. The MS spectra of the 17 selected peptides are shown in Fig. S1. For PL inhibitory peptides, all 12 peptides showed inhibitory activities, but 4 peptides (RFYLAG, RPRFY, SPHWNLN, SWDHY) did not reach 50% inhibition at the assayed concentrations (0.312 5–5 mg/mL). Among the remaining 8 peptides, AKDNVRFPL, LFCHM, LFCMH, RIPAGSPF, SMPLIR, and YFRPR exhibited relatively strong PL inhibitory activities, all below 3 $\mu\text{mol/mL}$. These results suggested that virtual screening could effectively reduce labor costs and time and is very effective for screening peptide inhibitors. It was reported that FYLGCDY, a peptide obtained from de-oiled rice bran, has shown PL inhibition with an IC_{50} of 0.47 $\mu\text{mol/L}$ ^[41]. SPFWNINAH, a peptide from oat bran, showed PL inhibition with an IC_{50} of 85.4 $\mu\text{mol/L}$ ^[6]. In addition, peptides TF, EW, QWM, NIF, AGY, and PIF from sesame protein showed IC_{50} were 751, 907, 986,

Table 2

Inhibitory activity of solid-phase synthetic peptides against PL and CE.

Enzyme type	Peptide sequence	IC_{50} ($\mu\text{mol/mL}$)
PL	AKDNVRFPL	1.51 $\pm$ 0.08
	LFCHM	2.68 $\pm$ 0.18
	LFCMH	2.02 $\pm$ 0.11
	QGALL	8.14 $\pm$ 0.50
	RFYLAG	–
	RGFFY	6.61 $\pm$ 0.26
	RIPAGSPF	2.42 $\pm$ 0.11
	RPRFY	–
	SMPLIR	1.78 $\pm$ 0.15
	SPHWNLN	–
	SWDHY	–
	YFRPR	2.24 $\pm$ 0.16
	FIEPPR	1.89 $\pm$ 0.09
	HGNQPLVF	1.73 $\pm$ 0.12
	LFCHM	2.05 $\pm$ 0.09
	LFCMH	1.03 $\pm$ 0.06
CE	NSFSMPILR	3.03 $\pm$ 0.15
	QGALL	1.44 $\pm$ 0.10
	RGFFY	2.34 $\pm$ 0.17
	RIPAGSPF	1.09 $\pm$ 0.09
	RPFLY	4.97 $\pm$ 0.36
	RPRFY	1.95 $\pm$ 0.24
	SPHWNL	1.39 $\pm$ 0.09
	YFRPR	1.22 $\pm$ 0.08

Note: – Not reach 50% inhibition at the assayed concentration.

1 044, 1 183 and 5 172 $\mu\text{mol/L}$, respectively^[13]. Considering the different experimental conditions in different studies, such as enzyme concentration, type, pH and concentration of buffer, substrate concentration and other conditions during the manipulation, the IC_{50} for peptide inhibition of PL has a great disparity. Therefore, it is necessary to introduce a positive control (e.g., orlistat) for

comparison in subsequent studies to better reflect the activity of the bioactive compounds. In addition, the use of methods closer to the real situation is particularly important to accurately measure the PL inhibitory activity of peptides. To assess the magnitude of IC_{50} of peptides against PL, orlistat was used as a positive control. The IC_{50} of orlistat against PL ($(1.08 \pm 0.07) \mu\text{mol/L}$) was much lower than that of the peptide, which is consistent with the study of Esfandi et al.^[6]. This is mainly attributed to the fact that the inhibition of PL by peptides as well as other biologically active compounds is mainly caused by non-covalent binding, whereas orlistat covalently binds to the PL active site. However, severe side effects as well as potential hepatotoxicity limit the application of orlistat. Therefore, the use of peptides to partially replace orlistat for combined inhibition of enzyme activity is an option to be explored in the future.

