Probing the binding mechanisms of α -tocopherol to trypsin and pepsin using isothermal titration calorimetry, spectroscopic, and molecular modeling methods

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Abstract α -Tocopherol is a required nutrient for a variety of biological functions. In this study, the binding of α -tocopherol to trypsin and pepsin was investigated using isothermal titration calorimetry (ITC), steady-state and time-resolved fluorescence measurements, circular dichroism (CD) spectroscopy, and molecular modeling methods. Thermodynamic investigations reveal that α -tocopherol binds to trypsin/pepsin is synergistically driven by enthalpy and entropy. The fluorescence experimental results indicate that α -tocopherol can quench the fluorescence of trypsin/pepsin through a static quenching mechanism. The binding ability of α -tocopherol with trypsin/pepsin is in the intermediate range, and one molecule of α -tocopherol combines with one molecule of trypsin/pepsin. As shown by circular dichroism (CD) spectroscopy, α -tocopherol may induce conformational changes of trypsin/pepsin. Molecular modeling displays the specific binding site and gives information about binding forces and α -tocopherol-tryptophan (Trp)/tyrosine (Tyr) distances. In addition, the inhibition rate of α -tocopherol on trypsin and pepsin was studied. The study provides a basic data set for clarifying the binding mechanisms of α -tocopherol with trypsin and pepsin and is helpful for understanding its biological activity in vivo.

Keywords Trypsin · Pepsin · α -Tocopherol · Isothermal titration calorimetry · Spectroscopy · Molecular modeling

1 Introduction

α -Tocopherol, the most biologically active form of vitamin E, is the major lipid-soluble radical-trapping antioxidant in human plasma and circulating lipoproteins [1]. The effective

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radical trapping ability of α -tocopherol is thought to underlie the strict biological requirement for this nutrient and its protective actions against a number of human pathologies such as mitigating cardiovascular disease [2], and slowing Alzheimer's disease progression [3]. α -Tocopherol has also attracted attention due to its non-antioxidant functions, in particular its emerging role as a cellular signaling molecule, including modulation of the activity of protein kinase C and phosphatidylinositol 3-kinase as well as regulation of a number of genes [4, 5]. Binding studies of α -tocopherol were carried out recently [6, 7]. However, an accurate and full basic data set, including thermodynamic parameters, binding parameters, the conformation changes, and the inhibition rate of proteases induced by α -tocopherol, has not yet been obtained. Proteases play an important role in many biologically relevant processes and are some attractive targets in therapy and pharmaceutical research [8].

Trypsin (EC 3.4.21.4) is secreted by the pancreas and converted from inactive trypsinogen [9]. It is the most abundant protease in nature and plays an essential role in digestion and deconstruction of food proteins and other physiological processes including hemostasis, apoptosis, signal transduction, reproduction, and immune response [10]. Trypsin belongs to the chymotrypsin superfamily of serine endopeptidases that are characterized by the catalytic triad His-57, Asp-102, and Ser-195 and cleaves peptide bonds only at the carboxylic groups of arginine and lysine [11]. The protein consists of 223 amino acid residues with a molecular weight of 23,300 Da. The individual chains are held together by six disulfide bridges. It is composed of two domains of nearly equal size, the major constituent of each domain being a set of six anti-parallel strands of polypeptide chain tied together into a β -sheet unit by a network of H-bonds [12]. Pepsin (EC 3.4.23.1) is an enzyme expressed as a prototype of pepsinogen and is released by the chief cells in the stomach to degrade food proteins into peptides [13]. It is an endopeptidase that catalyzes hydrolytic cleavage of peptic bonds in the vicinity of hydrophobic or aromatic amino acid residues [14]. Pepsin has been widely used in different processes such as food and pharmaceutical manufacture [15]. It has a single polypeptidic enzymatic chain of 326 amino acid residues and a molecular mass of 35,000 Da.

