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Study of the noncovalent interactions between phenolic acid and lysozyme by cold spray ionization mass spectrometry (CSI-MS), multi-spectroscopic and molecular docking approaches

Su Chen[a,b], Xin Gong[b], Hongwei Tan[b], Yang Liu[a], Lan He[a,**], Jin Ouyang[b,*]

[a] National Institutes for Food and Drug Control, Beijing, 102629, China
[b] College of Chemistry, Beijing Normal University, Beijing, 100875, China

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ABSTRACT

Elucidating the recognition mechanisms of the noncovalent interactions between pharmaceutical molecules and proteins is important for understanding drug delivery in vivo, and for the further rapid screening of clinical drug candidates and biomarkers. In this work, a strategy based on cold spray ionization mass spectrometry (CSI-MS), combined with fluorescence, circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), and molecular docking methods, was developed and applied to the study of the noncovalent interactions between phenolic acid and lysozyme (Lys). Based on the real characterization of noncovalent complex, the detailed binding parameters, as well as the protein conformational changes and specific binding sites could be obtained. CSI-MS and tandem mass spectrometry (MS/MS) technique were used to investigate the phenolic acid-Lys complexes and the structure-affinity relationship, and to assess their structural composition and gas phase stability. The binding affinity was obtained by direct and indirect MS methods. The fluorescence spectra showed that the intrinsic fluorescence quenching of Lys in solution was a static quenching mechanism caused by complex formation, which supported the MS results. The CD and FTIR spectra revealed that phenolic acid changed the secondary structure of Lys and increased the α-helix content, indicating an increase in the tryptophan (W) hydrophobicity near the protein binding site resulting in a conformational alteration of the protein. In addition, molecular docking studies were performed to investigate the binding sites and binding modes of phenolic acid on Lys. This strategy can more comprehensively and truly characterize the noncovalent interactions and can guide further research on the interactions of phenolic acid with other proteins.

1. Introduction

The noncovalent interaction between drugs and proteins is an important pathway for drugs to exert their biological effects and for proteins to perform their physiological functions, and it is also the molecular basis of drug therapy [1]. Therefore, the accurate characterization of protein-drug complexes and an in-depth study of the binding mechanism are of great significance. It is not only helpful to elucidate the mechanism of drug action at the molecular level, but also has guiding significance for promoting precise and rational drug use in clinical practice. In this regard, with the advent of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), the performance of mass spectrometry (MS) in structural identification and 4 S properties (speed, specific, sensitivity, stoichiometry) has been improved dramatically, which broaden its application in the field of biology [2,3]. The binding affinity, stoichiometries, stability, and dynamics of the complexes and protein conformational changes can be obtained directly by MS. Moreover, the latest proteomic techniques (i.e., “top-down” and “bottom-up” MS) yield spatial information on ligand binding [4,5] which cannot be obtained by traditional spectroscopic methods.

Ambient mass spectrometry (AMS) is a breakthrough in the MS development, playing a vital role in drug screening, doping control, agrochemical and explosive detection, as well as pharmaceutical analysis [6]. Recently, AMS techniques, such as desorption electrospray ionization mass spectrometry (DESI-MS) [7], liquid sample-desorption electrospray ionization mass spectrometry (LS-DESI-MS [8], venturi easy ambient sonic-spray ionization mass spectrometry (V-EASI-MS) [9] and laser ablation electrospray ionization (LAESI-MS) [10], have been widely applied to study the noncovalent interaction of drug-

* Corresponding author.
** Corresponding author.
E-mail addresses: helan1961@aliyun.com (L. He), jinoyang@bnu.edu.cn (J. Ouyang).

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protein. Due to its simple device and no need for complex sample pretreatment, especially the high-throughput capacity, their superiority in elucidating the mechanism of drug analysis and protein-ligand interactions are becoming increasingly prominent [11,12]. However, the high temperature of the desolvation chamber can cause damages to the weak interactions in ESI-MS [13], and the matrix-dependent effect of MALDI is interferential and difficult to select, which limits its application [1]. In some cases, the high energies and improper solvent systems may lead to the dissociation of complexes in the aforementioned AMS techniques [14]. In addition, some reports employ the MS signals of unbound drug molecules to investigate protein-drug interactions indirectly [15,16]. Therefore, the important binding parameters of the weak interactions between molecules cannot be obtained. There are also limitations on the real characterization of the noncovalent interactions in solutions using the aforementioned MS techniques.

Cold spray ionization mass spectrometry (CSI-MS) is a variant of ESI-MS that promotes ionization by increasing the polarizability of the compound at low temperature [17]. This native-MS technique allows the facile and precise characterization of weak noncovalent interactions in solutions compared to the conventional ESI method [18]. Over the past decades, many studies on the use of CSI-MS to characterize labile compounds such as organometallic complexes [19], supramolecules [20], reaction intermediates [21], protein conformational changes [22], amino acids, nucleotide clusters [23], and protein-ligand interactions [24], have been reported. In our laboratory, we have established a CSI-MS device and successfully detected the self-assembly behavior of an aggregation-induced emission enhancement system [25]. It provided more reliable experimental evidence and tool for studying the weak noncovalent interactions and the mechanism of aggregation-induced fluorescence.

In recent years, tumors, diabetes, cardiovascular and cerebrovascular diseases, bacteriosis and inflammation have affected human health and even threatened human life. Therefore, R&D for the above diseases has become the focus of today's social medicine field [26,27]. Phenolic acid compounds are one of polyphenols, which are widely distributed in traditional Chinese medicines such as honeysuckle, Tanacetum parthenium, perilla and fig [28–30]. Moreover, due to their multi-target effect in vivo, they have significant biological functions in anti-inflammatory, anti-allergy, anti-oxidation, anti-tumor, hypoglycemic and hypolipidemic, thus, they have high application potential. It has been reported that phenolic acids can bind to plasma proteins in vivo, thereby influencing the distribution, metabolism and biological activity of drugs [31]. Hence, the study of the interactions between phenolic acids and protein is of great significance in understanding its transport in vivo, improving its bioavailability and predicting the toxicity risk.

Lysozyme (Lys) is an important humoral immune factor and blood protein in the body. It not only has various biological functions such as antibacterial, antiviral and anti-tumor, but can also reversibly combine with many active molecules to exert therapeutic effects [32]. Studies have shown that changes in the Lys content of body fluids are associated with broncho-pulmonary dysplasia, conjunctivitis, nephropathy and hereditary non-neurogenic systemic amyloidosis, indicating that Lys can be used as a biomarker [32,33]. The W62, W63 and W108 residues are the main specific binding sites in Lys-small molecule interactions. All the previous reports are based on the specific binding of various small molecules to the W residues of Lys to perform physiological activities [32–34]. Many phenolic acids including chlorogenic acids can reversibly bind to Lys by the specific noncovalent interaction [31,34–36]. However, the action mechanism is still unclear and the structure-affinity relationships between them have not been reported. Furthermore, no breakthrough has been achieved in constructing the interaction model between small molecules and Lys.

In this study, a research strategy based on CSI-MS, combined with fluorescence, circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), and molecular docking techniques, was developed and applied to explore the noncovalent binding modes between phenolic acid and Lys, which can obtain comprehensive binding information. Firstly, the noncovalent binding mechanism of phenolic acid and Lys was studied by CSI-MS, and the stoichiometries, binding affinity and reactive groups of the interaction were obtained. The structural composition and gas phase stability of the complex were further verified by MS/MS technology. Thereafter, fluorescence, CD and FTIR spectroscopy were employed to explore the mechanism of the interaction between phenolic acid and Lys in solution, as well as the conformational change in Lys. Finally, the molecular docking method was used to confirm the binding site and binding type. The experimental and theoretical results were combined to further demonstrate the reliability of the method. The strategy adopted in this study and our previous reports provide new insights for a more comprehensive and authentic characterization of protein-drug interactions, and have great guiding significance in elucidating the biological activity mechanism and clinical use of phenolic acids.