For the CE inhibitory peptides, it was observed that all peptides showed good CE inhibitory activity with IC_{50} ranging from $(1.03 \pm 0.06) \mu\text{mol/mL}$ for LFCMH to $(4.97 \pm 0.36) \mu\text{mol/mL}$ for RPFLY. However, compared with orlistat ($IC_{50} = (0.82 \pm 0.08) \mu\text{mol/L}$), the IC_{50} of the peptides was still high. Ye et al.^[42] isolated and purified three small molecule peptides, FLF, IYF, and QIF, from tea protein hydrolysis products with good CE inhibitory effects. However, it was reported by Garzón et al.^[8] that the high molecular weight peptides were stronger inhibitors of CE, and they isolated the CE inhibitory peptide with the sequence WNIHMEHQDLTME from brewer's spent grain. Therefore, the isolation and identification of the CE inhibitory peptide and its molecular mechanism of action need to be further investigated. We could obtain from the *in vitro* validation results that the peptides LFCMH, RIPAGSPF, and YFRPR have good inhibitory effects on both PL and CE, which may be potential candidates for further studies as anti-obesity molecules.

3.6 Mechanism of interaction between PL and peptides

PL is a glycoprotein consisting of 449 amino acids, and it has two structural domains: a globular N-terminal structural domain generated by a central β -sheet core (amino acids 1–335) and a C-terminal structural domain consisting of a β -sheet (amino acids 336–449). The active site of PL consists of Ser152-His263-Asp176 together forming a catalytic triad^[43]. Three surface loops outside the active site wrap it to inhibit substrate binding to the active site. One of them, Cys237-Cys261, forms the largest loop by a disulfide bond, and residues 76–80 and 213–217 form another two smaller loops. These hydrophobic caps change conformation when they are at the water-oil interface, which opens the substrate binding site and thus activates the enzyme^[13]. Therefore, binding to either the binding site or to the 'loop' can inhibit lipase activity.

Based on the results of the virtual screen and the synthesis of the peptides, we can find that all PL inhibitory peptides contain hydrophobic amino acids such as leucine and proline. Similar results were observed by Baba et al.^[33] and Ngoh et al.^[44], who showed that most of the leucine and proline in the lipase inhibitory peptides were found to interact with lipase residues. In addition, the hydrophilic amino acid arginine is also a common feature of many PL inhibitory peptides, including the earlier identified enterostatin (VPDPR)^[45]. It is hypothesized that hydrogen bonding is the main factor for PL inhibition by inhibitory peptides containing Arg residues. Arginine contains an α -amino group, an α -carboxylic acid group and a guanidinium-containing side chain, and is therefore able to interact

with two charged residues through hydrogen bonding and with hydrophobic residues through hydrophobic interactions via its methylene group^[7].

For the peptide AKDNVRFPL, Asp3 can form hydrogen bonds with residues His263 within the active site. Thus, AKDNVRFPL can inhibit PL activity through hydrogen bonding interactions with the "lid" and the active site (Fig. 3A). For the peptide LFCMH, the hydrogen bonds formed by Phe2 and Cys3 with Ala259 and by His4 with Ser152 inhibit PL activity (Fig. 3B). LFCMH can prevent the unfolding of the loop by forming hydrogen bonds between Cys3/Met4 and the Ala259 residue in the loop (Fig. 3C). The peptides RIPAGSPF, SMPLIR and YFRPR all contain Arg residues, and it can be seen from Figs. 3D–F that the Arg residues of the three peptides can interact with the residues in PL. Ser1 of SMPLIR can form hydrogen bonds with Lys239 and Gln244 in the "lid", Leu4 and Ile5 form hydrogen bonds with Ala259 residue. The Tyr1 residue in YFRPR forms a hydrogen bond with Phe77 in the "lid", preventing exposure of the active site. It has been reported that Phe77 is an H-bond donor and electrostatic stabilizer and has formed the fifth hydrolysis of the oxygen anion pore^[46].

In addition to this, these peptides are enriched with hydrophobic amino acid residues, including Gly, Ala, Val, Leu, Ile, Pro and Phe. These amino acid residues can bind to PL through hydrophobic interactions, preventing the repositioning of the structural domain catalytic site as well as the unfolding of the lid, thereby limiting the function of PL^[13]. Several previously reported PL inhibitory peptides can also bind to residues in the active pocket and on the loop to occupy the groove of the enzyme active site, preventing the binding of the substrate to the enzyme active site^[2,13,42]. Fig. S2A demonstrates the non-covalent interactions of the positive control-orlistat binding to the major catalytic sites of PL. The β -lactone ring of orlistat can bind to Ser152 in the PL active site. In addition to this, orlistat can form hydrophobic interactions with many amino acid residues in the PL.

