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Differential mechanism of casein phosphopeptide-quercetin covalent/non-covalent complex on gastrointestinal response behavior

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ABSTRACT: Casein phosphopeptide-quercetin (CPP-QR) covalent and non-covalent complex were constructed, and the activity changes and difference mechanisms of these two complexes were explored after gastrointestinal digestion using dynamic simulation digestion and molecular dynamic (MD) simulation. Compared with CPP, the soluble calcium binding capacity of CPP-QR covalent and non-covalent complex increased by 44.35% and 34.57% respectively, and the DPPH· scavenging rate increased by 77.97% and 25.46% respectively after static simulation digestion. Thereinto, the activity of CPP-QR covalent complex was significantly better than that of CPP-QR non-covalent complex ($P < 0.05$). Under acidic conditions, the DPPH· scavenging rate of CPP-QR covalent and non-covalent complexes was higher than that of CPP ($P < 0.05$). While the antioxidant activities of two complexes decreased significantly at pH=8, they still exhibited markedly superior performance relative to CPP alone ($P < 0.05$). The CPP-QR covalent and non-covalent complex exhibited 66.72% and 70.72% higher inhibition rates than CPP against pepsin, and 83.85% and 46.60% higher inhibition against trypsin, respectively. MD simulation had revealed that the binding free energies were -60.71 kcal/mol for the non-covalent complex in the pepsin system versus -84.21 kcal/mol for the covalent complex in the trypsin system. The CPP-QR non-covalent complex has exhibited minimal radius of gyration (Rg) and root-mean-square deviation (RMSD) in pepsin systems, while the CPP-QR covalent complex had demonstrated superior stability with correspondingly minimal Rg and RMSD in trypsin systems. In conclusion, the soluble calcium binding capacity and DPPH· scavenging rate of the CPP-QR covalent complex were superior to those of the non-covalent complex and CPP. CPP-QR covalent complexes had higher stability in the intestinal phase due to their resistance to trypsin, while non-covalent complexes maintain higher activity during gastric digestion due to their resistance to pepsin and acid adaptation.

Keywords: Casein phosphopeptide, quercetin, Covalent/non-covalent complex, Gastrointestinal digestion stability, Molecular dynamics simulation

1. Introduction

Food-derived bioactive peptides, as essential components of life, exhibit diverse biological activities such as promoting mineral absorption, lowering blood pressure, antioxidation, and improving immune

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regulation, exerting health effects (1, 2) and have become a research focus in the field of health. Currently, there is a phenomenon of inconsistent health activities of foodborne bioactive peptides *in vivo* and *in vitro*, which seriously restricts their application in functional foods (3). The main reason for this is that bioactive peptides must undergo a strict digestion and absorption process to enter the systemic circulation and exert their effects (4). In general, bioactive peptides are readily hydrolyzed by gastric acid, pepsin, and trypsin into smaller peptides or free amino acids, compromising their bioactivity (5). The intestinal epithelial barrier, being the primary absorption site, constitutes the major obstacle to their systemic absorption into the bloodstream (6, 7). Addressing the susceptibility of bioactive peptides to digestive breakdown has thus been identified as a critical unresolved issue in current peptide studies.

Recently, the research on improving the gastrointestinal response behavior of bioactive peptides mainly focuses on external digestive and absorptive enzyme inhibitors, mucosal penetrants and delivery systems (3). Among these, the addition of enzyme inhibitors and permeation enhancers can significantly improve the bioavailability of polypeptides, while long-term use of these substances may disrupt normal digestive function (8). Linking peptides or proteins with polymeric functional groups through physicochemical modification techniques can significantly enhance their enzymatic stability and permeability, thereby improving absorption efficiency and prolonging half-life, though at a higher cost (9). Furthermore, advanced nanoparticle-based delivery platforms (e.g., nanoemulsions, lipid-based vesicles, and polymeric encapsulation) utilizing biodegradable materials enhance peptide stability against digestive enzyme breakdown. These systems facilitate controlled release mechanisms that substantially improve intestinal absorption efficiency and bioactive compound bioavailability (10). However, the interaction characteristics between nanocarriers and the organism, the controlled release process of polypeptides, and the subsequent absorption and metabolism mechanisms still need to be further studied (11). Exploring the interaction between food-derived functional factors with differentiated digestibility and absorption properties and peptides to establish a gastrointestinal homeostasis response system may provide an effective solution to this issue (12).

In recent years, interactions between bioactive peptides/proteins and polyphenols have emerged as a research hotspot in food science (13, 14). Polyphenols form covalent/non-covalent complexes with peptides/proteins through phenolic hydroxyl groups and amino/carboxyl groups, significantly enhancing system stability (15, 16). These interactions regulate physicochemical properties including solubility and bioavailability of bioactive components and influence nutrient digestion by inhibiting digestive enzymes and modifying protein conformations (17). Notably, polyphenols can interact with proteins, thereby initiating the inhibitory function on enzymatic activity and altering the digestibility of proteins as well as their absorption mechanisms (12, 18), offering novel strategies for controlled nutrient delivery. However, current research has mostly focused on studying single systems, and there have been few studies on comparative analysis of dual systems before. As a crucial calcium-promoting bioactive peptide, casein phosphopeptide (CPP) demonstrates significant potential in nutritional fortification. However, its chemical instability in gastrointestinal environments substantially restricts practical applications within functional food systems (19). Therefore, to

address the identified research gap, this study uniquely constructs both CPP-QR covalent and non-covalent complex systems for a direct and systematic comparison. The activity changes and difference mechanisms of these two complexes were explored from multiple dimensions such as structural characteristics, pH tolerance and enzyme inhibition, providing a theoretical basis for polyphenols to enhance the gastrointestinal stability of polypeptides and offering novel insights into the structure-activity relationship of interaction types on digestive fate.

2. Materials and methods

2.1 Materials and reagents

Casein phosphopeptide (CPP, purity 80%), quercetin (QR, purity 97%), Dialysis tubing (1000 kDa), pepsin (1:15000) and trypsin (1:250) were purchased from Shanghai Yuanye Biotechnology Co., LTD (Shanghai, China). Artificial gastric juices and artificial gastric juices were purchased from Beijing Regen Biotechnology Co., LTD (Beijing, China). Anhydrous calcium chloride, calcium standard solution, ascorbic acid were obtained from Sinopharm Chemical Reagent Co., LTD (Shanghai, China).

2.2 Preparation of CPP-QR covalent and non-covalent complex

The preparation method of the covalent complex was referred to the method of Jayaprakash (20) and slightly modified. The 10 mg/ml of CPP solution was prepared, H₂O₂ solution (30%) and ascorbic acid was added, mixed evenly, and left for 2 h at room temperature away from light. A certain concentration of QR solution was prepared, mixed evenly with the above CPP solution in equal volume, and the reaction was left for 24 h at room temperature away from light. The reaction solution was put into a 1000 Da dialysis bag, put into 3 times the volume of distilled water, and kept in a refrigerated environment at 4°C for 48 hours without light dialysis. The water was changed every 6 hours to remove the unreacted QR. And the post-dialysis solution was removed, pre-frozen and freeze-dried to obtain the CPP-QR covalent complex.

The preparation method of non-covalent complex was referred to the method of Ferraro et al. (21) and adjusted appropriately. The CPP powder was uniformly dispersed in a phosphate buffer (0.1 mol/L) with a specific pH value. Then, the magnetic stirrer was continuously stirred at 25°C with 400 r/min for 2 hours to ensure that the CPP was fully dissolved. Then the CPP solution (10 mg/mL) was kept overnight in a refrigerated environment at 4°C in order to promote complete hydration of the CPP for subsequent experimental use. A certain concentration of quercetin solution was prepared with phosphate buffer at corresponding pH, mixed with fully hydrated CPP solution in equal volume, and stirred at 25°C at 400 r/min with oxygen and light shielding magnetic force to fully react. The reaction solution was put into 1000 Da dialysis bag, put into 3 times the volume of the buffer corresponding to pH, refrigerated at 4°C away from light, and dialysis for 48 h. The buffer was changed every 6h to remove the unreacted QR. The post-dialysis solution was removed, pre-frozen and freeze-dried, and CPP-QR non-covalent complex was obtained.

2.3 Single factor experiment

The covalent complex single factor test considered quercetin concentration (1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL, 3.0 mg/mL) and ascorbic acid supplemental level (3.0 mg/mL, 4.0 mg/mL, 5.0 mg/mL, 6.0 mg/mL, 7.0 mg/mL) and reaction time (1.0h, 1.5h, 2.0h, 2.5h, 3.0h) on the soluble calcium binding capacity and antioxidant capacity of the covalent complex after digestion, so as to screen out the preparation conditions of the covalent complex with good anti-digestion ability. The single-factor experimental design of the covalent compound is shown in Table S1.

The single factor test of non-covalent complex considered quercetin concentration (1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL, 3.0 mg/mL), reaction pH (4.0, 5.0, 6.0, 7.0, 8.0), reaction time (1.0 h, 1.5 h, 2.0) h, 2.5h, 3.0h) on the soluble calcium binding capacity and antioxidant capacity of the non-covalent complex after digestion, so as to screen out the preparation conditions of the non-covalent complex with good anti-digestion ability. The single-factor experimental design of the covalent compound is shown in Table S2.

2.4 Orthogonal experiments and validation experiments

On the basis of single factor experiments, L9 (3⁴) orthogonal test method was used. The two complexes (CPP-QR covalent and non-covalent complex) of soluble calcium binding capacity and DPPH· scavenging rate were tested according to the factor level table design (Tables S3 and S4). For the importance difference of soluble calcium binding capacity (A) and DPPH· scavenging rate (B) in the evaluation system, weights of 0.7 and 0.3 were assigned, respectively. Then, the affiliation of the two indexes was calculated, and combined with the corresponding weights, the comprehensive score of the orthogonal test results was obtained. The calculation method of activity affiliation and comprehensive score was shown in formulas 1 and 2.

$$\text{Activity affiliation} = \frac{\text{Indicator value} - \text{Indicator Maximum}}{\text{Indicator maximum} - \text{Indicator Minimum}} \quad 1$$

$$\text{Comprehensive score} = A \times 0.7 + B \times 0.3 \quad 2$$

2.5 Static gastrointestinal simulated digestion in vitro

The CPP-QR covalent and non-covalent complex were placed 90 mg in a 100 mL centrifuge tube and fully dissolved with 18 mL distilled water, respectively. To simulate the digestive process in the stomach, the complex solution was adjusted to a pH 3.0 and 15 mL of simulated gastric juice was added. The mixture was then incubated in a shaker at a constant temperature of 37°C for 2 h, at which time¹⁵⁹, it was immediately removed and placed in an ice bath to terminate gastric digestive enzymes. Following gastric digestion, the pH of the mixture was neutralized to 8.0, and 15 mL of simulated intestinal fluid was introduced. The sample was subsequently maintained under controlled thermal conditions (37°C) with continuous agitation for 2 hours, followed by thermal treatment at 95°C for 15 minutes in a water bath to deactivate enzymatic activity and conclude the digestive simulation. The simulated digestion was put into a 1000 Da dialysis bag and dialysis was conducted at 4°C for 48 h. The post-dialysis solution was removed, pre-frozen and freeze-dried into powder.