Knowledge of interaction mechanisms between α -tocopherol and trypsin/pepsin is very important. First, the binding study of α -tocopherol with trypsin/pepsin can provide useful information for further study of pharmacokinetic and pharmacodynamic behavior of α -tocopherol [16]. The parameters, such as the mode of interaction, the binding constants, and the number of binding sites may provide some important theoretic information on the metabolism and distribution of α -tocopherol in vertebrates (including human beings) [17]. Second, α -tocopherol is considered to be a safe natural product. However, it may act as an antinutritional factor, in terms of the inhibition of proteases, when ingested in excess. To minimize the antinutritional effects and make full use of α -tocopherol, knowledge of the interaction between α -tocopherol and trypsin/pepsin is desirable [18]. Third, the conformational changes of trypsin/pepsin induced by interaction with α -tocopherol may reduce the activity of these proteins. Trypsin/pepsin are responsible for the hydrolysis of proteins into peptides and amino acids, the reduction in the activity of trypsin/pepsin may be harmful since it can contribute to a reduced absorption of nutrients [19]. In a word, the binding study of α -tocopherol with trypsin/pepsin is important in the field of pharmacology and biochemistry and is helpful for understanding its effect on trypsin/pepsin function and its

biological activity in vivo, and the clear and quantitative information on the nature of α -tocopherol and trypsin/pepsin interaction should provide a firm basis for its rational use in clinical practice.

2 Experimental

2.1 Materials

Trypsin from porcine pancreas, pepsin from porcine gastric mucosa, α -tocopherol, and casein were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and used without further purification. The trypsin solution (1×10^{-4} mol Γ^{-1}) was prepared in pH 7.4 phosphate buffer (0.01 mol Γ^{-1} PBS), which is to provide physiological conditions for trypsin [20]. A disodium hydrogen phosphate-citric acid buffer was used to keep the pH value at 2.0 for pepsin solution (1×10^{-4} mol Γ^{-1}), which provides physiological condition for pepsin [21]. α -Tocopherol was diluted with 99.5% (v/v) ethanol before use. The stock solutions of α -tocopherol were prepared and used immediately because of oxidation under light and air. Double-distilled water was used to prepare solutions. pH was determined using a pHs-2C pH-meter (Shanghai DaPu Instruments Co., Ltd, Shanghai, China) at ambient temperature. Sample masses were weighed accurately on a microbalance (Sartorius, BP211D) with a resolution of 0.01 mg. All other reagents were of analytical reagent grade and were used as purchased without further purification.

2.2 Isothermal titration calorimetry (ITC)

Titration of trypsin/pepsin with α -tocopherol was performed using a Model Nano-ITC 2G biocalorimetry instrument (TA instruments, USA) at 298 K. All these solutions were thoroughly degassed prior to the titrations to avoid the formation of bubbles in the calorimeter cell. The sample cell was loaded with the buffer or trypsin/pepsin solution and the reference cell contained double-distilled water. In a typical experiment, buffered trypsin/pepsin solution was placed in the 950- μ l sample cell of the calorimeter and α -tocopherol solution was loaded into the injection syringe. Injections were started after baseline stability had been achieved. α -Tocopherol was injected into the sample cell by means of syringes via 25 individual injections; the amount of each injection was 10 μ l. The first injection of 10 μ l was ignored in the final data analysis. The contents of the sample cell were stirred throughout the experiment at 200 rpm to ensure thorough mixing. Raw data were obtained as a plot of heat (μ J) against injection number and featured a series of peaks for each injection. These raw data peaks were transformed using the instrument's software to obtain a plot of enthalpy change per mole of injectant against molar ratio. Control experiments included the injection of α -tocopherol solution into buffer, buffer into trypsin/pepsin, and buffer into buffer, controls were repeated for the same trypsin/pepsin concentration used. The last two controls resulted in small and equal enthalpy changes for each successive injection of buffer and therefore were not further considered in the data analysis [22]. Corrected data refer to experimental data after subtraction of the α -tocopherol into buffer control data.