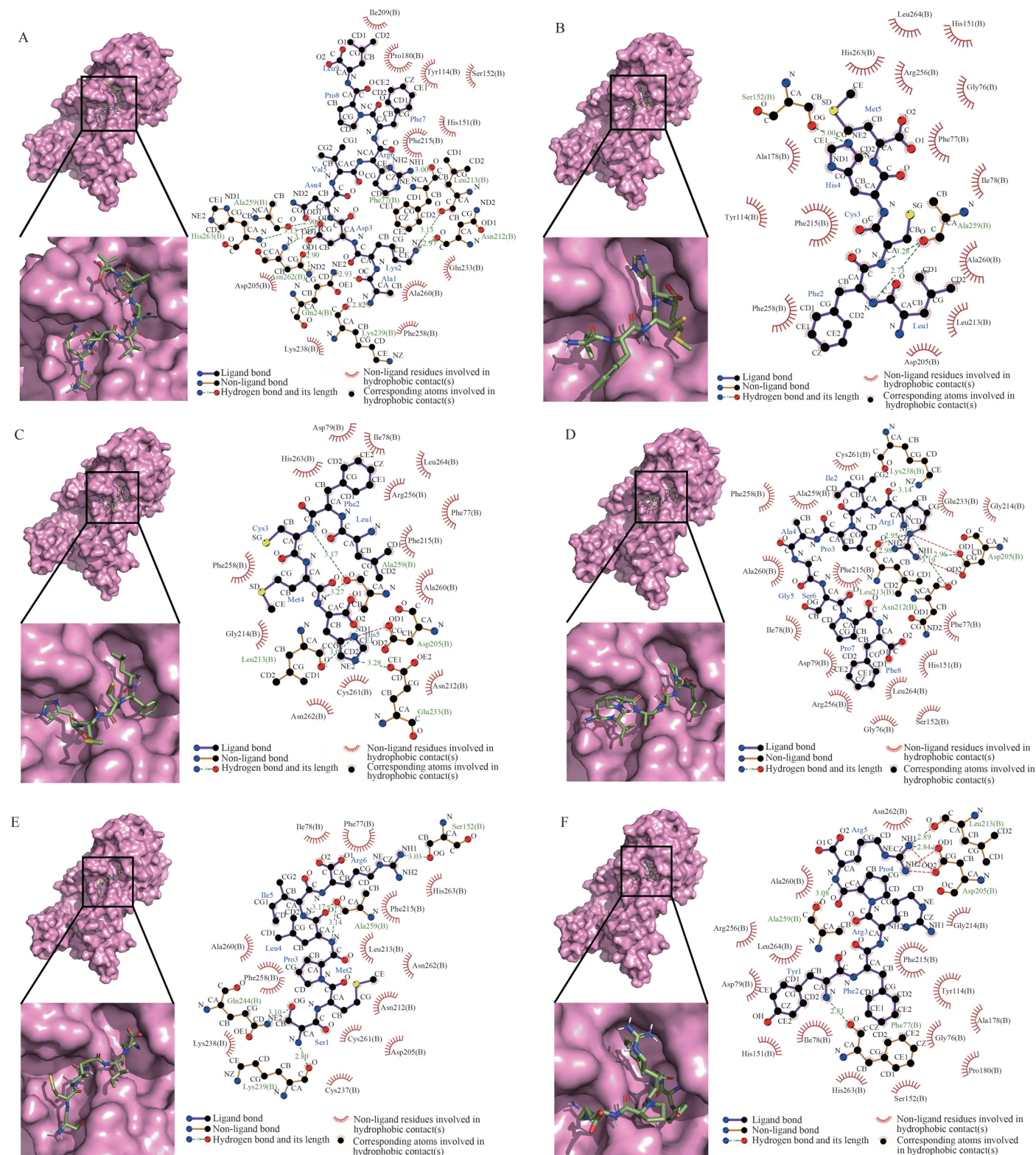
3.7 Mechanism of interaction between CE and peptides

CE is a serine hydrolase that belongs to the α/β -hydrolase family. The catalytic triad consisting of residues Ser194, Asp320 and His435 and the oxygen anion vacancy consisting of residues Gly107, Ala108 and Ala195 together form the active site^[2]. In addition to this, the ring formed by the salt bridge between Cys64 and Cys80 covering the active site also affects its activity^[38].