The determination of the soluble calcium binding capacity referred to the laboratory preliminary test method (22). Briefly, phosphate buffer (20 mM, pH 7.8) and CaCl₂ solution (5 mM) was evenly mixed. To

induce calcium phosphate precipitation, the reaction system was maintained at 37°C through water bath heating for 15 minutes. After introducing the composite sample powder, the solution underwent additional thermal incubation at 37°C for 2 hours. Subsequent separation was achieved through centrifugation (10,000 rpm, 15 minutes), yielding the final supernatant for analysis. The amount of soluble calcium binding was determined by colorimetric method of o-cresolphthalein complexone. In addition, the DPPH· scavenging rates of all the above samples were determined according to previous methods (23).

2.6 pH Stability in vitro

CPP, CPP-QR covalent and non-covalent complex sample solutions were prepared with phosphate buffers of pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 at concentrations of 0.1 M and 0.02 M, respectively. And the determination of DPPH· scavenging and soluble calcium binding amount of these samples were using the same methods as 2.5.

2.7 Inhibition rate of digestive protease

(1) Determination of pepsin inhibition rate

CPP, QR, CPP-QR covalent and non-covalent complex solutions with concentrations of 2, 4, 6, 8, 10 mg/mL, phosphate buffer (pH 2.2), pepsin solution (10 mg/mL), and hemoglobin solution (0.5 mg/mL) were prepared, respectively. The group was as follows: experimental group: 0.2mL sample solution with different concentrations + 0.2mL pepsin solution + 1mL hemoglobin solution; experimental blank group: 0.2mL sample solution with different concentrations + 0.2mL phosphate buffer + 1mL hemoglobin solution; control group: 0.2mL phosphate buffer + 0.2mL pepsin solution + 1mL hemoglobin solution; control blank group: 0.4 mL phosphate buffer + 1 mL hemoglobin solution. Following a 5-minute incubation at 37°C, the absorption intensity of sample solutions was measured at 275 nm. The recorded values were categorized into four groups: A1 represented the experimental test group, A2 the experimental blank group, A3 the control test group, and A4 the control blank group, respectively. The inhibition rate of pepsin in different samples was calculated according to formula 3.

$$\text{Pepsin inhibition rate} = \frac{(1-(A_1-A_2))}{(A_3-A_4)} \times 100\% \quad 3$$

(2) Determination of trypsin inhibition rate

CPP, QR, CPP-QR covalent and non-covalent complex solutions with concentrations of 2, 4, 6, 8 and 10 mg/mL, phosphate buffer (pH 8), trypsin solution (5 mg/mL) and BAPNA solution (0.5 mg/mL) were prepared, respectively. The group was as follows: experimental group: 0.2mL sample solution with different concentrations + 0.2mL trypsin solution + 1mL BAPNA solution; experimental blank group: 0.2mL of different concentrations of sample solution + 0.2mL phosphate buffer + 1 mL BAPNA solution; control group: 0.2 mL phosphate buffer + 0.2 mL trypsin solution + 1 mL BAPNA solution; control blank group: 0.4 mL phosphate buffer + 1 mL BAPNA solution. The absorbance value of each group were measured at 420 nm wavelength after reaction at 37°C for 5 min. The value of the experimental group was denoted as A₁, the value of the experimental blank group was denoted as A₂, the value of the control group was denoted as A₃, and the

value of the control blank group was denoted as A_4 . The inhibition rate of trypsin of different samples was calculated according to formula 4.

$$\text{Trypsin inhibition rate} = \frac{(1-(A_1-A_2))}{(A_3-A_4)} \times 100\% \quad 4$$

2.8 *In vitro* dynamic gastrointestinal digestion

The method of *in vitro* dynamic gastrointestinal digestion was according to the reported study (24) with several modification. CPP, CPP-QR covalent and non-covalent complex solutions of 0.075 g/mL were prepared with ultra-pure water, respectively. 6 mL sample solution and 1.2 mL artificial gastric juice were added to the simulated rat stomach, and then the switch was closed. Appropriate amount of artificial gastric juice, artificial intestinal juice, 1 mol/L hydrochloric acid (simulated bile) and 1 mol/L NaHCO_3 (simulated pancreatic juice) were added to the syringe. The dynamic mouse gastrointestinal tract was connected step by step to simulate the digestive system (Xiaodong Yijian Instrument Equipment Co., LTD, Suzhou, China). The operation parameters were set as follows: gastric fluid injection pump: 30 $\mu\text{L}/\text{min}$, 180 min; pancreatic fluid injection pump: 10 $\mu\text{L}/\text{min}$, 180 min; bile injection pump: 12 $\mu\text{L}/\text{min}$, 180 min; empty-injection pump: 60 $\mu\text{L}/\text{min}$, 120 min; gastric tilt angle motor: $0.03^\circ/\text{min}$, time 180 min; gastric extrusion motor: 3 rpm, 180 min; gastric roller motor: 12 rpm, 180 min; intestinal roller motor: 36 $\mu\text{L}/\text{min}$, time 180 min. After 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 h of simulated digestion in the system, samples from the simulated stomach and intestines were taken out, boiled for 15 min to eliminate enzymes, lyophilized for 48 h, and DPPH $\cdot$ scavenging rate and soluble calcium binding amount were measured using the same methods as 2.5

2.9 Molecular docking

Pepsin (subtype 5, Uniprot ID: P0DJJD9) and trypsin (subtype 1, Uniprot ID: P07477) were download from Uniprot database (<https://www.uniprot.org/>), respectively. The crystal structure of pepsin protein subtype 5 has not yet been resolved, so the three-dimensional structure predicted by alphafold2 was used. The A-chain of the 2RA3 crystal structure with the highest resolution was used for trypsin subtype 5. The core sequence of CPP had been derived from the highly calcium-binding casein phosphopeptide sequence (RELEELNVPGEIVESLSSEESITR) identified in Luo's research (25). Using the 'Build Biopolymer from Sequence' module in Schrödinger software, the sequence had been modeled and the structure had been saved in PDB format. The protein had then been prepared using the Protein Preparation Wizard module within the same software. The 3D structures of the proteins had been assigned bond orders and hydrogenated respectively. The water molecules and cofactors had been removed, and subsequently, the hydrogen bond network had been optimized. Following protein energy minimization utilizing the OPLS_4 force field, Molecular docking was performed using the Protein Protein docking (Piper) module in Schrödinger. The standard docking protocol was implemented with rotational flexibility parameters configured to 70,000 ligand probes, facilitating exhaustive sampling of ligand-protein conformational space. This computational strategy ultimately generated 30 distinct molecular conformations for subsequent analysis. Pepsin and trypsin proteins were defined as receptors and named as A chain. CPP, CPP-QR covalent and non-covalent complex were

defined as ligands and are named B-chains. Piper utilizes RMSD values of atomic positions to categorize the initial 1000 rotational conformations through cluster analysis, where the most centrally located conformation (demonstrating highest neighbor density) within each cluster is designated as the representative structure. Subsequently, conformations are prioritized according to cluster population density, with the conformation from the most populated cluster receiving the highest priority for subsequent binding site characterization and interaction analysis.

2.10 Molecular dynamics simulation

As a computational biology tool, MD simulations enable systematic investigation of biomolecular motions and conformation transitions at temporal resolution (26). Throughout the simulation period, the RMSD values of protein α -carbon atoms were computed against the original configuration to quantify structural deviations over time. The protonation state of amino acid residues in proteins and peptides was calculated using PROPKA 3.0. OPLS4 force field is used to parameterize proteins and small molecules, TIP3P is used for solvation water model, and the boundary thickness is 10.0 Å. In order to further evaluate the stability of the binding mode of the system, the Desmond module in the Schrodinger drug design software package was used to test the pepsin-CPP, pepsin-CPP-QR complex, pepsin-CPP-QR non-covalent complex, trypsin-CPP, trypsin-CPP-QR covalent complex and trypsin-CPP-QR non-covalent complex systems were simulated by molecular dynamics.

(1) System construction

The protonation states of amino acid residues in proteins and polypeptides were determined using the PROPKA 3.0 software. The original crystal water molecules within the chosen protein structure were preserved. The complete atomic model was subsequently generated through Schrodinger's System Builder toolkit. The OPLS_4 force field was applied to assign parameters to both protein and small molecule components, while the TIP3P model was implemented to represent the solvent environment with a buffer region thickness set at 10.0 Å. The protein-ligand complex was immersed in a cubic aqueous environment for solvation treatment. Following system volume optimization, electrical neutrality was established through the addition of sodium or chloride ions counterions.

(2) Simulation process

The computational framework was established using molecular dynamics (MD) simulations. Structural relaxation was achieved through 50,000 iterations of energy minimization employing the steepest descent algorithm. The heavy atom positions were constrained prior to conducting 50,000-step equilibration simulations under constant temperature (NVT) and pressure (NPT) conditions. Thermal regulation at 310 K and atmospheric pressure stabilization at 1 atm were implemented throughout the simulation process. After the completion of the two equilibrium stages, the unrestricted dynamic simulation of 100 ns was carried out. The energy and coordinates of the trajectory was saved every 10 ps. After simulating trajectories, Schrodinger 2023 was used to draw interactive patterns and dynamic trajectory animations. After 100 ns molecular

dynamics simulation, RMSD, RMSF and Rg of the whole process of the complex were calculated. And a snapshot of the stable state was taken for MM-GBSA binding free energy analysis and energy decomposition.

2.11 Data processing and statistical analysis

Experimental data were analyzed using Origin 2022 and GraphPad Prism 8.0 for visualization, while statistical significance ($P < 0.05$) was determined via Duncan's multiple range test in SPSS 26.0. Error bars in all figures represented the standard deviation (SD) from the mean of three independent replicates.

3. Results and discussion

3.1 Preparation of CPP-QR covalent complex

When the quercetin concentration was 2.0 mg/mL, the soluble calcium binding capacity (4.43 $\mu\text{g/mL}$) and DPPH $\cdot$ scavenging rate (66.19%) of the CPP-QR covalent complex both reached the highest level after simulated gastrointestinal digestion, which was significantly higher than that of other treatment groups ($P < 0.05$, Figure 1a-b). As shown in Table S5, when the concentration of quercetin was 2.0 mg/mL, the comprehensive affiliation was 1.00. Therefore, 2.0 mg/mL quercetin was selected for the subsequent orthogonal experiments. After 2.5h of reaction, the soluble calcium-binding capacity (4.04 $\mu\text{g/mL}$) and DPPH $\cdot$ scavenging rate (74.45%) of the CPP-QR covalent complex reached the maximum values after simulated digestion, which were significantly higher than those of other treatment groups ($P < 0.05$, Figure 1c-d). Taking into account these two indicators, the comprehensive affiliation was 1.00 when the reaction time was 2.5 (Table S6). Therefore, the reaction time of 2.5h was selected for the subsequent orthogonal experiment. When the addition amount of ascorbic acid was 6.0 mg/mL, the soluble calcium binding capacity of CPP-QR covalent complex after simulated digestion was 4.63 $\mu\text{g/mL}$, and its DPPH $\cdot$ scavenging rate reached 76.80 % (Figure 1e-f). As shown in Table S7, the comprehensive affiliation was 0.94 when the addition amount of ascorbic acid was 6.0 mg/mL, which was the highest among all groups. Therefore, 6.0 mg/mL of ascorbic acid was selected for the subsequent orthogonal experiments.