2. Experimental

2.1. Materials and sample preparation

Lysozyme and ammonium acetate (molecular biology grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Caffeoylquinic acid (chlorogenic acid, CQA), rosmarinic acid (RA), 1,3-O-dicaffeoylquinic acid (1,3-CQA), 4,5-dicaffeoylquinic acid (4,5-CQA), caffeic acid, p-coumaric acid, ferulic acid, protocatechuic acid, vanillic acid, quinic acid, caffeic acid ethyl ester and caffeic acid phenethyl ester were obtained from the National Institutes for Food and Drug Control (Beijing, China). Danshensu was bought from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). N,N′,N″-triacetyl chitotriose (NAG_3) was purchased from Toronto Research Chemicals (North York, Canada). Deionized water (18.2 MΩ·cm) was produced using a Milli-Q water purification system (Millipore, USA). Methanol (HPLC grade) was acquired from Fisher Chemical Company (Fair Lawn, NJ, USA). All the reagents were used without further purification.

Lysozyme was dissolved in water and further diluted with 5 mM ammonium acetate (~pH 6.9). Phenolic acids were first dissolved in methanol and then diluted with 5 mM ammonium acetate (~pH 6.9) to the final concentration (the content of methanol $<$ 10%, v/v). For the protein-ligand system, Lys (final concentration of 35 μM) and phenolic acid were mixed at various molar ratio of 2:1, 1:1, 1:2, 1:5. and 1:10 in 5 mM ammonium acetate. All the mixed solutions were incubated at 37 °C for 30 min before determination.

2.2. CSI-MS experiment

The CSI-MS device used in this experiment was built according to previous study [25]. All the MS experiments were performed on Thermo LTQ linear ion trap mass spectrometer (Thermo Scientific, USA). Data acquisition and processing were performed by Xcalibur 2.0 software (Thermo) in the positive ion mode over the m/z range of 100–2000. The spray voltage, capillary voltage and tube lens were 3.0 kV, 100 V, and 250 V respectively. Samples were introduced into the mass spectrometer using a microsyringe pump at a flow rate of 30 μL/min. The duration of infusion time was about 1 min to ensure the balance of the equipment, after which data acquisition was initiated. The distance between the spray jet and mass inlet was 6 mm and the spray temperature was about 10 °C. The structural composition of the complexes was verified by collision induced dissociation (CID) in the MS/MS experiments. The isolation width was selected as 2–2.5 mass units and the activation time was 30 ms. The normalized collision energy was optimized to ensure that the fragments could be observed at an adequate signal-to-noise ratio.

2.3. Fluorescence spectroscopy

The binding mechanism between Lys and phenolic acid was investigated by fluorescence spectroscopy on an RF-6000 spectrophotometer (Shimadzu, Japan). The emission spectrum was recorded from 290 nm to 450 nm at an excitation wavelength of 280 nm at 298 K. The excitation and emission slit widths were both 10 nm with a scanning speed of 600 nm/min. The concentration of Lys was fixed at 35 μM, while that of the phenolic acid solution was varied from 0 to 35 μM. The fluorescence quenching mechanism and dissociation constant (K_d) were investigated using the well-known Stern-Volmer equation (1) and Eqs. (2) and (3) [37,38].

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

$$\log[(F_0 - F)/F] = \log(K_a) + n\log[Q] \quad (2)$$

$$K_d = \frac{1}{K_a} \quad (3)$$

where F_0 and F are the fluorescence intensities of Lys in the absence and presence of the phenolic acid. K_q and [Q] are the bimolecular quenching rate constant and concentration of the phenolic acid, respectively. τ_0 is the average fluorescence lifetime of the fluorescence molecules without Q. For the ordinary biomolecule, τ_0 is 10^{-8} s. K_{sv} is the Stern-Volmer quenching constant, which can be obtained from the slope of the plot. K_a and n are the association constants and the number of binding sites, respectively.

2.4. CD spectroscopy

The far-UV CD spectra of Lys in the absence and presence of phenolic acid were recorded by a J-810 spectropolarimeter (JASCO, Japan) in a nitrogen atmosphere over the range of 200–260 nm. Each average spectrum was obtained from two successive runs in a quartz cell (0.1 cm path length). The secondary structure content of the protein e.g., α-helix, β-sheet, β-turn, and random coil, were analyzed by CDNN software [39]. The molar ratios of phenolic acid to Lys used for CD measurements were 0:1, 1:1 and 2:1. The final concentration of Lys was 17.5 μM.

2.5. FTIR measurements

A FTIR Affinity-1 spectrometer (Shimadzu, Japan) was used to record the infrared spectra of Lys and its complexes in the range of 1800–1100 cm^{-1} at room temperature with a resolution of 4 cm^{-1} and 40 scans. The FTIR spectrum of the complex was obtained by subtracting the spectrum of the buffer solution containing each ligand alone with the same instrumental parameters.

2.6. Molecular docking studies

Molecular docking studies were performed using the AutoDock 4.2 [40] and AutoDock Tools-1.5.6 (ADT) with an embedded Lamarckian genetic algorithm [41]. The docking conformation of the Lys-ligand complexes was visualized using the PyMol software [42]. Before the docking, the geometries of the four ligands, CQA, RA, 1,3-CQA, and 4,5-CQA were optimized by the DFT B3LYP functional with 6-311G (d,p) basis set using the Gaussian 09 program package [43]. The optimized structures are shown in Fig. S1. The structure of Lys was obtained from the Protein Data Bank (PDB ID: 6LYZ) [44]. The entire crystalline water from the PDB structure was deleted, and the polar and non-polar hydrogen atoms of protein and the ligands were added and merged using the ADT's hydrogen module. During the docking procedure, the protein was set to be rigid, while all the torsional bonds were set to be flexible for the ligands. During docking process, the grid sizes along the X-, Y- and Z-axes were set to 40 × 40 × 40 with a spacing of 0.375 Å. The grid box was centered at the coordinates of x = 3.551, y = 22.086, z = 34.249 of Lys. The number of docking runs was set as 100 for each ligand [45]. The conformation with the minimum binding free energy was further analyzed.

3. Results and discussions

3.1. Characterization of the noncovalent complex of Lys with phenolic acid by CSI-MS and condition optimization

CQA is the most common individual compound of the phenolic acid family in some foods and traditional Chinese injections or tablets such as coffee, green tea, Mailuoning injection and Shuanghua Baihe tablets [46]. It has been reported that the substitution of the hydrogen on the aromatic ring and the esterification of the carboxylic unit play important roles in their bioactivities [47]. However, the mechanism of the interactions between CQAs with Lys, and the structure-affinity relationships are unknown. Thus, we selected CQA (*m/z* 354) as an example to determine the noncovalent complex formed with Lys by CSI-MS. It was found that Lys showed a relatively narrow charge distribution. The ions at *m/z* 1789.35, 1590.84 and 1431.93 corresponded to P^{8+}, P^{9+} and P^{10+} respectively, indicating a natural folded state (Fig. 1a) [48,49]. After incubation for 30min, the new mass spectrum peaks at *m/z* 1832.68 and 1876.77 denote the 1:1 and 1:2 complexes of CQA with Lys in the +8 charge state (Fig. 1b). The peak at *m/z* 1629.02 is representative of the +9 charge state of the 1:1 complex of CQA with Lys. From the CSI-MS spectra, the intact noncovalent complexes can be easily observed and the stoichiometric ratio can be seen directly. We also conducted a test on the complexation of the well-known specific ligand (NAG_3) to Lys by CSI-MS to confirm the specific interaction