Figs. 3G–L show the results of the molecular docking of six peptides with high CE inhibitory activity validated by chemical synthesis. For HGNQPLVF, the hydrogen bond formed between Asn3 with His435 and the hydrophobic interactions between Val7 with Gly107 and Ala108, Phe8 with Ser194 binds the peptides tightly to CE, thereby occupying the groove in the CE active site and inhibiting the enzyme activity. For LFCMH, hydrogen bonds are formed mainly by Phe2 and Cys3 at the active sites of Ala108, Ser194, and His435, resulting in stable complexes. For QGALL, hydrogen bonds are mainly formed by Ala3 with Ala108 and Ser194. Besides, Gly107 and Ala195 can also form hydrophobic interactions with the peptide, thus further enhancing the binding of the peptide to CE. RIPAGSPF is a peptide that has the inhibitory ability for both PL and CE. For CE, we can see that Gly5 and Arg1 formed hydrogen bonds with Ser194 and His435, affecting the active site's function. Fisayo Ajayi et al.^[4] also found that some peptides containing Arg residues have the potential to inhibit CE. While for SPHWNL, Ser1 formed hydrogen bonds with

His435 and Trp4 with Ser194. For YFRPR, Arg3 and Arg5 formed hydrogen bonds with Glu193, Tyr125 and His435, Asp437, respectively, and Tyr1 formed hydrogen bonds with Ser194.

In conclusion, the most important role played by these peptides in binding to CE is the hydrogen bonds formed by Ser194 and His435 with the peptide residues. Fisayo Ajayi et al.^[4] also found that the