As shown in Table S11, the order of the influence of factors on the soluble calcium binding capacity and DPPH $\cdot$ scavenging rate is as follows: ascorbic acid amount > reaction time > quercetin concentration. Through orthogonal experiments and verification tests, the optimal preparation process conditions for the CPP-QR covalent complex were determined as follows: quercetin concentration: 1.5 mg/mL, reaction time: 2.0 h, ascorbic acid amount: 5.0 mg/mL. Under these conditions, the soluble calcium binding amount of the complex was 4.52 $\mu\text{g/mL}$, and the DPPH $\cdot$ scavenging rate was 70.66%.

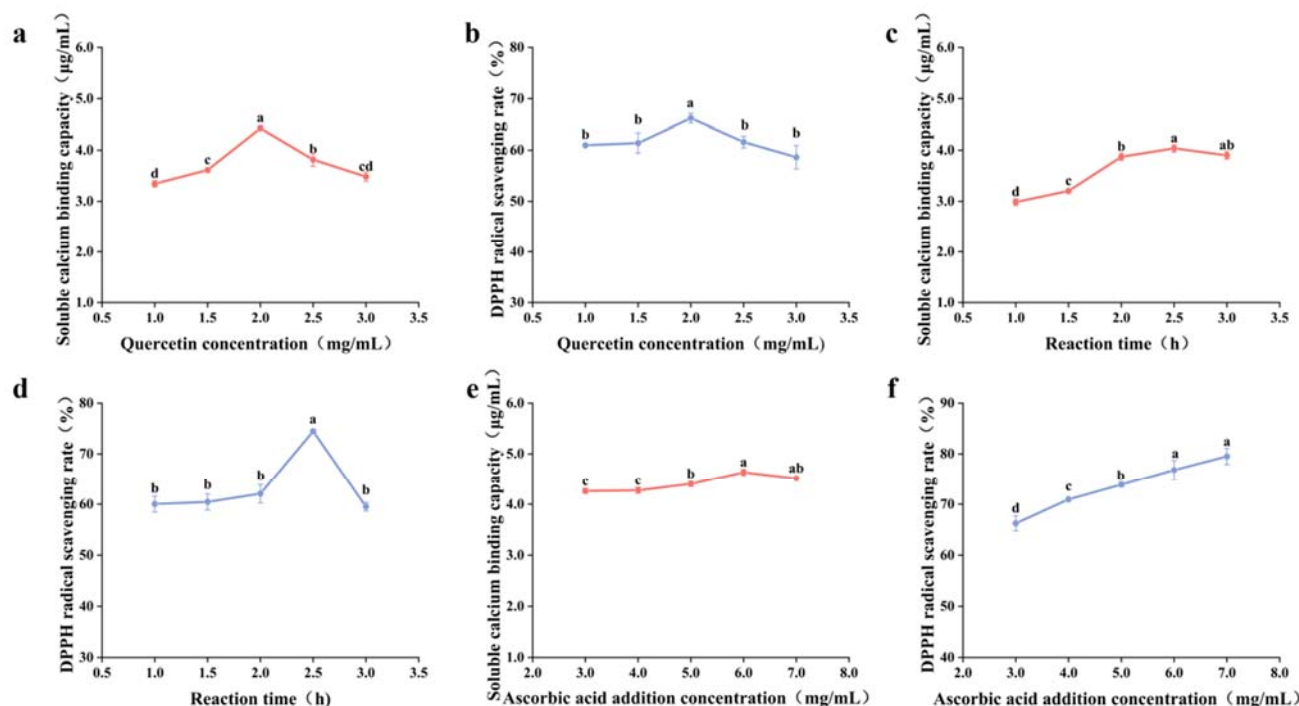


Figure 1. Effects of quercetin concentration (a: soluble calcium binding capacity, b: DPPH radical scavenging rate), reaction time (c: soluble calcium binding capacity, d: DPPH radical scavenging rate), and ascorbic acid dosage (e: soluble calcium binding capacity, f: DPPH radical scavenging rate) on CPP-QR covalent complex after digestion. Data are presented as mean $\pm$ SD (n=3). Different lowercase letters above the bar chart indicate significant differences ($P < 0.05$).

3.2 Preparation of CPP-QR non-covalent complex

As shown in Figure 2a-b, when the quercetin concentration was 2.0 mg/mL, the soluble calcium binding capacity of the CPP-QR non-covalent complex reached the highest level (2.19 μ g/mL) after digestion, and its DPPH $\cdot$ scavenging rate was 39.48%, which showed no significant difference compared with 2.5 mg/mL ($P > 0.05$). Taking these two indicators into account, when the quercetin concentration was 2.0 mg/mL, the comprehensive affiliation was 0.95 (Table S8). Hence the concentration of 2.0 mg/mL of quercetin was selected for the subsequent orthogonal experiment. When the reaction time was 2.5h, the soluble calcium binding capacity and DPPH $\cdot$ scavenging rate of the CPP-QR non-covalent complex after simulated digestion were significantly higher than those of the other treatment groups ($P < 0.05$, Figure 2c-d). Taking into account two indicators, the comprehensive affiliation was calculated as 1.00 when the reaction time was 2.5 (Table S9). Therefore, the reaction at 2.5h was selected for the subsequent orthogonal experiment. When the reaction pH was 6.0, the soluble calcium binding capacity and DPPH $\cdot$ scavenging rate of the CPP-QR non-covalent complex in the simulated digestion were significantly higher than those of the other treatment groups ($P < 0.05$, Figure 2e-f). Based on the affiliation calculation, the comprehensive affiliation was 1.0 when the reaction pH was 6.0 (Table S10). Therefore, the subsequent orthogonal experiments were conducted with the reaction pH set at 6.0.

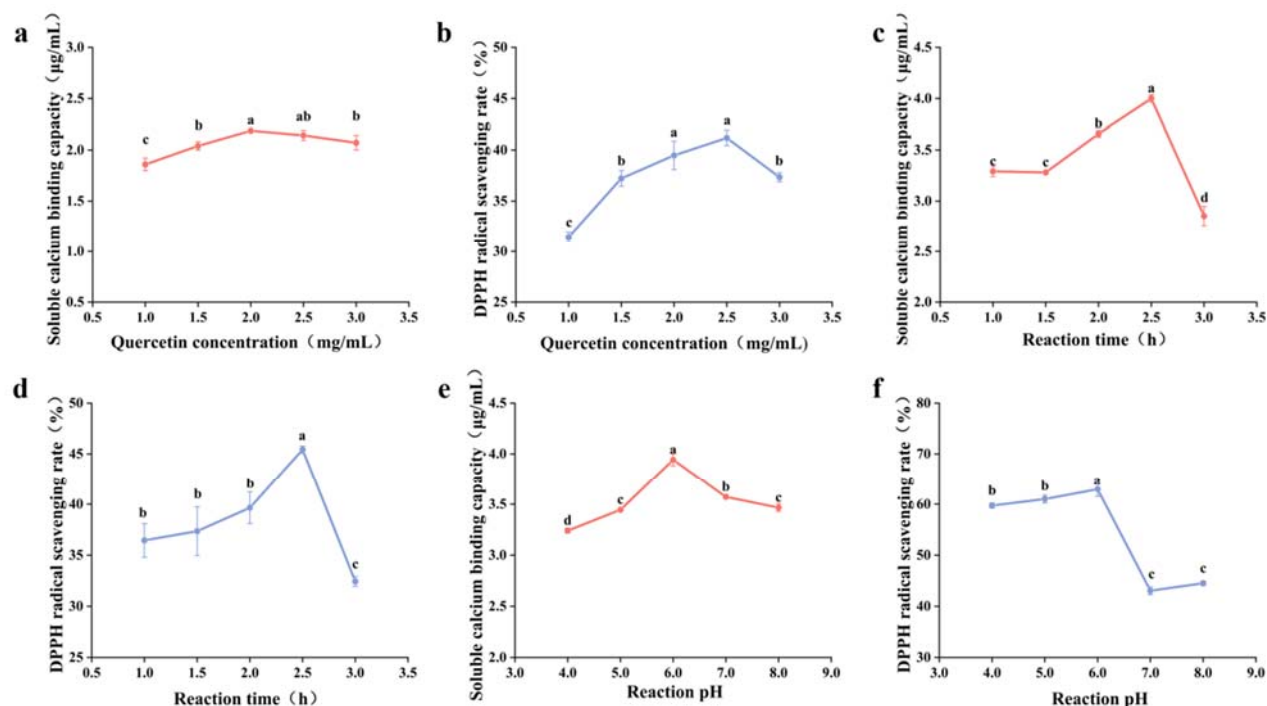


Figure 2. Effects of quercetin concentration (a: soluble calcium binding capacity, b: DPPH radical scavenging rate), reaction time (c: soluble calcium binding capacity, d: DPPH radical scavenging rate), and ascorbic acid dosage (e: soluble calcium binding capacity, f: DPPH radical scavenging rate) on CPP-QR non-covalent complex after digestion. Data are presented as mean $\pm$ SD ($n=3$). Different lowercase letters above the bar chart indicate significant differences ($P < 0.05$).

As shown in Table S12, the order of the influence of factors on the soluble calcium binding capacity and DPPH $\cdot$ scavenging rate was reaction pH > reaction time > quercetin concentration. Through orthogonal experiments and verification experiments, the optimal preparation process conditions for the CPP-QR non-covalent complex were determined as follows: quercetin concentration: 2.5 mg/mL, reaction time: 3.0 h, reaction pH: 5.0. Under these conditions, the soluble calcium binding capacity of the complex after digestion was 4.21 $\mu\text{g/mL}$, and the DPPH $\cdot$ scavenging rate was 49.82%.

3.3 Static simulation of complex digestion

As shown in Figure 3a, the soluble calcium binding capacity of CPP-QR covalent and non-covalent complex were significantly higher than that of CPP after static simulation of gastrointestinal digestion ($P < 0.05$), increasing by 44.35% and 34.57% respectively. This might be due to the formation of covalent or non-covalent bonds between CPP and QR, which enhanced the stability of the complex structure and effectively resisted the action of digestive enzymes (27), thereby maintaining a high calcium binding capacity. Additionally, the soluble calcium-binding capacity of the CPP-QR covalent complex was significantly higher than that of the non-covalent complex after static simulation of gastrointestinal digestion ($P < 0.05$). It is speculated that the covalent complex system was more stable than the non-covalent complex system and had stronger anti-digestion ability, thereby increasing the soluble calcium binding capacity.