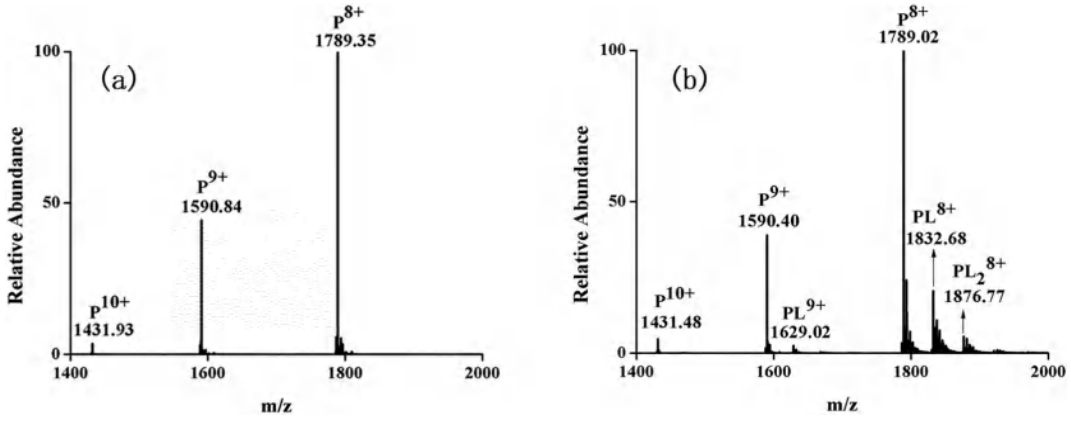

Fig. 1. CSI-MS spectra of the multiply charged state of Lys (a) and the complex of Lys with chlorogenic acid (CQA) at a 1:2 concentration ratio (b). PL and PL_2 represent the 1:1 and 1:2 complex respectively.

between Lys and CQA. NAG_3 formed a 1:1 complex with Lys (Fig. S2), which was consistent with the result by ESI-MS [50]. Moreover, the fraction of Lys bound to NAG_3 decreased when incubating CQA with the mixture of Lys-NAG_3, indicating the specific interaction of CQA with Lys [51].

Suitable experimental conditions are very important to improve the detection sensitivity of the weak interaction and reduce the interference of non-specific binding [51]. These conditions often include instrumental parameters, sample preparation processes, and the drug-protein concentration ratio, etc [52,53]. Therefore, we carefully optimized these conditions. Firstly, the relative intensity of the 1:1 complex (*m/z* 1832.68) formed by Lys and CQA was used to optimize the parameters of the mass spectrometer, such as spray voltage, capillary voltage, tube lens and flow rate. The results show that the spray voltage of 3.0 kV and the flow rate of 30 μL/min were optimal for the detection of the complex (Fig. S3). A higher tube lens contributed to improve the sensitivity and reduce interference in the low mass region, so 250 V was selected (Fig. S4). It has been reported that a high concentration of ligand will increase the probability of non-specific binding [54]. Then, the concentration of the ligand–protein complex was carefully optimized to investigate the interaction. Fig. 2 shows that the 1:1 and 1:2 complexes always existed under different Lys-CQA molar ratios from 2:1 to 1:2, proving the specificity of noncovalent binding. However, when the molar ratio of Lys-CQA increased to 1:5 or higher, 1:3 and 1:4 complexes appeared indicating the non-specific interaction. Therefore, to minimize the non-specific binding during the CSI process, the protein-drug concentration was maintained at 1:2 in all the experiments. The organic solvent effect on the protein conformation and noncovalent complexes was also investigated and the methanol concentration was maintained below 10% in the following experiments (Figs. S5 and S6).

3.2. Study of the structure-affinity relationship

Exploring the influence of ligands with different structures on the affinity can effectively clarify the active groups and binding modes of the interaction, which is helpful for drug molecular design and R&D. The structure of phenolic acid is generally composed of caffeic acyl and ester parts. Therefore, we explored them separately to determine if the carboxyl and hydroxyl groups contribute to the interaction, and to verify the effect of different types of esters. The different phenolic acid structures investigated are shown in Fig. S7.

It was found that except CQA (*m/z* 354), only RA (*m/z* 360), 1, 3-CQA (*m/z* 516), and 4, 5-CQA (*m/z* 516) bound to Lys and formed 1:1, 1:2 and 1:3 complexes (Fig. 3), while the typical mass peaks indicative of other phenolic acids were not observed (Table S1). Furthermore, by analyzing the structure of phenolic acid, the noncovalent interaction was significantly enhanced after ester formations indicating that the ester part is the reactive group, while the carboxylic unit provided no effective binding site. Moreover, when the ester group is larger and contains benzene ring, hydroxyl group and carboxyl group, the interaction is strengthened. The number and position of the side chains of the caffeic acyl group also had an effect on the interaction, suggesting that the caffeic acyl part was also an active group. Many studies on the interactions between small molecules and Lys have shown that the hydrophobic interaction is the main mechanism and that an electrostatic interaction and hydrogen bonding also exist [55–57]. Therefore, we speculated that the molecular size and hydrophobicity increased after ester formation and that the hydrophobic interaction between the ligand and the protein was enhanced. Moreover, the carboxyl group can dissociate into COO^-, which can interact with Lys through electrostatic interaction. Additionally, the number of hydroxyl groups increased

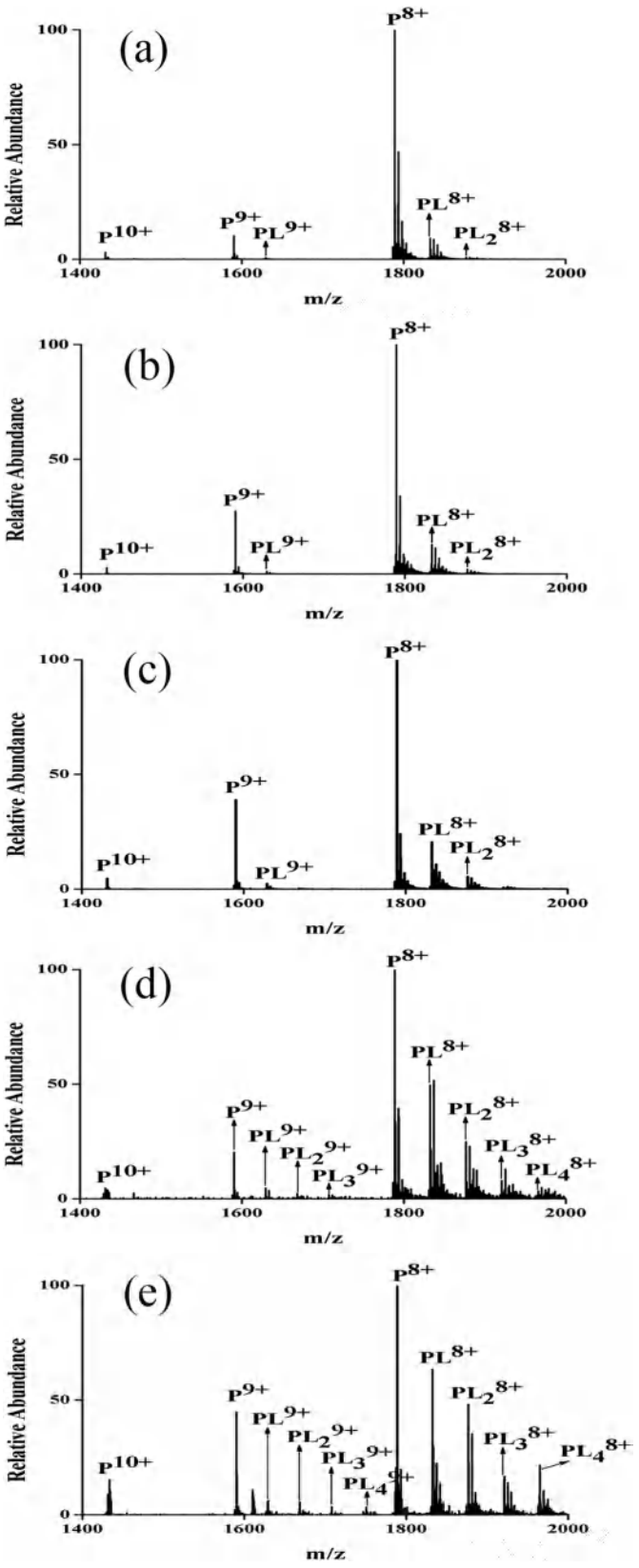

Fig. 2. CSI-MS spectra of the complexes of Lys with CQA at different concentration ratio: (a) 2:1, (b) 1:1, (c) 1:2, (d) 1:5, (e) 1:10.