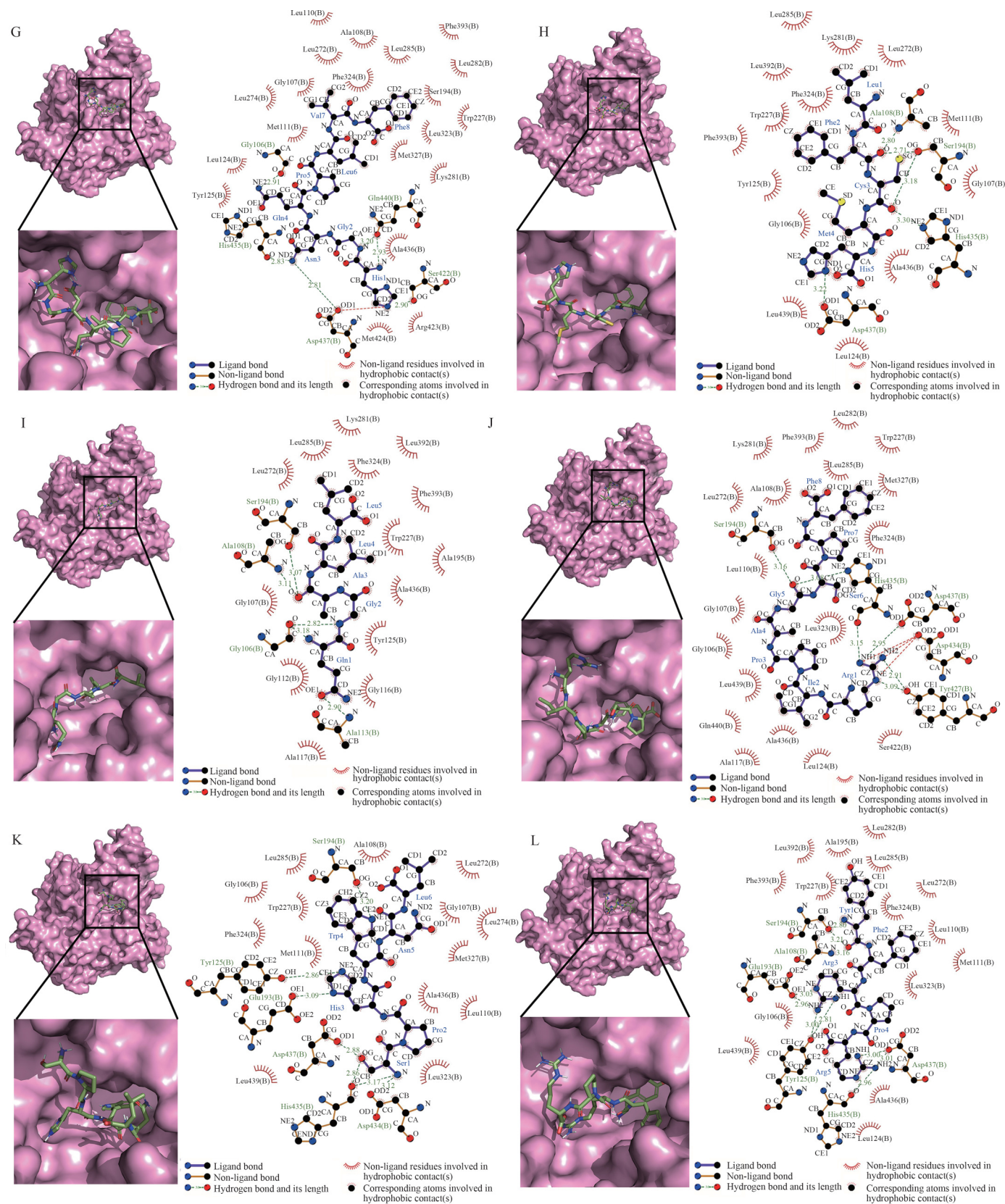


Fig. 3 (Continued)

peptide PPFVPAPT could form hydrogen bonds with these two residues. Besides, the hydrogen bonds formed by Ala108, Asp437, and hydrophobic interactions between hydrophobic amino acids also

play an important role. Although Asp437 is not an active site, the hydrogen bond formed by it was also found to play an important role in the binding of peptides to CE in the study of Baba et al.^[33].

Residues Tyr270-Leu285 belong to the part of the N-terminal structural domain in the coiled-coil ring. Interaction with this part can also prevent the enzyme from releasing the catalytic product and thus inhibit the enzyme activity^[2]. Fig. S2B demonstrates the non-covalent interactions of the positive control-orlistat binding to the major catalytic sites of CE. The β -lactone ring of orlistat forms a hydrogen bond with Arg63 of CE, thus occupying the active site of CE.

3.8 Inhibition mode of PL and CE inhibitory peptides

The inhibition patterns of PL and CE inhibitory peptides on PL and CE were analyzed by double reciprocal Lineweaver-Burk plots. LFCMH, RIPAGSPF and YFRPR, which inhibited both PL and CE activities most strongly, were selected for inhibition kinetic analysis. Figs. 4A–C demonstrate the Lineweaver-Burk plots of the three peptides against PL. The fitted curves for different concentrations of LFCMH intersected in the second quadrant, indicating that the inhibition of PL by LFCMH was of a mixed-type inhibition^[47]. The K_m value of LFCMH increased significantly with increasing concentration, whereas the v_{max} value decreased with increasing concentration (Table S3), which further validated the above results. The same mixed-type inhibition was observed for peptides TF, QWM, and NIF identified from sesame proteins^[13] and three lipid-lowering peptides (NLYYKVV, WWYVK, and EGLLMWPY) identified from Sacha inchi meal^[47]. Molecular docking revealed that LFCMH can inhibit PL activity through multiple mechanisms. As for the peptides RIPAGSPF and YFRPR, the K_m values increased with increasing peptide concentration, whereas the v_{max} values did not change significantly (Table S3). Figs. 4B–C also show that the intersection of Lineweaver-Burk plot fitting curve is located on the Y-axis, indicating that the peptides inhibit PL activity mainly through competitive inhibition. FPFYGVWVK found in Sacha inchi meal similarly inhibits PL activity through competitive inhibition^[47].

Figs. 4D–F demonstrate the kinetic profiles of the inhibition of CE by the three peptides. It can be seen that the double reciprocal curves of substrate concentration and reaction rate at different three peptides all intersected the Y-axis, indicating that the inhibition of CE by all three peptides was competitive^[13]. This is consistent with the results of molecular docking.

Since the identification of inhibitory peptides in this study was achieved by screening the binding ability of the enzyme active site to the peptides, most of the screened inhibitory peptides can inhibit the enzyme activity by binding to the active site and thus restricting substrate entry. In addition, it can be seen that the catalytic efficiency of PL and CE decreases with increasing peptide concentration in the presence of these three peptides. Among them, the catalytic efficiency of LFCMH for PL and CE decreased more, indicating that LFCMH has a stronger inhibitory effect on PL and CE within this concentration^[13].