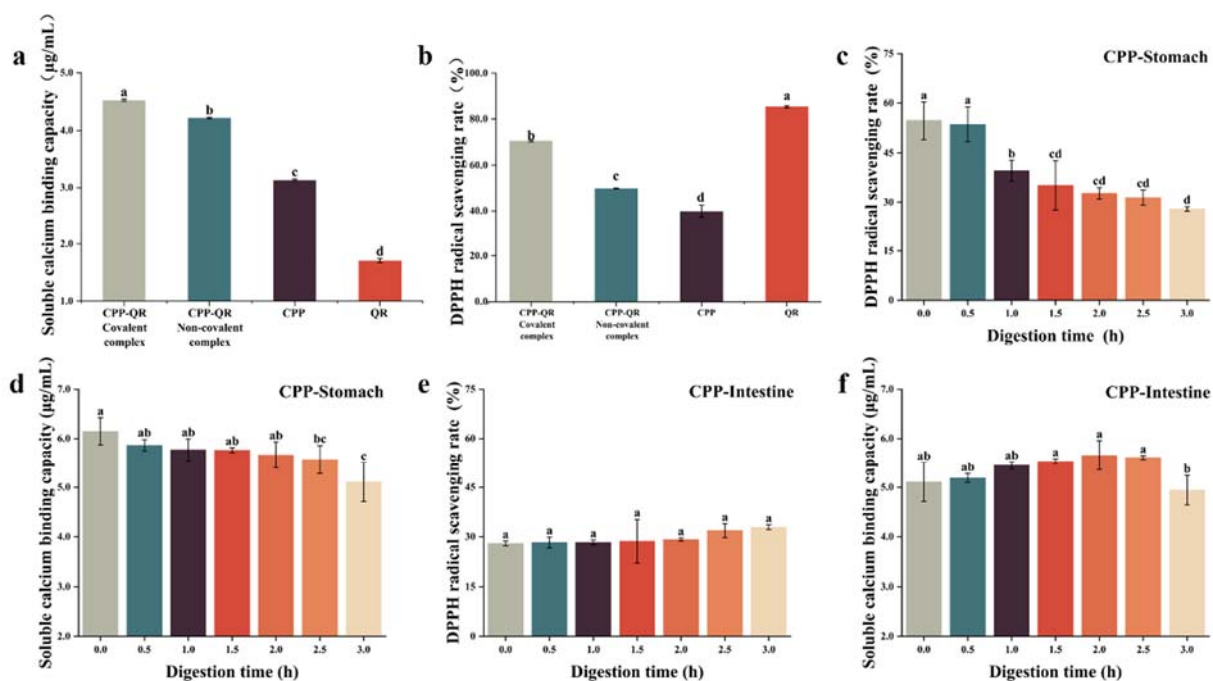


Figure 3. Comparative analysis of soluble calcium binding capacity (a) and DPPH radical scavenging rate (b) between CPP-QR covalent and non-covalent complex after digestion. The variation patterns of DPPH radical scavenging rate (c) and soluble calcium binding capacity (d) of CPP during gastric digestion, and the variation patterns of DPPH radical scavenging rate (e) and soluble calcium binding capacity (f) of CPP gastric digestion products during intestinal digestion. Data are presented as mean $\pm$ SD ($n=3$). Different lowercase letters above the bar chart indicate significant differences ($P < 0.05$).

As shown in Figure 3b, compared with CPP, the DPPH $\cdot$ scavenging rates of CPP-QR covalent complexes and non-covalent complexes were significantly increased after static simulation of gastrointestinal digestion ($P < 0.05$), while the DPPH $\cdot$ scavenging rate of CPP-QR covalent and non-covalent complex was significantly lower than that of QR after digestion. This might be due to the fact that both the covalent and non-covalent interactions between CPP and QR could potentially mask or restrict the exposure of phenolic hydroxyl groups in the QR molecule to a certain extent. This would reduce the opportunities for them to come into contact with DPPH free radicals, thereby lowering the antioxidant performance of the complex (28). The DPPH $\cdot$ scavenging rate of the CPP-QR covalent complex was significantly higher than that of its non-covalent complex after digestion ($P < 0.05$). This difference might be that the CPP and QR in the covalent complex were closely linked through covalent bonds, allowing more stable retention of antioxidant-related functional groups and showing higher DPPH radical clearance (27). Static experiments ignore mechanical shear caused by gastric peristalsis and duodenal emptying, so a dynamic gastrointestinal simulation system needs to be introduced.

3.4 Dynamic digestion of CPP-QR covalent/non-covalent complex

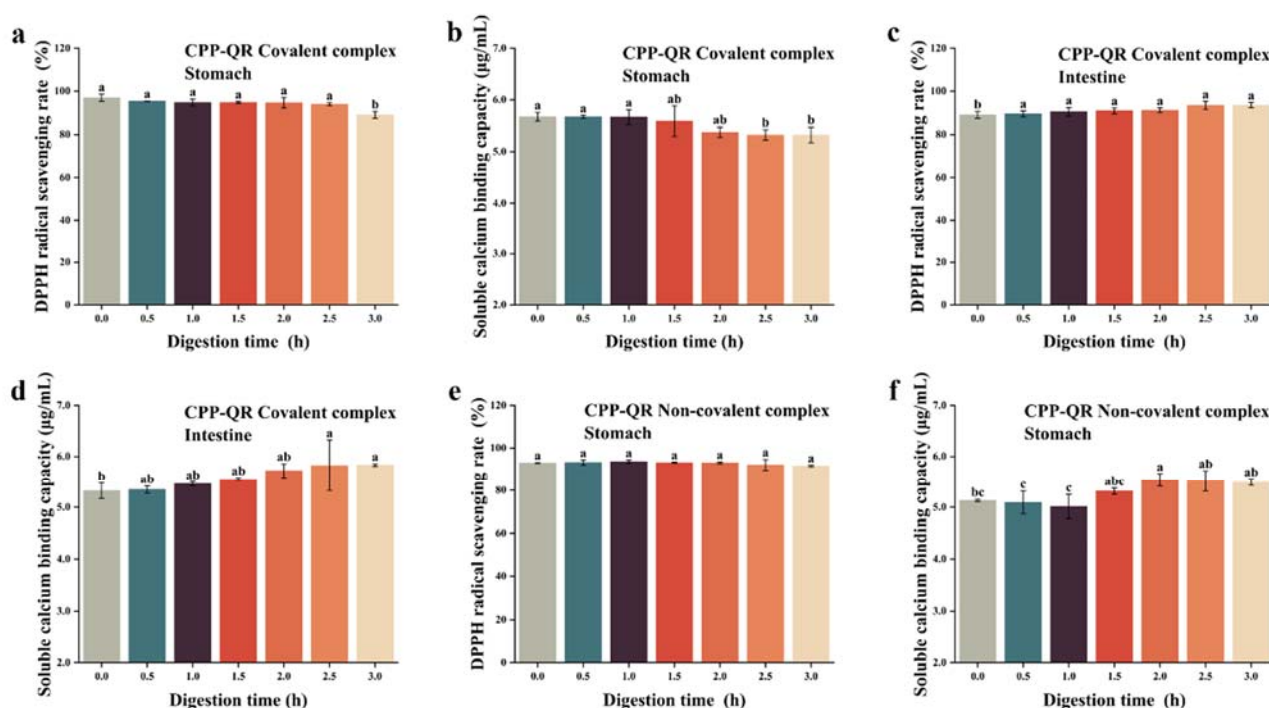
(1) Dynamic digestion of CPP

As shown in Figure 3c-d, the DPPH $\cdot$ scavenging rate and the soluble calcium binding capacity of CPP decreased by 54.49% and 16.91% after gastric digestion, respectively ($P < 0.05$). The acidic environment in digestive juices may cause some active groups of CPP to undergo protonation, aggregation or conformational change, thereby affecting the exertion of its activity (29). During the intestinal digestion stage, the

DPPH $\cdot$ scavenging capacity and the soluble calcium binding capacity of CPP after being processed in the stomach did not show significant changes after 2 hours of intestinal digestion ($P > 0.05$, Figure 3e-f). This might be that the neutral/weakly alkaline environment of the intestinal tract caused the phosphate groups of CPP to lose protons and regain negative charges, thereby resisting pancreatic enzymes and preventing further degradation (19). Compared with the untreated CPP samples, the DPPH $\cdot$ scavenging rate and soluble calcium binding capacity of the CPP products after gastrointestinal digestion decreased by 39.87% and 19.60%, respectively.

(2) Dynamic digestion of CPP-QR covalent complex

As depicted in Figure 4a-b, the untreated CPP-QR covalent complex exhibited a DPPH radical scavenging rate of 96.92% and soluble calcium binding capacity of 5.67 $\mu\text{g/mL}$. After gastric digestion, the DPPH $\cdot$ scavenging rate and the soluble calcium binding capacity of the CPP-QR covalent complex decreased by 8.21% and 6.12%, respectively. Compared to CPP after gastric digestion, the DPPH $\cdot$ scavenging rate and soluble calcium binding capacity of the CPP-QR covalent complex after gastric digestion were increased by 218.06% and 3.95% respectively. This indicated that the CPP-QR covalent complex can maintain higher bioactivity even in the gastric digestion environment compared to CPP. According to Figure 4c-d, the DPPH $\cdot$ scavenging rate of the gastric digestion products of the CPP-QR covalent complex decreased to 93.57% after intestinal digestion, representing a 3.46% reduction compared to its pre-digestion level. Additionally, the soluble calcium binding capacity of the gastric digestion products of the CPP-QR covalent complex increased to 5.83 $\mu\text{g/mL}$ after gastrointestinal digestion, showing a 2.74% enhancement compared to its pre-digestion state. Notably, the CPP-QR covalent complex demonstrated significantly higher DPPH radical scavenging capacity and soluble calcium binding capacity after gastrointestinal digestion compared to CPP, with increases of 63.01% and 15.23% respectively. This indicates that the CPP-QR complex exhibits superior resistance to gastrointestinal degradation, particularly during intestinal digestion.



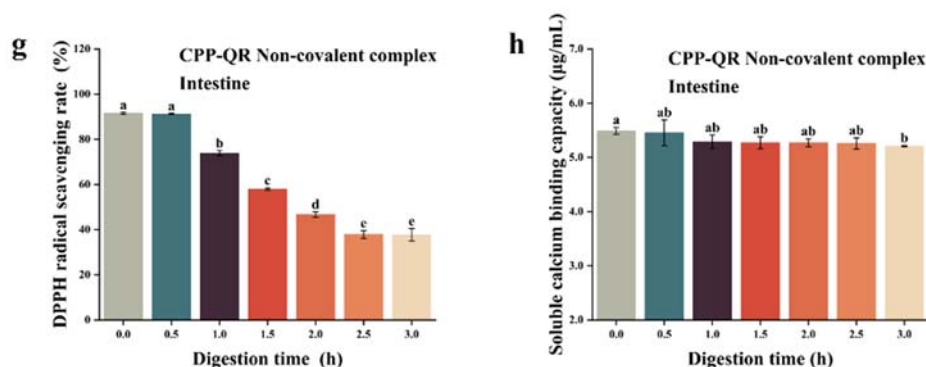


Figure 4. The variation patterns of DPPH radical scavenging rate (a) and soluble calcium binding capacity (b) of CPP-QR covalent complex during gastric digestion, and the variation patterns of DPPH radical scavenging rate (c) and soluble calcium binding capacity (d) of CPP-QR covalent complex gastric digestion products during intestinal digestion. The variation patterns of DPPH radical scavenging rate (e) and soluble calcium binding capacity (f) of CPP-QR non-covalent complex during gastric digestion, and the variation patterns of DPPH radical scavenging rate (g) and soluble calcium binding capacity (h) of CPP-QR non-covalent complex gastric digestion products during intestinal digestion. Data are presented as mean $\pm$ SD ($n=3$). Different lowercase letters above the bar chart indicate significant differences ($P < 0.05$).