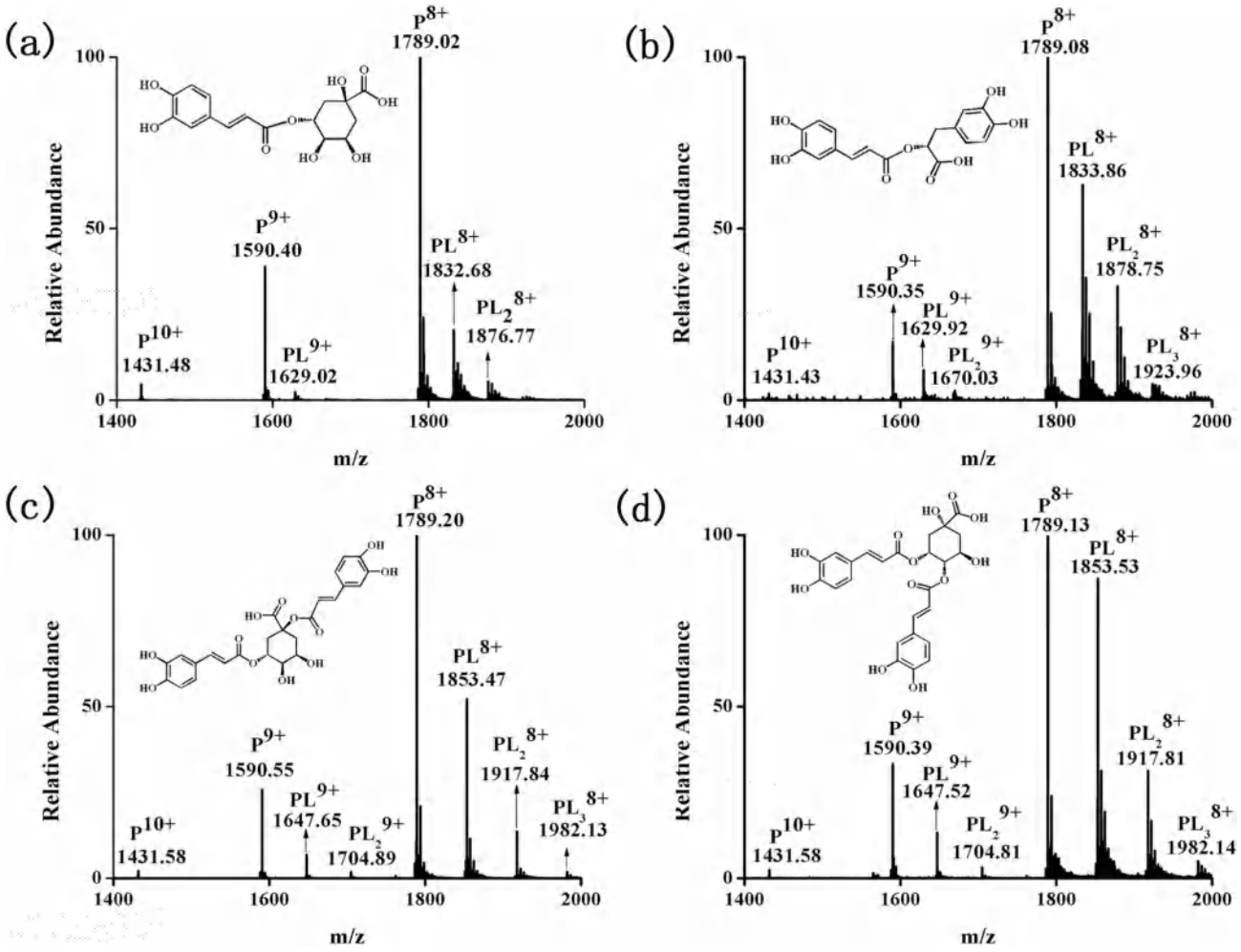

Fig. 3. CSI-MS spectra of the complexes of Lys with (a) CQA, (b) RA, (c) 1,3-CQA, and (d) 4,5-CQA at a 1:2 protein/ligand molar ratio.

after ester formation, and the hydrogen bonds may have a certain contribution. The results of the ITC thermodynamic experiments (Fig. S8) showed positive ΔH andΔS values, implying that the binding type between phenolic acid and Lys was mainly a hydrophobic interaction [58], which confirmed the MS results.

3.3. Competition experiment and determination of binding constants using CSI-MS

We investigated the relative binding affinity of the phenolic acids screened above, which interacted with Lys, by direct and indirect MS methods. Among them, the direct competitive experiment is an intuitive and simple method, which has been widely used successfully [59]. It involves the mixing of two or more ligands with the same protein in equal proportions, and the binding strength is reflected according to the peak intensity of each complex. Fig. 4 displays the mass spectra of the four phenolic acids mixed each other with Lys. The results of the relative binding affinities are as follows: 1,3-CQA > CQA (Fig. 4a), RA > CQA (Fig. 4b), RA > 1,3-CQA (Fig. 4c), 4,5-CQA > RA (Fig. 4d). Therefore, we can summarize the order of the total binding affinities as 4,5-CQA > RA > 1,3-CQA > CQA. The results suggested that the caffeic acyl and ester parts were the reactive groups and the number and location of the caffeic acyl group have great effects on the affinity. Further, the binding force was enhanced as the number of caffeic acyl groups increased. However, we speculated that due to the positions of the two side chains of 1, 3-CQA, which are far from each other in space, it is difficult for it to bind to the active sites of Lys; consequently, it has a lower affinity than RA.

Next, we used the MS titration assay to determine the dissociation constant (K_d), based on the quantification of the relative abundance (Ab) of protein-ligand complex (PL^{n+}) and unbound protein ions (P^{n+}) in the mass spectra [60]. So far, it is controversial that the titration assay does not precisely reflect the hydrophobic-based protein-ligand interaction for the argument that the dehydrated complexes are less stable in the gas phase than in solutions [61]. However, recent kinetic data have confirmed that the hydrophobic interaction is more stable in the gas phase under mild experimental conditions or by adding stabilizers, so that the meaningful K_d values of the complexes can be obtained [62,63]. In this study, a milder CSI-MS method was adopted to effectively avoid in-source dissociation and disaggregation of the protein-ligand complex. Moreover, the formation of non-specific binding was minimized by appropriate experimental conditions. The applied field of 3 kV did not influence the complex formation and the evaluated K_d, because Lys has no characteristic ions over the *m/z* range of 1000–2000 in the negative ion mode (Fig. S9) [49]. Zenobi et al. [64] has reported the analysis of model supramolecular complexes with a purely hydrophobic association in solution using CSI-MS. The following equations (4)–(7) were introduced to calculate the K_d and evaluate the binding affinity [51]. Since the 1:3 complexes are very low in abundance compared to the 1:1 and 1:2 complexes, we only calculated the K_d values of the 1:1 and 1:2 complexes. The calculated K_d and their relative standard deviations (RSD) are displayed in Table 1.