3.9 Effect of peptides on HDL-C and LDL-C in 3T3-L1 preadipocytes

The peptides must first be absorbed into the circulation in order to modulate 3T3-L1 preadipocyte lipid metabolism. The PAMPA assay was used to examine the passive permeation efficiency of three peptides in artificial membranes. PAMPA, a cell-free method that uses a lipid-infused artificial membrane to determine the passive permeability of substances from a donor compartment to an acceptor compartment, is a widely used assay to predict oral absorption that exhibits a high degree of correlation with Caco-2 assay^[30]. All three peptides were permeable through the artificial membrane. LFCMH and RIPAGSPF displayed a moderate permeability ($(2.52 \times 10^{-6} \pm 3.35 \times 10^{-7})$ and $(1.55 \times 10^{-6} \pm 5.50 \times 10^{-8})$ cm/s), and YFRPR displayed a moderate permeability ($(5.84 \times 10^{-7} \pm 6.68 \times 10^{-8})$ cm/s) but still permeates through the artificial membrane at a lower rate^[30].

In recent years, 3T3-L1 preadipocytes have become one of the most exemplary cell lines for studying the molecular mechanisms of lipid accumulation and lipid-lowering^[42]. The effects of these three peptides on the cell viability of preadipocytes were determined by the CCK-8 assay, as shown in Fig. 5A. It can be seen that RIPAGSPF and YFRPR had no significant effect on cell viability in the range of 50–600 μ g/mL. However, the concentration of LFCMH over 400 μ g/mL caused a significant decrease in cell viability. Therefore, the concentration of the peptide for its effect on cellular lipid lowering was subsequently determined to be 100–400 μ g/mL.

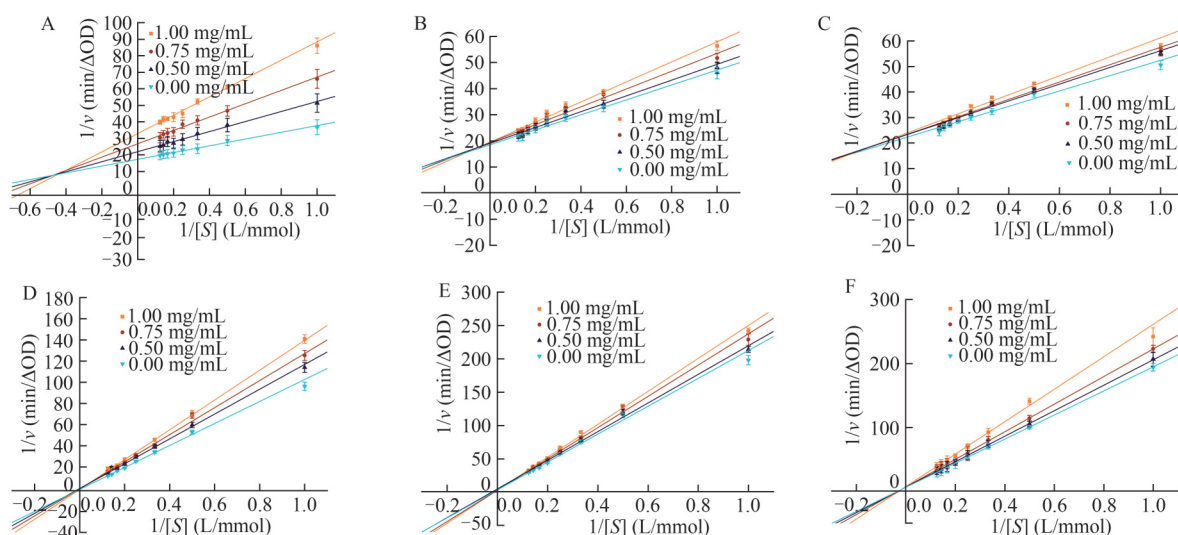


Fig. 4 Lineweaver-Burk double-reciprocal plots for (A–C) PL and (D–F) CE inhibitory peptides (A, D) LFCMH, (B, E) RIPAGSPF, and (C, F) YFRPR. Data were represented as means $\pm$ SEM.