Estimated binding parameters were obtained from ITC data using NanoAnalyze software provided by the manufacturer. Fitting the data according to the independent binding model resulted in the stoichiometry of binding (n), the equilibrium binding constant (K_a^θ), and

enthalpy of complex formation ($\Delta_r H_m^\theta$). The standard changes in free energy ($\Delta_r G_m^\theta$) and entropy ($\Delta_r S_m^\theta$) are calculated using the following equations:

$$\Delta_r G_m^\theta = -RT \ln K_a^\theta \quad (1)$$

$$\Delta_r G_m^\theta = \Delta_r H_m^\theta - T \Delta_r S_m^\theta \quad (2)$$

2.3 Steady-state fluorescence measurements

The steady-state fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian, USA) equipped with a 1.0-cm quartz cell holder and a thermostat bath. A certain volume of the stock solution of trypsin/pepsin and various volumes of the stock solution of α -tocopherol solution were transferred to 0.01-liter volumetric flasks. The mixture was diluted to the experimental concentrations with phosphate buffer solution of pH 7.4 or disodium hydrogen phosphate-citric acid buffer of pH 2.0 as the working solutions. The trypsin concentration was kept at $1 \times 10^{-5} \text{ mol l}^{-1}$. The pepsin concentration was kept at $2 \times 10^{-5} \text{ mol l}^{-1}$. The excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was set at 280 nm (both Trp and Tyr are excited), and the emission spectra were read at 300–450 nm at a scan rate of 100 nm min^{-1} . Triplicate samples were measured.

The fluorescence measurements are hindered by the inner-filter effect, which is that small ligands absorb light at the excitation and emission wavelengths of proteins, leading to unreliable results [23]. Thus, it is very important to subtract such an effect from the raw quenching data. The extent of this effect can be roughly estimated with the following equation [24]:

$$F_{\text{cor}} = F_{\text{obsd}} 10^{(A_{\text{ex}} + A_{\text{em}})/2} \quad (3)$$

where F_{cor} and F_{obsd} are the corrected and observed fluorescence intensities, respectively, whereas A_{ex} and A_{em} are the sum of the absorbance of protein and ligand at the excitation and emission wavelengths, respectively. The fluorescence intensity utilized in this study is the corrected intensity.

2.4 Time-resolved fluorescence measurements

The fluorescence lifetimes of trypsin and pepsin in the absence and presence of α -tocopherol were measured on the FLS 980 spectrometer (Edinburgh Instruments Ltd., England). The excitation of the sample was carried out by a nano-second diode laser as a light source. The fluorescence decay was analyzed using the software provided with the FLS 980 spectrometer. The trypsin/pepsin and α -tocopherol concentrations were both kept at $1.0 \times 10^{-4} \text{ mol l}^{-1}$.

2.5 Circular dichroism (CD) measurements

The CD measurements were carried out on a Chirascan spectropolarimeter (Applied Photophysics Ltd., England) under constant nitrogen flush. The CD spectra of trypsin/pepsin

solution in absence and presence of α -tocopherol were recorded from 200 to 260 nm. During the experiment, a quartz cell holder with a path length of 0.2 cm was used. The bandwidth and the scan speed were set at 1.0 nm and 20 nm min⁻¹, respectively. The sample temperature was maintained at 298 K. The trypsin/pepsin concentration was fixed to 1.0×10^{-5} mol l⁻¹ and the α -tocopherol concentration used was 1.0×10^{-4} mol l⁻¹. Corresponding blanks (without trypsin/pepsin) were recorded and subtracted from the sample spectra, and results were taken as CD ellipticity in mdeg.