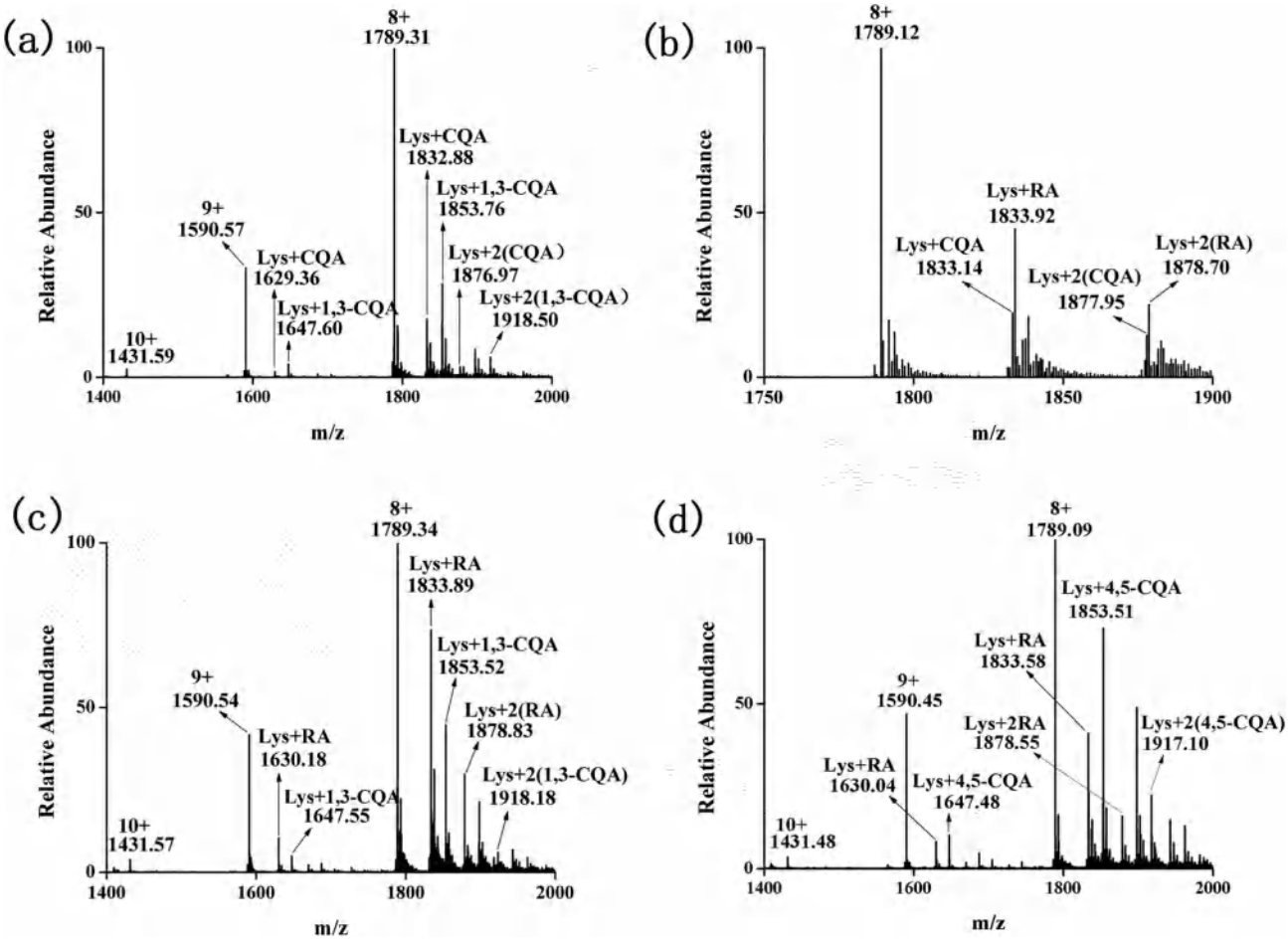

Fig. 4. The mass spectra for competition experiments of Lys binding with two ligands at a 1:2:2 ratio: (a) CQA + 1,3-CQA, (b) RA + CQA, (c) RA + 1,3-CQA and (d) RA + 4,5-CQA.

Table 1
K_{d1} and K_{d2} values of the noncovalent complexes formed between Lys and CQA/RA/1,3-CQA/4,5-CQA.

Complexes	$K_{d1} \times 10^{-5}$ (mol/L)	RSD (n = 6)	$K_{d2} \times 10^{-9}$ (mol/L)^2	RSD (n = 6)
Lys + 4,5-CQA	5.976	0.033	8.795	0.170
Lys + RA	6.253	0.024	9.143	0.075
Lys + 1,3-CQA	16.287	0.016	43.212	0.198
Lys + CQA	38.267	0.037	84.121	0.129

$$\frac{[PL_1]_{eq}}{[P]_{eq}} = \frac{\sum_n Ab(PL_1^{n+})}{\sum_n Ab(P^{n+})} = R_1 \quad (4)$$

$$\frac{[PL_2]_{eq}}{[P]_{eq}} = \frac{\sum_n Ab(PL_2^{n+})}{\sum_n Ab(P^{n+})} = R_2 \quad (5)$$

$$K_{d1} = \frac{1}{R_1} \cdot \left[L_0 - P_0 \cdot \frac{R_1 + 2R_2}{1 + R_1 + R_2} \right] \quad (6)$$

$$K_{d2} = \frac{1}{R_2} \cdot \left[L_0 - P_0 \cdot \frac{R_1 + 2R_2}{1 + R_1 + R_2} \right]^2 \quad (7)$$

where $Ab(P^{n+})$ $Ab(PL_1^{n+})$, and $Ab(PL_2^{n+})$ denote the abundance of protein, protein bound with one and two ligands in the +n charge states, respectively. P_0 and L_0 represent the initial concentrations of the protein and ligand.

As shown in Table 1, the orders of K_{d1} and K_{d2} for the complexes are all CQA > 1,3-CQA > RA > 4, 5-CQA, and the RSD of K_{d1} is lower than K_{d2}. This may because the 1:1 complexes are more stable than the 1:2 complexes and hydrophobic interaction plays a major role in the gas phase. Since a lower K_d value indicates stronger binding affinity, the order of the relative binding affinity obtained by calculation is consistent with the above competition experiment.

3.4. Collision induced dissociation (CID) experiments by CSI-MS

MS/MS is a technique that can provide detailed structural information and the dissociation pathways of a target analyte. It can provide further insights into how ligands binding to the target protein and the strength of the affinity [65]. In order to further confirm the structural composition of the noncovalent complexes formed by phenolic acid and Lys and to evaluate their stability, CID experiments were conducted. Firstly, the 1:1 (*m/z* 1832.46) and 1:2 (*m/z* 1877.38) complexes formed by CQA and Lys were selected as precursor ions. It was found that when the CID energy was 30%, the 1:1 complex lost one CQA molecule and produced Lys in the +8 charge state (*m/z*1788.69) (Fig. 5a). Similarly, the 1:2 complex initially lost one CQA molecule to produce a 1:1 complex (*m/z*1832.50), and then it lost another CQA molecule to generate Lys (*m/z*1789.24) (Fig. 5b). Further, as the CID energy was increased to 40%, the parent complex ions disappeared and only the Lys ion existed, finally. A similar result was reported by Oldham's group using ESI-MS. The signal of the complex decreased until it disappeared as the collision energy increased, while the P^{8+} ions of Lys increased gradually [66]. The results showed that the complexes were stable in the gas phase and confirmed that the neutral loss of drug molecule was the main dissociation pathway in the CID mode.