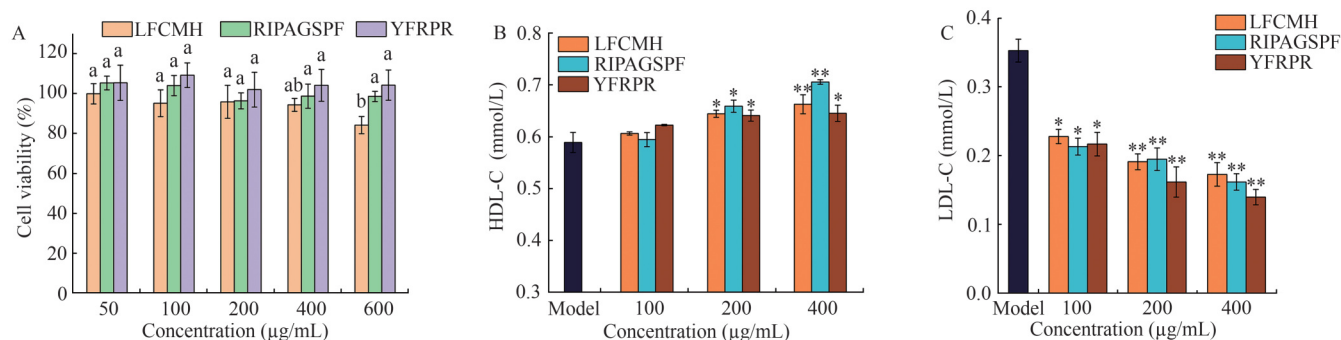


Fig. 5 Effects of peptides LFCMH, RIPAGSPF and YFRPR on (A) cell viability, (B) HDL-C and (C) LDL-C in 3T3-L1 cells. Data were expressed as means $\pm$ SEM. Different lowercase letters indicate significant differences between concentrations ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$, compared with the model group.

Cholesterol level is an important contributor to dyslipidemia. Maintaining high levels of HDL-C and LDL-C are essential for the prevention of dyslipidemia^[48]. Figs. 5B–C show the effects of three peptides, LFCMH, RIPAGSPF and YFRPR, on HDL-C and LDL-C levels in 3T3-L1 preadipocytes. All three peptides increased HDL-C levels. Among them, LFCMH and RIPAGSPF could increase HDL-C content extremely significantly ($P < 0.01$) at 400 $\mu\text{g/mL}$. Meanwhile, all three peptides could significantly ($P < 0.01$) reduce LDL-C levels, which were reduced by 51.04%, 54.17% and 60.42%, respectively, at a concentration of 400 $\mu\text{g/mL}$. Ye et al.^[42] also demonstrated that the peptides FLF, IYF and QIF had the effect of lowering the levels of intracellular lipid and cholesterol in the 3T3-L1 preadipocytes.

4. Conclusion

We explored the inhibitory activity of *R. roxburghii* seed proteins against PL and CE under traditional and simulated intestinal conditions. Seventeen peptides with inhibitory potential against PL and CE were screened by combining ultrafiltration, peptidomics, and virtual screening. Among them, AKDNVRFPL, LFCHM, LFCMH, RIPAGSPF, SMPLIR, and YFRPR showed high inhibitory activity against PL, and molecular docking showed that they mainly inhibit PL activity by forming hydrogen bonds and hydrophobic interactions with Ser152 and His263 in the active site and Ala259 and Phe77 in the surface ring. The peptides HGNQPLVF, LFCMH, QGALL, RIPAGSPF, SPHWNL, and YFRPR showed high inhibitory activity against CE, and molecular docking showed that they inhibited CE activity mainly by forming hydrogen bonds and hydrophobic interactions with Ser194 and His435 in the active site. LFCMH, RIPAGSPF, and YFRPR possess good inhibitory activity against both enzymes and thus may have an effect in intervening obesity. This study only identified and validated the activity of the peptides at the *in vitro* level and explored their potential to regulate lipid metabolism at the cellular level. Future work should focus on the molecular mechanism of the PL and CE inhibitory peptides. Their stability under simulated gastrointestinal conditions and the lipid-lowering effects *in vivo* should be further explored from practical applications as well.

Conflict of interest

All authors have no competing interests to disclose.

Acknowledgements

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2024.9250163>.

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