(3) Dynamic digestion of CPP-QR non-covalent complex

As shown in Figure 4e-f, the CPP-QR non-covalent complex exhibited a DPPH $\cdot$ radical scavenging rate of 92.97% and demonstrated a soluble calcium binding capacity of 5.13 $\mu\text{g/mL}$. After gastric digestion, the DPPH $\cdot$ scavenging rate and soluble calcium binding capacity of the CPP-QR non-covalent complex showed no significant changes compared to pre-digestion levels ($P < 0.05$). The CPP-QR non-covalent complex exhibited significantly higher resistance to gastric digestion compared to CPP and CPP-QR non-covalent complex. This phenomenon may be associated with its resistance to degradation by pepsin or its conformational stability in low pH environments (30). After the CPP-QR non-covalent complex gastric digestion products entered the intestinal digestion process, the DPPH $\cdot$ scavenging rate and soluble calcium-binding capacity decreased by 59.46% and 1.54% compared to the undigested sample, respectively (Figure 4g-h). Compared with the digestive products of CPP, the CPP-QR non-covalent complex demonstrated a 12.68% higher DPPH $\cdot$ scavenging rate and a 5.18% greater soluble calcium-binding capacity after gastrointestinal digestion.

In summary, the covalent/non-covalent complexation of CPP with QR significantly enhanced the digestive stability of CPP, which was consistent with the results of static digestion. These structural characterization results directly support the hypothesis that the nature of the interaction dictates digestive stability. Notably, the CPP-QR covalent complex demonstrated superior digestive resistance than CPP-QR non-covalent complex in the intestinal tract. This may be attributed to two mechanisms: firstly, the structural rigidity from covalent cross-linking could shield trypsin cleavage sites (Lys/Arg residues), whereas non-covalent complexes tend to expose these susceptible sites upon dissociation. Secondly, the elevated intestinal pH (6.5-7.5) weakens ionic and hydrogen bonding interactions, leading to structural disintegration of non-covalent complexes, while covalent bonds maintain structural integrity due to their pH-independent stability (31, 32). To further verify its mechanism, subsequent studies respectively simulated the effects of the pH environment and digestive enzymes during the digestion process on the activities of these two complexes.

3.5 Activity changes of CPP-QR covalent/non-covalent complex under pH environmental stress

(1) Activity changes of CPP under pH environmental stress

To further investigate the molecular mechanisms underlying the differential digestive stability between covalent and non-covalent CPP complexes, this study systematically examined the stability variations of digestion-resistant complexes under simulated gastrointestinal pH environments (excluding enzymatic action). As shown in Figure 5a, the soluble calcium binding capacity of CPP showed no significant difference under varying pH conditions ($P > 0.05$), suggesting that its calcium-binding ability was less affected by environmental acidity/alkalinity. This phenomenon may be attributed to the structural stability of phosphate groups in CPP molecules across different pH environments (33). The clearance rate of DPPH $\cdot$ in untreated CPP was 54.73%, 35.38% at pH 3.0, and decreased to 18.25% at pH 8.0. These findings indicated that weakly alkaline environments are more likely to impair DPPH $\cdot$ scavenging rate of CPP compared to acidic conditions. This phenomenon may be attributed to the fact that acidic (pH 3.0) and weakly alkaline (pH 8.0) conditions could alter the charge, solubility, and conformation of CPP, thereby reducing its effective contact with DPPH free radicals. Furthermore, structural damage and aggregation effects became more pronounced under alkaline conditions, resulting in the lowest scavenging rate (34).

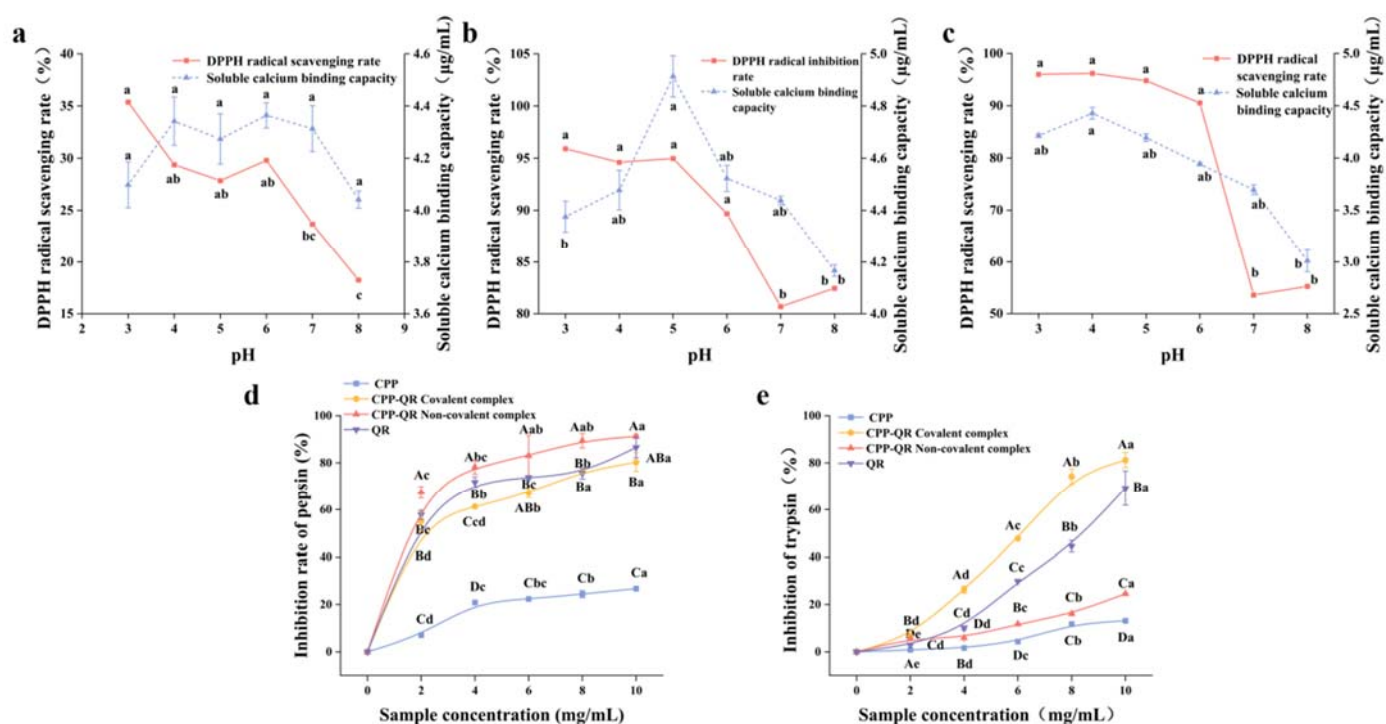


Figure 5. Activity changes of CPP (a), CPP-QR covalent complex (b), and CPP-QR non-covalent complex (c) in DPPH radical scavenging rate and soluble calcium binding capacity under different pH conditions. Effect of different concentrations of complexes on the inhibitory effect of pepsin (d) and trypsin (e). Data are presented as mean $\pm$ SD ($n=3$). The different uppercase letters above the line chart indicate significant differences between groups ($P < 0.05$), while the different lowercase letters indicate significant differences within groups ($P < 0.05$).

(2) Activity changes of CPP-QR covalent complex under pH environmental stress

As shown in Figure 5b, the CPP-QR covalent complex exhibited no significant changes in soluble calcium binding capacity under condition of pH 3 and pH 8 ($P > 0.05$), while its DPPH $\cdot$ scavenging rate was 95.91 % at pH 3, which decreased to 82.45% under pH 8 and decreased to 82.45% at pH 8. It decreased

significantly by 14.03%. This suggested that the soluble calcium binding capacity of the CPP-QR covalent complex exhibited insensitivity to pH variations, whereas the DPPH $\cdot$ scavenging activity significantly decreased under weakly alkaline conditions. This phenomenon may be attributed to the rigid structure formed by covalent bonds, which stabilized the spatial conformation of calcium-binding sites and thereby reduced sensitivity to pH changes (35). Conversely, the decline in DPPH $\cdot$ scavenging activity under weakly alkaline conditions was likely closely associated with the deprotonation of phenolic hydroxyl groups in QR (36) or structural degradation (37).

(3) Activity changes of CPP-QR non-covalent complex under pH environmental stress

As shown in Figure 5c, the soluble calcium binding capacity of CPP-QR non-covalent complex remained stable across pH 3-8 ($P > 0.05$). And the DPPH $\cdot$ scavenging rate of the complex reached 96.01% at pH 3, but significantly decreased to 55.25% at pH 8, showing a 42.45% reduction in antioxidant activity. The pH-insensitive calcium binding capacity of CPP-QR non-covalent complex may be attributed to the low pH-dependence of its binding sites or the structural stability of the complex. In contrast, the observed decline in DPPH $\cdot$ scavenging rate with increasing pH could be associated with the deprotonation of antioxidant groups (37) or chemical degradation of the QR component (36).

Overall, the dynamic changes in pH environment significantly affected the soluble calcium binding capacity and DPPH $\cdot$ scavenging rate of CPP-QR covalent and non-covalent complex, and this result aligned with the experimental results obtained from the dynamic digestion test.

3.6 Digestive enzyme inhibition rate

(1) Pepsin inhibition rate

Pepsin inhibition rate refers to the percentage of pepsin activity being suppressed.

According to Figure 5d, when the sample concentration was 10 mg/mL, the inhibition rate of the CPP-QR covalent and non-covalent complex on pepsin was 80.27% and 91.22%, respectively. And the inhibition rate of CPP on pepsin was 26.71%. Compared to CPP, the pepsin inhibition rate of CPP-QR covalent and non-covalent complex showed increases of 66.72% and 70.72%, respectively. The IC₅₀ values of CPP, CPP-QR covalent complex, CPP-QR non-covalent complex, and QR were 17.40 mg/mL, 4.01 mg/mL, 2.58 mg/mL, and 3.44 mg/mL, respectively. The CPP-QR covalent and non-covalent complex showed superior anti-pepsin digestion stability. Notably, CPP-QR non-covalent complex demonstrated a higher inhibitory rate against pepsin compared to CPP-QR covalent complex, which aligned with the results in dynamic digestion that the CPP-QR non-covalent complex exhibited superior resistance to gastric digestion. These results demonstrated that the CPP-QR non-covalent complex effectively inhibited pepsin activity, thereby significantly enhancing its resistance to the gastric digestive environment. This further validated the unique advantages of this complex in terms of gastrointestinal stability.