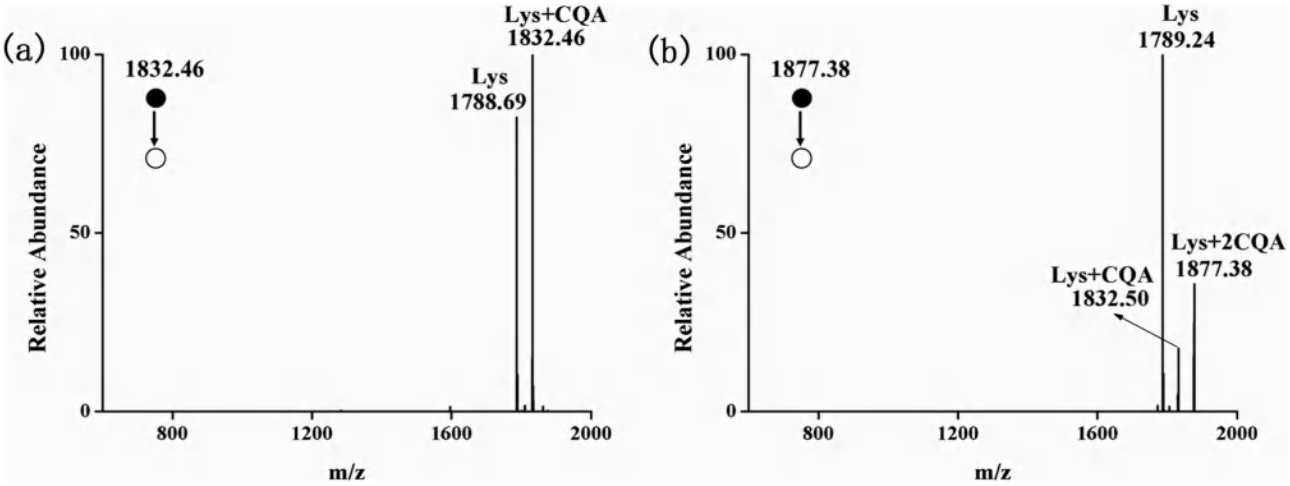

Fig. 5. Collision induced dissociation (CID) mass spectra of the 1:1 complex (a) and 1:2 complex (b) formed by Lys and CQA at a 1:2 molr ratio when CID energy is 30%.

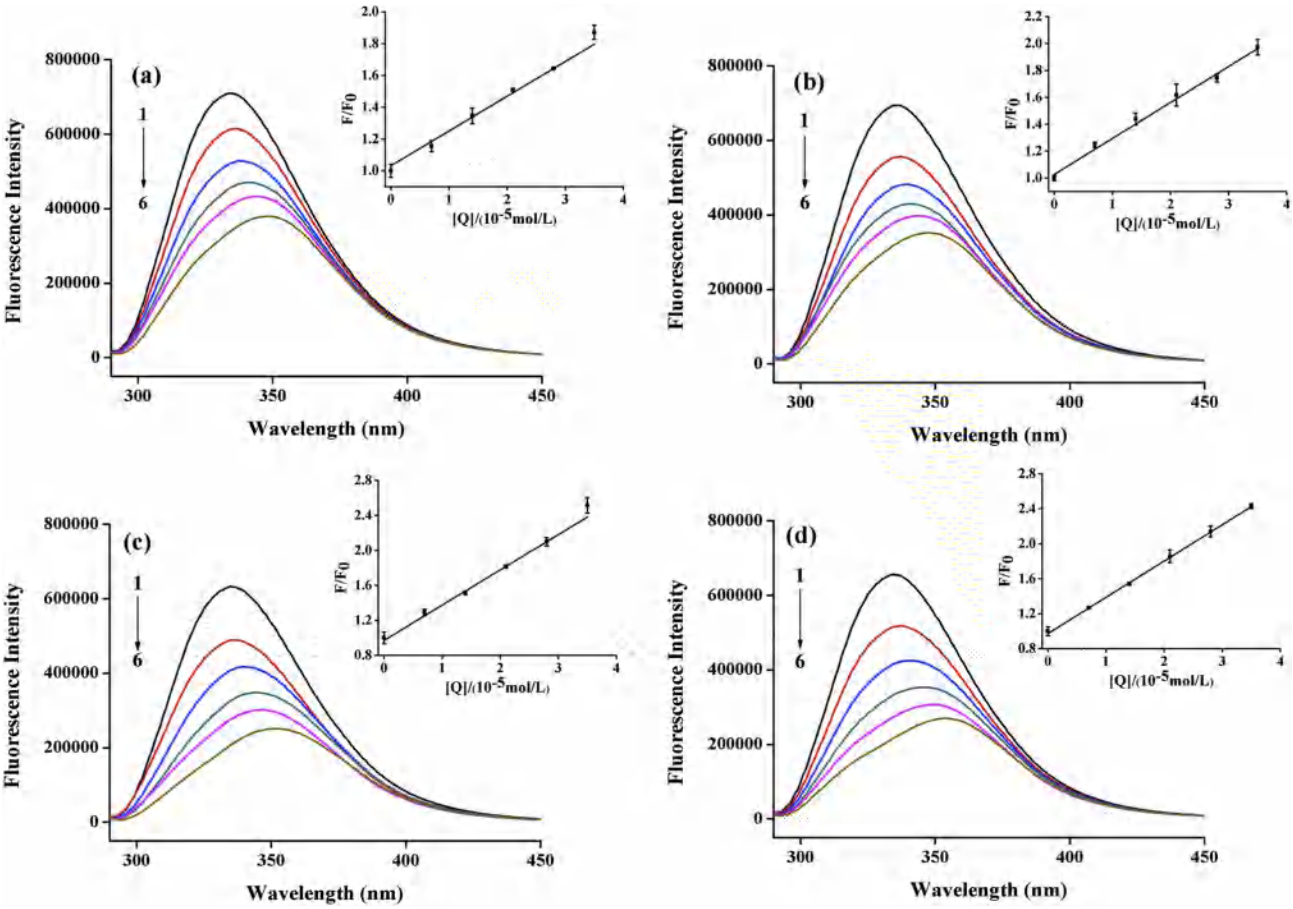

Fig. 6. Fluorescence spectra of Lys in the presence of different concentrations of phenolic acid and the inserted Stern-Volmer plots in 5 mM ammonium acetate buffer, pH 6.9: (a) CQA, (b) RA, (c) 1,3-CQA, and (d) 4,5-CQA. $C_{Lys} = 3.5 \times 10^{-5}$ mol L^{-1}; $C_{phenolic\ acid}$ ($\times 10^{-5}$ mol L^{-1}) (1–6): 0; 0.7; 1.4; 2.1; 2.8; 3.5.

Furthermore, the results confirmed that the composition of the Lys-CQA complexes were 1:1 and 1:2. The other phenolic acids obtained the consistent results (Figs. S10–S12).

3.5. Characterization of the interaction between phenolic acid and Lys by fluorescence spectroscopy

Fluorescence spectroscopy is a simple and sensitive approach for studying the protein-ligand interaction. It can provide information about the fluorescence quenching mechanism and the conformational changes of natural protein in solution, which can be a support for MS research. As shown in Fig. 6, native Lys, which had a strong emission peak at 334 nm after being excited at 280 nm, is mainly attributed to its W62, W63 and W108 residues [55]. The fluorescence signal of Lys decreased with the addition of phenolic acid, indicating that phenolic acid was bound to Lys and quench its intrinsic fluorescence. In addition, as the ligand concentration increased, the emission peak of Lys shown a red shift (from 334 to 348, 348, 352, and 353 nm for CQA, RA, 1,3-CQA

Table 2
The binding parameters for the interaction of Lys with CQA/RA/1,3-CQA/4,5-CQA.

Phenolic acid	Eqs	K_{sv} (10^4, M^{-1})	K_q (10^{12}, $M^{-1}S^{-1}$)	R^2
CQA	$F_0/F = 2.44 \times 10^4 [Q] + 0.99$	2.44 ± 0.11	2.44	0.9962
RA	$F_0/F = 2.67 \times 10^4 [Q] + 1.04$	2.67 ± 0.04	2.67	0.9925
1,3-CQA	$F_0/F = 4.20 \times 10^4 [Q] + 0.97$	4.20 ± 0.16	4.20	0.9917
4,5-CQA	$F_0/F = 4.12 \times 10^4 [Q] + 0.98$	4.12 ± 0.08	4.12	0.9995

and 4,5-CQA, respectively). Moreover, considering that phenolic acids have no absorbance at λ_{max} (334 nm) of Lys, the influence of ligands can be considered negligible (Fig. S13). The results showed that the interaction between phenolic acid and Lys altered the microenvironment where W62 is located. The hydrophobicity of tryptophan near the binding site increased and the structure of protein underwent noticeable changes that were further verified by CD and FIIR spectroscopy. Furthermore, the Stern-Volmer equation was used to calculate the K_q and to explore the quenching mechanism. The Stern-Volmer plots are given in Fig. 6 and the calculated parameters are summarized in Table 2. The results showed that the K_q values were two orders of magnitude larger than the limiting diffusion rate constant (2×10^{10} L mol^{-1} s^{-1}) [56] suggesting that the quenching mechanism was mainly a static quenching process due to the complex formation rather than a dynamic collision process, which supported the MS results. The plot of $\log[(F_0–F)/F]$ vs $\log[Q]$ is shown in Fig. S14. The values of K_d determined by CSI-MS and fluorescence spectroscopy are compared and listed in Table S2. It is shown that the K_d values of the Lys-phenolic acid complexes were estimated to be 10^{-4}–10^{-5} mol/L [34,35]. The phenomenon of the deviations in the determination of K_d by MS and other methods has also been observed by other groups [67,68]. The order of the K_d values determined by fluorescence spectroscopy is consistent with the results obtained by CSI-MS, which further confirmed the order of the binding affinity.

3.6. Conformational investigation by CD spectroscopy

CD spectroscopy can be employed to rapidly and sensitively study the protein secondary structure in solution, and it has been widely used to monitor the conformational alteration of protein induced by its interaction with biologically active molecules. It is reported that the specific binding of small molecules with Lys often leads to the increase or decrease of its α-helix content [32,57]. This indicates that small molecules mainly affect the stability of α-helix in the secondary structure of Lys. The far-UV CD spectra of Lys in the absence and presence of phenolic acid (1:0, 1:1 and 1:2) are shown in Fig. 7. There are two negative peaks at 208 nm and 222 nm, which resulted from n–π* and π–π* transition respectively. They represent the α-helical feature of Lys. Following the binding with phenolic acids, the peak intensity increased without any significant shift of the peaks. The alterations of the secondary structures of Lys, e.g., α-helix, β-sheet, β-turn, and random coil, were analyzed and the results are summarized in Table 3. It was found that the gradual increase in the concentration of CQA, RA, 1, 3-CQA, and 4, 5-CQA resulted in an increase in the α-helix content of Lys from 27.9% to 28.7%, 30.1%, 31.0% and 28.7% respectively. From the above results, we can conclude that the complex formation between phenolic acids and Lys resulted in the alteration of the secondary structure of the protein, as well as an increase in the stability of α-helix. And this led to the changes in microenvironment of the protein with the exposure of hydrophobic amino acid residues, which ultimately affected the physiological function of Lys. A similar result in naringin and naringenin binding with Lys was reported recently [55].

3.7. FTIR characterization of the Lys-phenolic acid system

The alteration of the protein conformation before and after interacting with phenolic acids has been investigated by FTIR spectroscopy. Protein has many characteristic absorption bands in the infrared region, mainly including the amide I band (1700-1600 cm^{-1}) and amide II band (1575-1480 cm^{-1}). The amide I band is due to the stretching vibration of the C═O bond, which is more sensitive to conformational changes and is usually used to reflect the secondary structure alteration of protein. The amide II band is owing to the C–N stretching and N–H bending modes [56]. When the environment of the protein is altered, such as binding with a bioactive molecule, the band intensity and position will change. From this, we can infer the binding site and the protein conformational change. As shown in Fig. 8, the 1539 and 1651 cm^{-1} bands can be attributed to amide I and II of Lys. We found that the position of the amide I band shifted to 1659, 1657, 1657, and 1659 cm^{-1}, and that of the amide II band shifted to 1535, 1532, 1532, and 1535 cm^{-1} with the addition of phenolic acids, respectively. There was also a slight change in peak intensities. These results suggested that phenolic acids can bind with the C═O and C–N groups of the protein and lead to the rearrangement of the polypeptide carbonyl hydrogen bonding network, which can further elucidate the conformational change due to the formation of the complexes.

3.8. Molecular docking results

To substantiate the experimental results and reveal the probable binding locations of the four ligands within Lys, molecular docking studies were performed. Gu et al. [33] reported that three ligands are all located at the deep cleft of Lys in their work and we obtained the same results (Fig. S15). The docking complexes revealed that residues W62, W63, R73, I98, V99, D101, G102, N103, G104 and W108 from Lys were involved in the interaction with these phenolic acid ligands. In the docking complexes, the aromatic rings from these four ligands showed different extents of hydrophobic interaction with the nonpolar residues, such as W, V, and I around the cleft, suggesting that hydrophobic force plays a major role during the complex formation, which is supported by MS and ITC results. For 1,3-CQA, the aromatic ring of the ligand has π–π stacking interactions with W62, as shown in Fig. 9 (c). For CQA and 4,5-CQA, there are π–π stacking interactions between the aromatic rings of the ligand and W108, as shown in Fig. 9 (a) and (d). The participation of the W residues in the binding sites further demonstrated the observed fluorescence quenching and the conformational changes of Lys. Besides the hydrophobic interactions, there are also hydrogen bonds formed between the hydroxyl groups of the phenolic acid ligands and Lys. The red dashed lines in Fig. 9 showed the hydrogen bonding of the residues (I98, D101) from Lys with the four ligands. In addition, the docking shows that there is approximately one binding site for the ligands binding to Lys via electrostatic forces. The electrostatic interaction between the carboxyl anions of phenolic acids and the positively charged residues of Lys also facilitates their binding. The ligands were identified to bind to residue R73 via electrostatic interactions, as shown in Fig. 9. These computational findings correlated closely with the experimental results.

4. Conclusions

This work reported a strategy based on CSI-MS combined with multi-spectroscopic and molecular docking methods to study the