(2) Trypsin inhibition rate

As shown in Figure 5e, the CPP-QR covalent complex demonstrated a trypsin inhibition rate of $81.19 \pm 3.19\%$, which was significantly higher than those of the CPP-QR non-covalent complex

(24.55±0.36%) and the CPP non-covalent complex (13.11%) at a concentration of 10 mg/mL. The IC₅₀ values of CPP, CPP-QR covalent complex, CPP-QR non-covalent complex, and QR were 36.06 mg/mL, 6.19 mg/mL, 22.27 mg/mL, and 8.41 mg/mL, respectively. This result was consistent with the results in dynamic digestion that CPP-QR covalent complex exhibits excellent anti-intestinal digestive ability. This indicated that the significant inhibitory effect of CPP-QR covalent complex on trypsin activity was an important mechanism for enhancing its anti-digestive performance.

In summary, the CPP-QR non-covalent complex demonstrated superior resistance to pepsin digestion, whereas the covalent CPP-QR complex exhibited enhanced resistance to trypsin digestion. This discrepancy may be attributed to the fact that non-covalent interactions and covalent bonding could potentially reduce cleavage efficiency by masking or disrupting the specific recognition sites for pepsin or trypsin (31). The mechanisms underlying these differential effects will be systematically investigated in subsequent studies through molecular docking and molecular dynamics simulation analyses.

3.7 Molecular docking

(1) Molecular docking with pepsin

Molecular docking, as a computational simulation method, can predict how small molecule ligands bind to biological macromolecular receptors, where they bind and how strong they bind. As shown in the Figure 6a and Table 1, two key hydrogen bonds were formed between pepsin and CPP: one involving the Asp40 residue of pepsin with Arg1 of CPP, and the other established a hydrogen bond network between Tyr176 of pepsin and Glu5 of CPP. The total binding free energy between pepsin and CPP was calculated to be −22.25 kcal/mol, indicating that CPP showed a certain inhibitory effect on pepsin. This finding was consistent with the pepsin inhibition rate of CPP observed from the results of digestive enzyme inhibition rate. As shown in Figure 6b and Table 1, the binding of the CPP-QR covalent complex to pepsin was mainly achieved through hydrogen bond and salt bridge. Specifically, the key interactions between pepsin and CPP included: Lys25 of pepsin and Glu11 of CPP formed hydrogen bond, Tyr79 of pepsin and Ile12 of CPP formed hydrogen bond, and Tyr175 of pepsin and Arg1 of CPP formed hydrogen bond. Asn114 of pepsin simultaneously established dual hydrogen bond with both Glu5 of CPP and QR, and Gly224 of pepsin also formed hydrogen bond with QR. Additionally, a stable salt bridge structure was formed between the Arg35 of pepsin and the Glu4 of CPP. According to calculation results, the total binding free energy of the pepsin-CPP-QR covalent complex (−26.33 kcal/mol) was lower than that of the CPP system, indicating stronger binding affinity and higher thermodynamic stability. This results suggested that the CPP-QR covalent complex exhibited an enhanced pepsin inhibitory effect compared to CPP, which was consistent with the findings presented in digestive enzyme inhibition rate. The CPP-QR non-covalent complex bound with pepsin through hydrogen bond and salt bridge: hydrogen bond and salt bridge were simultaneously formed between Arg35 of pepsin and Glu2 of CPP, and Lys44 of pepsin and Glu14 of CPP. QR formed a hydrogen bond with Ser172 of pepsin and established a salt bridge with Lys44 of pepsin (Figure 6c and Table 1). Additionally, the binding free energy of the CPP-QR non-covalent complex with pepsin (−30.49 kcal/mol) was significantly lower than that of CPP

and CPP-QR covalent complex. This suggested that the CPP-QR non-covalent complex had a superior inhibitory effect on pepsin compared with the CPP-QR covalent complex and CPP. This phenomenon was consistent with the experimental results of digestive enzyme inhibition rate.

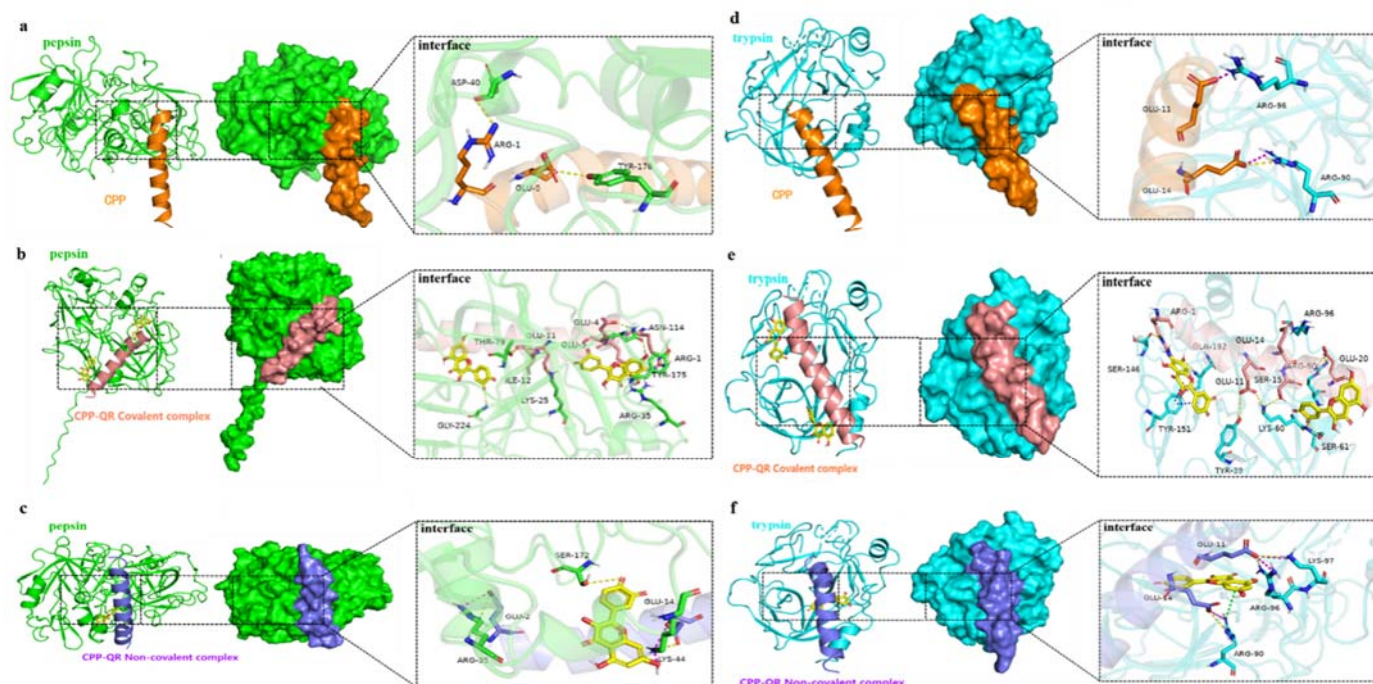


Figure 6. The molecular docking of pepsin and CPP (a), CPP-QR covalent complex (b) and CPP-QR non-covalent complex (c), and the molecular docking of pepsin and CPP (d), CPP-QR covalent complex (e) and CPP-QR non-covalent complex (f).

Among them, pepsin was green, trypsin was blue, CPP was orange, CPP-QR covalent complex was pink, CPP-QR non-covalent complex was purple, and quercetin was yellow. The green and purple sticks were the amino acid residues (interactions sites) formed by pepsin/ trypsin and ligands, respectively. The yellow dashed line represented hydrogen bond, and the purple dashed line represented salt bridges, and the green dashed line showed the cation- π interaction.

Table 1 Intermolecular force between pepsin and ligand

Ligand	Amino acids in pepsin (receptor)	Amino acids in ligand	Distance (Å)	Number	Intermolecular force
CPP	Tyr 176	Glu 5	3.0	1	hydrogen bond
	Asp 40	Arg 1	2.8	1	hydrogen bond
	Lys 25	Glu 11	3.0	1	hydrogen bond
	Thr 79	ILE 12	2.9	1	hydrogen bond
	Asn 114	Glu 5	2.8	1	hydrogen bond
CPP-QR covalent complex	Asn 114	quercetin	2.6	1	hydrogen bond
	Tyr 175	Arg 1	3.5	1	hydrogen bond
	Gly 224	quercetin	2.8	1	hydrogen bond
	Arg 35	Glu 4	3.0	1	salt bridge
	Arg 35	Glu 2	3.2	1	hydrogen bond
CPP-QR non-covalent complex	Lys 44	Glu 14	2.6	1	hydrogen bond
	Ser 172	quercetin	3.6	1	hydrogen bond
	Arg 35	Glu 2	3.2	1	salt bridge
	Lys 44	Glu 14	2.8	1	salt bridge
	Lys 44	quercetin	1.5	1	salt bridge

In this study, a single representative peptide sequence (RELEELNVPGEIVESLSSEESITR) was used for molecular docking and kinetic simulation. Although this simplified model cannot fully reflect the structural diversity and potential synergistic effects of all components in the commercial CPP mixture, the selected peptide segment was determined based on the core phosphopeptide fragments with clear strong

calcium-binding ability reported in the literature. The conformational characteristics and calcium binding behavior of the peptide in docking were highly consistent with the experimental observations, indicating that it can better represented the main structural pattern of CPP playing key biological activities, thus supported the rationality of the mechanism conclusions obtained in this study. In summary, CPP bound to pepsin through two hydrogen bonds, the CPP-QR covalent complex formed six hydrogen bonds and one salt bridge, and the CPP-QR non-covalent complex adopted the synergistic effect of three hydrogen bonds and three salt Bridges. The binding stability of the CPP-QR non-covalent complex with pepsin was greater than that of the CPP-QR covalent complex and CPP. This might be attributed to the fact that the strength of hydrogen bond was weaker than that of salt bridge, and hydrogen bond were susceptible to competition from water molecules or interference from polar environments, and salt bridges had lower sensitivity to pH and ionic strength and could remain stable during dynamic binding (38).