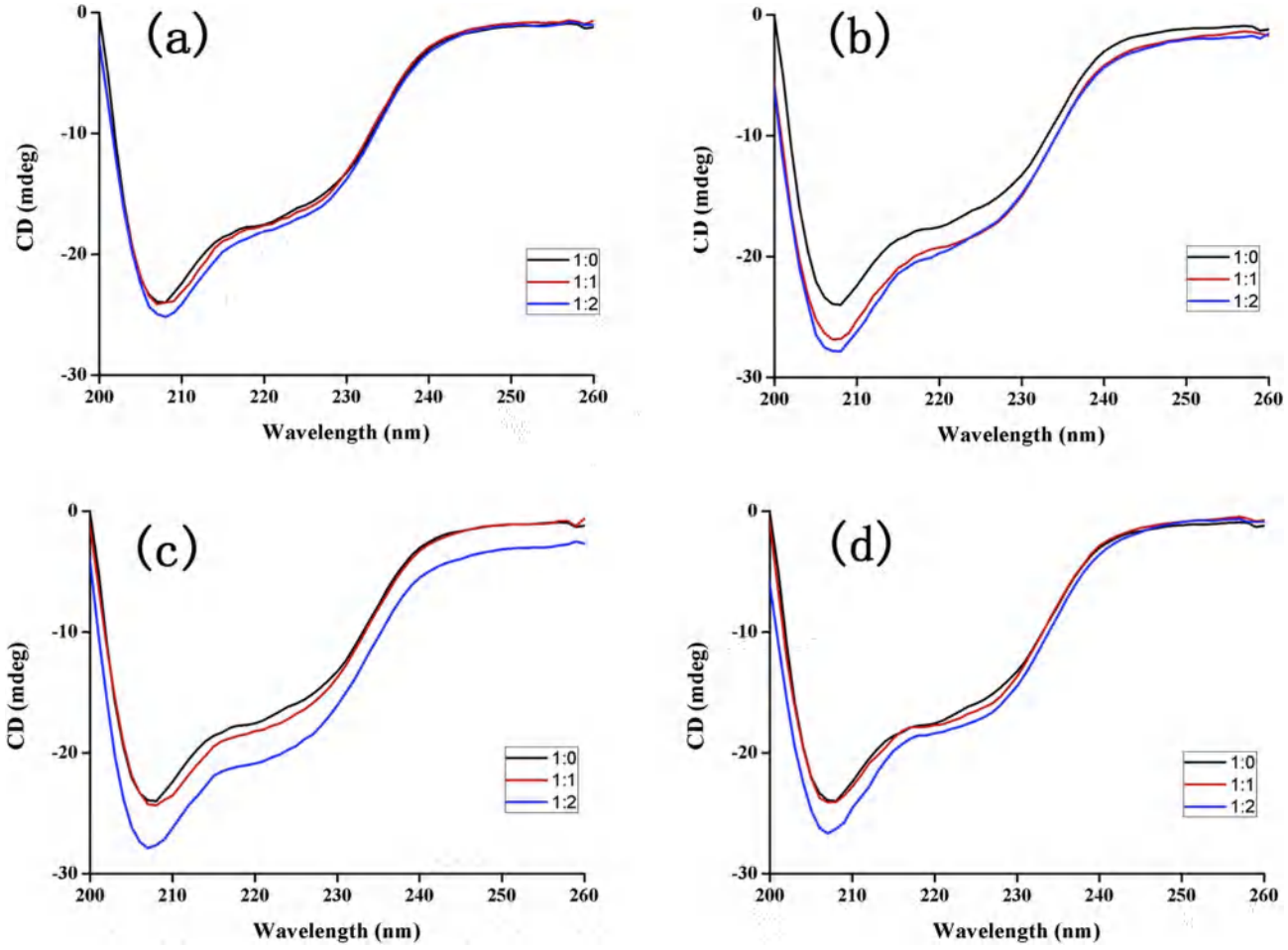

Fig. 7. Far-UV CD spectra of Lys in the absence and presence of (a) CQA, (b) RA, (c) 1,3-CQA, and (d) 4,5-CQA in 5 mM ammonium acetate of pH 6.9.

Table 3
Effects of phenolic acid on the percentage of secondary structure in Lys.

Complex	Secondary structure contents of Lys, %			
	α-Helix	β-Sheet	β-Turn	Unordered
Native Lys	27.9	20.6	18.2	34.6
Lys + CQA (1:1)	27.9	20.6	18.2	34.4
Lys + CQA (1:2)	28.7	20.0	18.0	33.7
Lys + RA (1:1)	29.5	19.4	17.9	32.6
Lys + RA (1:2)	30.1	19.0	17.8	31.9
Lys + 1,3-CQA (1:1)	28.5	20.2	18.0	34.1
Lys + 1,3-CQA (1:2)	31.0	18.4	17.6	31.6
Lys + 4,5-CQA (1:1)	28.0	20.5	18.1	34.4
Lys + 4,5-CQA (1:2)	28.7	20.0	18.1	33.2

noncovalent binding mechanisms of phenolic acid with Lys. The intact noncovalent complexes were preserved and characterized using a mild CSI-MS technique, and the stoichiometries, binding constants, structure-affinity relationships and other binding information were obtained. The results showed that the ester group and the number and location of the caffeic acyl group have a great influence on the affinity. The MS/MS experiments revealed the structural composition and gas phase stability of the complex. The competition experiment and MS titration assay all showed that the relative binding affinity was in the order of: 4,5-CQA > RA > 1,3-CQA > CQA. The results of fluorescence demonstrated the formation of ground-state phenolic acid-Lys complexes, and the quenching mechanism was static in nature. The CD and FTIR spectra indicated that the addition of phenolic acid would change the secondary structure and microenvironment of Lys.

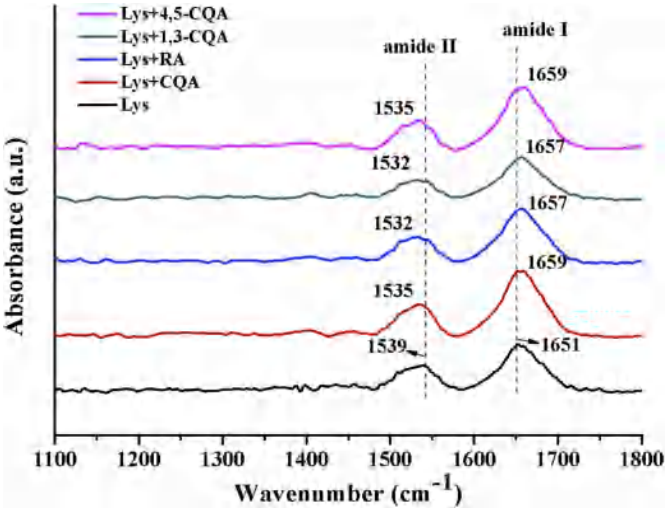

Fig. 8. FTIR spectra of Lys and Lys-CQA/RA/1,3-CQA/4,5-CQA complexes at the molar ratio of 1:2 in the region 1800–1100 cm^{-1} in 5 mM ammonium acetate buffer at pH 6.9.

Molecular docking results indicated that phenolic acids can bind with tryptophan amino acid residues of Lys and hydrophobic force plays a major role in stabilizing the complex. The information obtained in this study could provide a better understanding of the bioactivities of phenolic acids. The developed strategy could be applied to study the noncovalent interactions between natural products and other proteins.

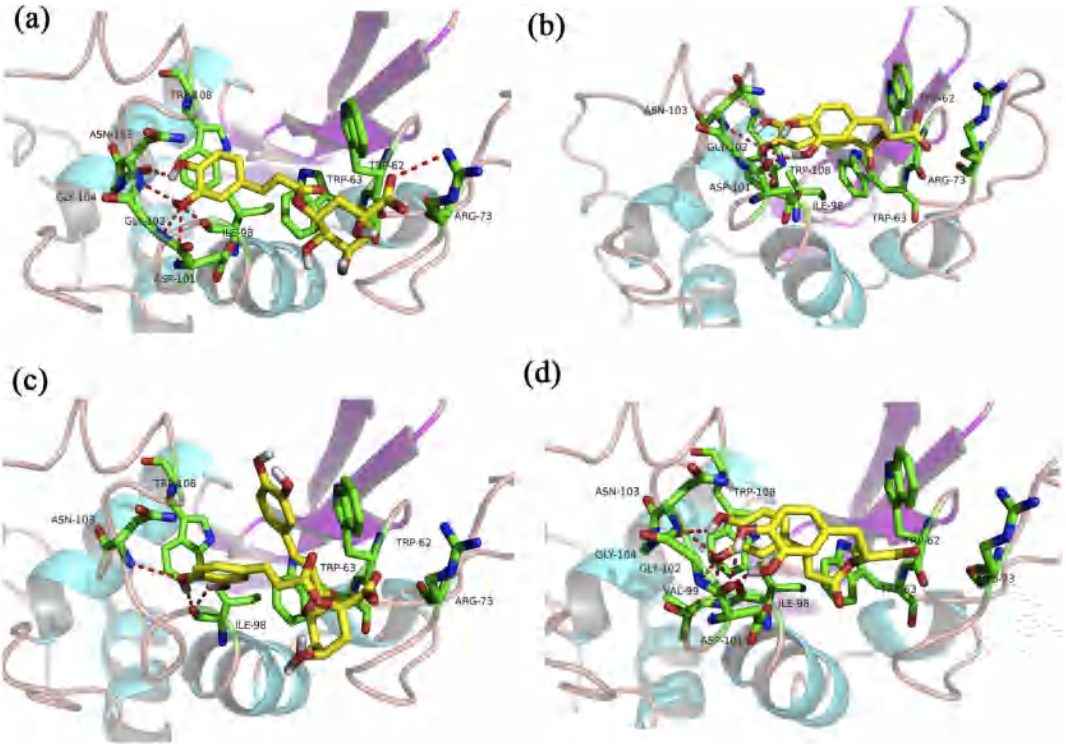

Fig. 9. The docked pose of (a), (b), (c) and (d) represent the participation of amino acid residues in the interactions with CQA, RA, 1,3-CQA and 4,5-CQA respectively. Trp:W, Arg:R, Ile:I, Val:V, Asp:D, Gly:G, Asn: N.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.120762.

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