(2) Molecular docking with trypsin

As shown in the Figure 6d and table 2, the CPP mainly bound to trypsin through hydrogen bond and salt bridge. Specifically, the Arg90 of trypsin and the Glu14 of CPP formed hydrogen bond and salt bridge, and the Arg96 of trypsin and the Glu11 of CPP formed salt bridge. And the total binding free energy of trypsin docking with CPP was -24.60 kcal/mol. The CPP-QR covalent complex bound to trypsin mainly through hydrogen bond, salt Bridges and π - π stacking interaction (Figure 6e and Table 3). Specifically, the Try39 of trypsin formed hydrogen bond with Glu11 of CPP, Lys60 formed hydrogen bond with Glu 11 and Ser15 of CPP, Ser61 formed hydrogen bond with QR, and Arg90 formed hydrogen bond with Glu20 of CPP. Arg96 of trypsin formed hydrogen bond and salt bridge with Glu14 of CPP, Ser146 of trypsin formed hydrogen bond with Arg1 of CPP, Gln192 of CPP formed hydrogen bond with QR, and QR formed π - π stacking interaction with Tyr151 of trypsin. The total binding free energy of trypsin-CPP-QR covalent complex docking (-33.97 kcal/mol) was lower than that of trypsin-CPP docking, demonstrating a more stable binding interaction between trypsin and CPP-QR covalent complex. As shown in Figure 6f and Table 2, the CPP-QR non-covalent complex bound to trypsin mainly through hydrogen bond, salt bridge and π - π stacking interaction. Structural analysis revealed that trypsin's Arg90 established dual interactions (hydrogen bonding and salt bridge) with CPP's Glu14, while Arg96 and Lys97 similarly formed hydrogen-bond/salt-bridge pairs with Glu11 of CPP. Notably, a π - π stacking effect was observed between QR and trypsin's Arg90. Molecular docking calculations demonstrated a total binding free energy of -29.80 kcal/mol for the trypsin/CPP-QR non-covalent complex system.

Table 2 Intermolecular force between trypsin and ligand

Ligand	Amino acids in trypsin (receptor)	Amino acids in ligand	Distance (Å)	Number	Intermolecular force
CPP	Arg 90	Glu 14	2.9	1	hydrogen bond
	Arg 96	Glu 11	1.8	1	salt bridge
	Arg 90	Glu 14	3.2	1	salt bridge
CPP-QR covalent complex	Tyr 39	Glu 11	3.6	1	hydrogen bond
	Lys 60	Glu 11	3.1	1	hydrogen bond
	Lys 60	Ser 15	2.9	1	hydrogen bond

	Ser 61	quercetin	3.3	2	hydrogen bond
	Arg 90	Glu 20	2.8; 3.1	2	hydrogen bond
	Arg 96	Glu 14	2.8; 3.0	1	hydrogen bond
	Ser 146	Arg 1	3.3	1	hydrogen bond
	Gln 192	quercetin	3.4	1	hydrogen bond
	Arg 96	Glu 14	3.5	1	salt bridge
	Tyr 151	quercetin	4.4; 4.4	2	π - π stacking
	Arg 90	Glu 14	3.6	1	hydrogen bond
	Arg 96	Glu 11	1.6	1	hydrogen bond
CPP-QR	Lys 97	Glu 11	3.3	1	hydrogen bond
non-covalent	Arg 90	Glu 14	4.1	1	salt bridge
complex	Arg 96	Glu 11	3.2	1	salt bridge
	Lys 97	Glu 11	3.3	1	salt bridge
	Arg 90	quercetin	3.2	1	π - π stacking

Table 3 Binding free energy and energetic decomposition of pepsin to CPP, CPP-QR covalent and non-covalent complex.

MM-GBSA	Pepsin-CPP (kcal/mol)	Pepsin-CPP-QR covalent complex (kcal/mol)	Pepsin-CPP-QR non-covalent complex (kcal/mol)
ETotal	-20.85	-33.64	-60.71
EEEL	-24.18	-36.72	-31.82
ECov	0.59	0.39	7.30
EH-bond	-2.03	-0.04	-1.97
ESA	-5.64	-6.93	-16.35
EPacking	-0.03	-4.16	-2.62
EPB	29.76	48.52	36.37
EvdW	-19.32	-34.71	-51.62

E_{Total} , E_{EEL} , E_{Cov} , E_{H-bond} , E_{SA} , $E_{Packing}$, E_{PB} , E_{vdW} , represented the total binding free energy, electrostatic energy, covalent bonds, hydrogen bond, SASA energy, van der waals packing, polar solvent energy, van der Waals energy, respectively

To sum up, CPP bound to trypsin through one hydrogen bond and two salt bridges. The CPP-QR covalent complex formed eight hydrogen bonds, one salt bridge and one π - π packing with trypsin. The CPP-QR non-covalent complex adopted the synergistic effect of three hydrogen bonds, three salt bridges and one π - π packing with trypsin. The binding stability of the CPP-QR covalent complex with trypsin was greater than that of the CPP-QR non-covalent complex and CPP.

3.8 Molecular dynamics (MD) simulation

3.8.1 MD with pepsin

(1) Root mean square deviation (RMSD) analysis

The variation of RMSD over time is a key indicator for evaluating the equilibrium state of the system and its structural stability (39). The MD simulation water box constructed for pepsin-CPP, pepsin-CPP-QR covalent complex and pepsin-CPP-QR non-covalent complex was shown in Figure 7a-c. As shown in the figure 7d, the RMSD fluctuation values of three systems remained below 0.1 nm at 100 ns. It can be inferred that the simulation system had reached a stable state of dynamic equilibrium. The RMSD values for the three groups were as followed: pepsin-CPP > pepsin-CPP-QR covalent complex > pepsin-CPP-QR non-covalent complex. And the RMSD value of pepsin -CPP-QR non-covalent complex exhibited the minimal fluctuation, indicating that the structure of the CPP-QR non-covalent complex in pepsin system was more stable compared

with that of the CPP-QR covalent complex and CPP. This phenomenon may be attributed to the non-covalent binding of CPP and QR was the synergistic effects of multi-site dynamic binding and retention of natural conformation. While CPP-QR covalent binding enhanced local stability, it may introduced a new dynamic imbalance through rigid constraints (40). Based on the analysis of the results in digestive enzyme inhibition, the inhibitory effect of the CPP-QR non-covalent complex on pepsin was significantly better than that of CPP and the CPP-QR covalent complex. This result was consistent with the research result in dynamic digestion that the CPP-QR non-covalent complex showed better stability during gastric digestion.

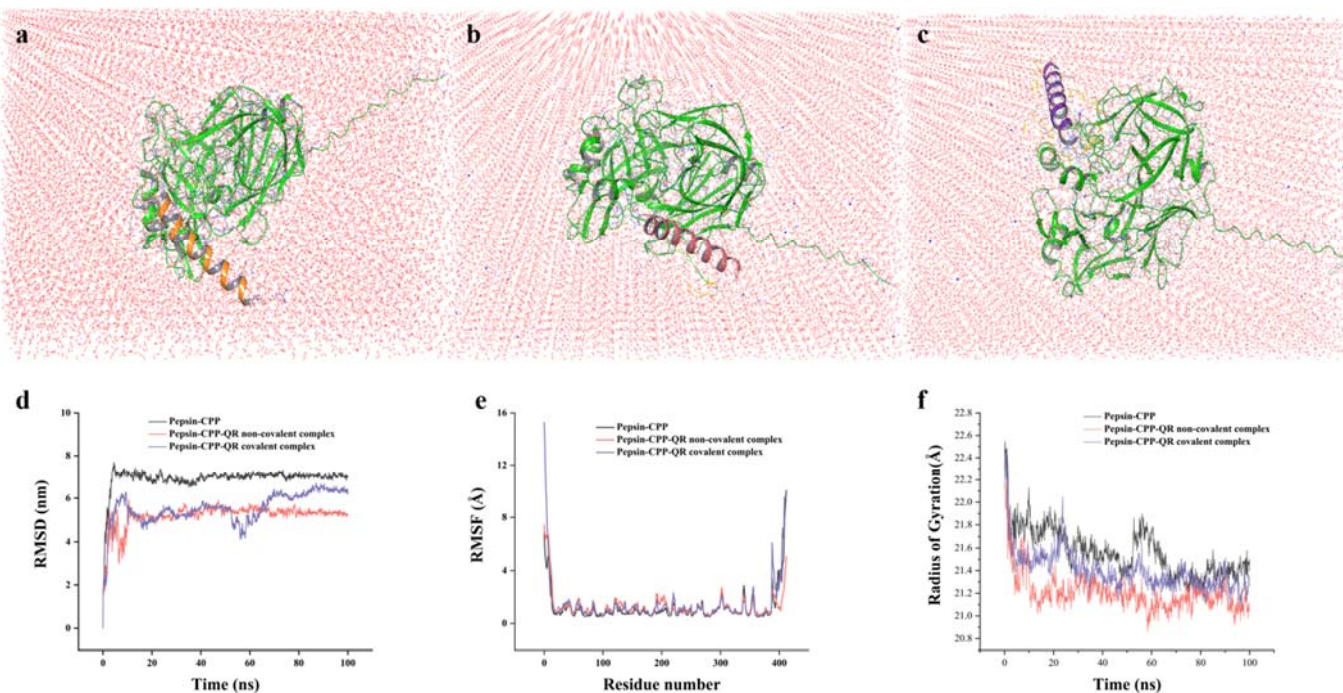


Figure 7. The molecular dynamic simulations of pepsin-CPP (a), pepsin-CPP-QR covalent complex (b), and pepsin-CPP-QR non-covalent complex (c) simulated a water box, where pepsin was green, CPP was orange, CPP-QR covalent complex was pink, and CPP-QR non-covalent complex was purple. The changes in root mean square deviation (RMSD, d), root mean square fluctuation (RMSF, e), and radius of gyration (Rg, f) before and after the binding of pepsin with CPP, CPP-QR covalent/non-covalent complex.

(2) Root mean square fluctuation (RMSF) analysis

RMSF is typically used to analyze the degree of freedom of amino acid residue movement to represent the conformation of macromolecules and the differences in atomic positions between conformations at specific times (41). As shown in Figure 7e, In the 0-250 cavity of the pepsin active site, the root mean square deviation (RMSF) of the CPP-QR non-covalent/covalent complex was slightly higher than that of CPP. This may be due to the complex disrupting local stability in pepsin, resulting in a loosened and unstable local structure in this region. In the CPP-QR non-covalent complex system, the RMSF of the terminal loop area (350-400) of the pepsin active site was significantly lower than that of both CPP and the CPP-QR covalent complex. This indicated that the non-covalent complex reduced the flexibility of amino acid residues in the pepsin active site, leading to a contracted enzyme active domain, impaired substrate binding, and decreased pepsin activity. Combined with the results of molecular binding, it is speculated that the addition of the CPP-QR covalent/non-covalent complex may indirectly induce the microenvironment disturbance of the pepsin active center through hydrogen bonds, hydrophobic interactions or steric hindrance, thereby increasing

its conformational flexibility. The results indicated that the activity of pepsin was reduced (42). However, CPP and QR might non-covalently form a stable complex through cross-linking or blocking the digestion site, thereby improving its structural resistance and ultimately demonstrating stronger anti-digestive ability (43).

(3) Radius of gyration (Rg) analysis

It was commonly accepted that the Rg quantified molecular structural compactness. An increased Rg indicated protein expansion, looser conformation, and potentially reduced stability (44). At 0-100 ns, the Rg of these three systems were all within the range of 20.8-22.6 nm (Figure 7f), and the overall rigid structure of the protein remained stable. The Rg values demonstrated the following order: Pepsin-CPP > Pepsin-CPP-QR covalent complex > CPP-QR non-covalent complex. Notably, this rank order inversely parallels the experimental IC₅₀ trend, implying that the smaller the Rg the tighter the complex and the stronger the pepsin inhibition. This indicated that the CPP-QR non-covalent complex had a more stable structure compared to the CPP-QR covalent complex and CPP in the pepsin reaction system, thereby demonstrating superior anti-pepsin digestion ability. This result was consistent with the results of static and dynamic digestion study.

(4) Binding free energy and energetic decomposition analysis

By calculating the energy change of the system before and after the ligand binding to the receptor and decomposing it into the contribution of different physical interactions, the driving force and key residues of the binding process are revealed quantitatively. As shown in Table 3, the binding free energy of the pepsin-CPP-QR non-covalent complex system (-60.71 kcal/mol) was lower than that of the pepsin-CPP-QR covalent complex (-33.64 kcal/mol) and pepsin-CPP (-20.85 kcal/mol). This result indicated that the CPP-QR non-covalent complex was relatively stable in the pepsin system, reflecting its good anti-gastric protein digestion stability and facilitating the maintenance of activity. The CPP-QR non-covalent complex had the smallest E_{vdw} value, which indicated that the van der Waals force interaction between the CPP-QR non-covalent complex and pepsin contributed the most to the total binding free energy. Additionally, the CPP-QR covalent complex and pepsin were mainly constrained by electrostatic energy and polar solvent energy compared with other ligands. The van der Waals energy and electrostatic energy were conducive to the binding of complex to the pepsin, while the solvation energy showed an unfavorable effect on the binding (45).

3.8.2 MD with trypsin

(1) RMSD analysis

As shown in Figure 8d, the RMSD value of the trypsin-CPP-QR covalent complex was significantly lower than that of trypsin-CPP and trypsin-CPP-QR non-covalent complex, and the structural fluctuation was reduced, indicating that the CPP-QR covalent complex has better structural stability in the trypsin reaction system. This might be attributed to the fact that the bond energy of covalent bonds was much higher than that of non-covalent interactions, which significantly reduced the structural displacement and conformational fluctuations within the complex (46). It was demonstrated that the CPP-QR covalent complex had superior

anti-trypsin digestion stability, which was conducive to maintaining its activity in the intestinal tract. This was consistent with the results of the dynamic digestion experiment of the CPP-QR covalent complex.

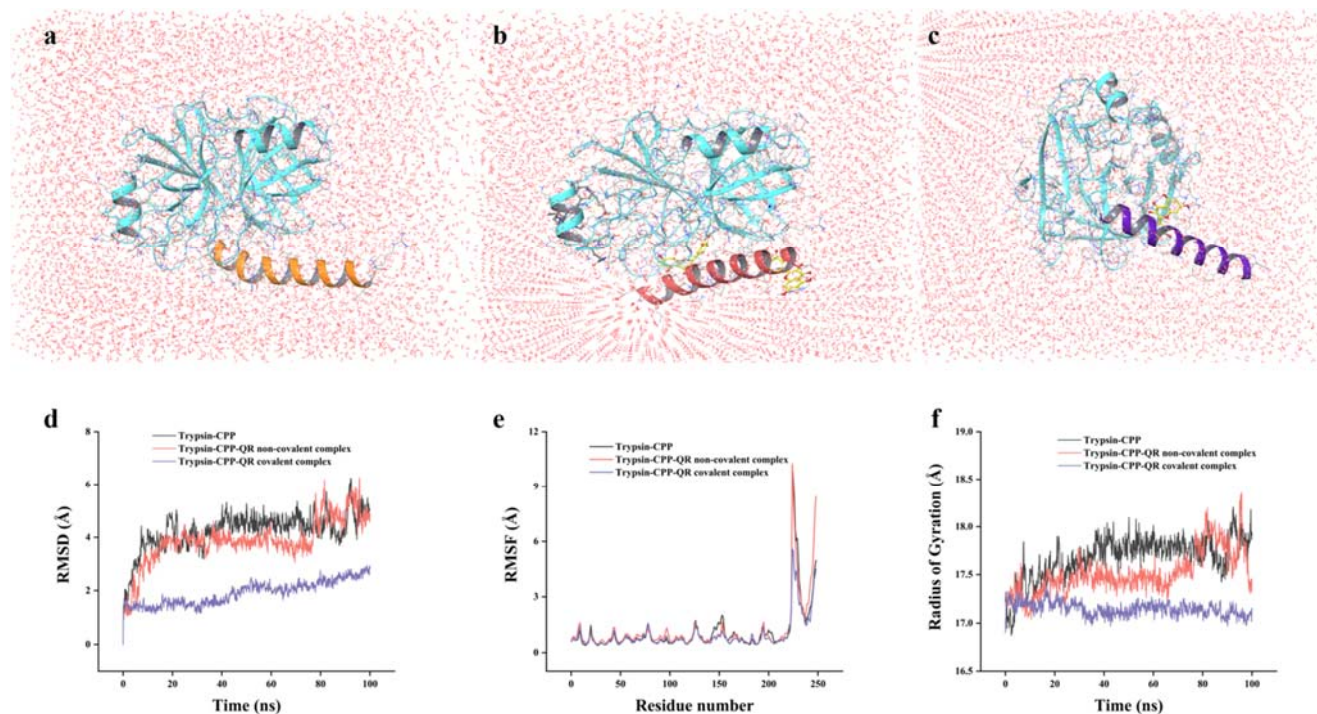


Figure 8. The molecular dynamic simulations of trypsin-CPP (a), trypsin-CPP-QR covalent complex (b), and trypsin-CPP-QR non-covalent complex (c) simulated a water box, where trypsin was blue, CPP was orange, CPP-QR covalent complex was pink, and CPP-QR non-covalent complex was purple. The changes in root mean square deviation (RMSD, d), root mean square fluctuation (RMSF, e), and radius of gyration (Rg, f) before and after the binding of trypsin with CPP, CPP-QR covalent/non-covalent complex.

(2) RMSF analysis

The RMSF of the three systems all fluctuated slightly within the range of 0 to 225, and the trends were similar with little difference (Figure 8e). The RMSF of amino acid residues at the cavity 145-155 of trypsin active sites in the CPP-QR covalent/non-covalent complex system was significantly lower than that of CPP. This indicated that the addition of the complex reduced the flexibility of amino acid residues at the trypsin active site, thereby causing the active domain of the enzyme to contract tightly. This may lead to trypsin being less likely to bind to the substrate, thereby reducing the activity of enzyme (47). In the terminal loop area (225-250), the flexibility was relatively large, hence the fluctuations of the three systems increased. Among them, the fluctuation of the trypsin-CPP-QR covalent complex was the smallest, indicating that this system was relatively stable. This result was consistent with the RMSD result. This further indicated that the CPP-QR covalent complex could maintain structural stability under the trypsin reaction system, reflecting its good anti-intestinal digestion ability and activity retention.

(3) Rg analysis

As shown in Figure 8f, the Rg of the three systems all fluctuated within the range of 16.5-18.5 nm at 0-100 ns. The Rg value of trypsin-CPP was higher than that of the trypsin-CPP-QR non-covalent complex and the trypsin-QR covalent complex, and the fluctuation of the trypsin-CPP-QR covalent complex was significantly smaller. It indicated that the CPP-QR covalent complex can maintain structural stability in the

trypsin reaction system, which was conducive to the maintenance of its activity. This was consistent with the results of the previous dynamic and static simulation digestion experiments. Given that a smaller R_g reflects a tighter structure, the minimal fluctuation of R_g in the trypsin CPP-QR covalent complex means that its conformation is least easily recognized by enzymes, thereby directly reducing the cleavage efficiency of trypsin on QR and achieving higher levels of enzyme inhibition.

(4) Binding free energy and energetic decomposition analysis

As shown in Table 4, the binding free energy of trypsin with the CPP-covalent complex (-84.21 kcal/mol) was lower than that with the CPP-QR non-covalent complex (-69.89 kcal/mol) and CPP (-39.69). This indicated that, the CPP-QR covalent complex maintained structural stability in the trypsin system, reflecting its superior anti-trypsin digestion ability and the retention of its activity. The E_{vdw} value of CPP-QR covalent and non-covalent complexes was lower than that of CPP, which indicated that the van der Waals force interaction between the complex system and trypsin contributed significantly to the binding free energy. In addition, the CPP-QR covalent complex was mainly constrained by electrostatic energy and polar solvent energy with trypsin compared with other ligands. Lu et al. found that electrostatic interaction, including hydrogen bonds, made the highest energy contribution in the binding. Lu et al. also reported that electrostatic interactions, particularly hydrogen bonding, made the most significant energetic contribution to the binding process between β -lactoglobulin and rosmarinic acid (48).

Table 4 Binding free energy and energetic decomposition of trypsin to CPP, CPP-QR covalent and non-covalent complex.

MM-GBSA	Trypsin-CPP (kcal/mol)	Trypsin-CPP-QR covalent complex (kcal/mol)	Trypsin-CPP-QR non-covalent complex (kcal/mol)
E_{Total}	-39.69	-84.21	-69.89
E_{EEL}	139.68	250.21	195.73
E_{Cov}	-0.31	3.45	5.07
E_{H-bond}	-2.85	-4.25	-3.14
E_{SA}	-6.71	-15.74	-22.86
$E_{Packing}$	-2.75	-3.33	-3.91E-14
E_{PB}	-105.69	-229.16	-160.49
E_{vdW}	-61.16	-85.31	-84.48

E_{Total} , E_{EEL} , E_{Cov} , E_{H-bond} , E_{SA} , $E_{Packing}$, E_{PB} , E_{vdW} , represented the total binding free energy, electrostatic energy, covalent bonds, hydrogen bond, SASA energy, van der waals packing, polar solvent energy, van der Waals energy, respectively

4. Conclusion

This study explored the differential mechanism in gastrointestinal response behavior of CPP-QR covalent and non-covalent complex during the digestive process and under environmental stress. Compared with the CPP, the soluble calcium binding capacity and DPPH $\cdot$ scavenging rate of the CPP-QR covalent/non-covalent complex were significantly enhanced, and the synergistic effect of the covalent complex was more significant. It was revealed that the CPP-QR covalent complex had higher activity stability in the intestinal stage, while the non-covalent complex maintained strong activity in the gastric digestion stage. That is, the non-covalent complex had superior anti-gastric protease stability ($IC_{50}=2.58\text{mg/mL}$) and

the covalent complex had superior anti-trypsin stability ($IC_{50}=6.19\text{mg/mL}$). The binding free energies non-covalent complex: -60.71 kcal/mol (pepsin system) versus covalent complex: -84.21 kcal/mol (trypsin system). This difference was attributed to the fact that CPP-QR covalent complex had stronger resistance to trypsin digestion, while CPP-QR non-covalent complex resulted from their better tolerance to pepsin digestion and adaptability to acidic environments. This study reveals the targeted stability mechanism of covalent and non-covalent complexes in different stages of the digestive tract, which is suitable for intestinal targeted applications requiring continuous release of nutrients and high biological activity, and provides a theoretical basis for the rational design of functional complexes in the nutrient delivery system. In future research, the interaction between 2-4 high-abundance core phosphopeptide fragments in CPP and the target will be evaluated to compare the similarities and differences in their binding modes and energies, and to verify whether the proposed mechanism is conserved among different representative peptide segments.

Declaration of Competing Interests

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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