2.6 Trypsin/pepsin activity assay

Trypsin/pepsin activity was determined by UV–Vis absorption spectroscopy with casein used as a substrate as described [25], and recorded with a TU-1810 spectrophotometer (Puxi Analytic Instrument Ltd., Beijing, China). In brief, the working solution of trypsin was prepared with pH 7.4 phosphate buffer (0.01 mol l⁻¹ PBS), and the working solution of pepsin was prepared with a disodium hydrogen phosphate-citric acid buffer. Trypsin/pepsin activity assay involved a reaction medium containing 4.0×10^{-5} mol l⁻¹ trypsin/pepsin solution and 10 mg casein in the presence and absence of α -tocopherol. α -Tocopherol solution was added to the reaction mixtures just before the reaction (α -tocopherol:trypsin/pepsin at a mass ratio of 0:1, 0.1:1, 0.2:1, 0.4:1, 0.6:1, 0.8:1, 1:1 and 1.2:1). An amount of 5 ml 0.4 mol l⁻¹ Na₂CO₃ and 1 ml folin-phenol reagent were added to the reaction mixture and colored for 20 min at 313 K. The hydrolysis of casein to tyrosine was monitored at 680 nm. Control trypsin/pepsin activity was determined without α -tocopherol. All samples were analyzed in triplicate. The inhibition rate (%) of trypsin/pepsin activity was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{A-a}{A} \times 100 \quad (4)$$

where A and a were the absorbance of the control group and the experimental group, respectively.

2.7 Molecular modeling studies

Calculations were carried out using AutoDock Vina (The Scripps Research Institute, La Jolla, CA, USA). The three-dimensional structure of α -tocopherol was generated and was optimized at DFT/B3LYP/6-311G by Gaussian 03. The native structures of trypsin (PDB ID 2ZQ1) and pepsin (PDB ID 3PEP) were taken from the Protein Data Bank. All water molecules were removed and the polar hydrogen and the Gasteiger charges were added at the beginning of the modeling study. To recognize the binding sites in trypsin/pepsin, docking was carried out with setting of grid box size 40 Å × 40 Å × 40 Å. The grid centers of the α -tocopherol-trypsin system and α -tocopherol-pepsin system were set at -24.562, -0.483, 17.216 Å and 1.957, 15.092, -24.599 Å, respectively. The geometries of α -tocopherol were subsequently optimized using the Tripos force field with Gasteiger–Hückel charges, and non-polar hydrogen was then merged. FlexX program was applied to calculate the possible conformation of α -tocopherol that binds to trypsin/pepsin. During the modeling process, a maximum of ten conformers were considered for α -tocopherol. The conformer with the lowest binding free energy was used for further analysis. As is addressed above, a computational model of the target receptor was built. The output from AutoDock Vina was rendered with Discovery Studio software (Accelrys, San Diego, CA, USA).

3 Results and discussion

3.1 Isothermal titration calorimetry (ITC) studies

Isothermal titration calorimetry (ITC), which measures directly the heat evolved during a reaction, is the method of choice for obtaining thermodynamic information. This is because only ITC allows researchers to obtain directly the variations of enthalpy $\Delta_r H_m^\theta$ and of entropy $\Delta_r S_m^\theta$, as well as the binding constant K_a^θ and the stoichiometry of binding n , for an association process [26].

A representative calorimetric titration profile of $2 \times 10^{-3} \text{ mol l}^{-1}$ α -tocopherol with $2 \times 10^{-4} \text{ mol l}^{-1}$ trypsin/pepsin at 298 K is shown in Fig. 1a₁ and a₂, respectively. Each peak in the binding isotherm represents a single injection of α -tocopherol into the trypsin/pepsin solution. As the sites available on trypsin/pepsin become progressively occupied during titration, the exothermicity of the peaks decreases and eventually saturates. Figure 1b₁ and b₂ show the plot of enthalpy change against $[\alpha\text{-tocopherol}]/[\text{trypsin}]$ and $[\alpha\text{-tocopherol}]/[\text{pepsin}]$ molar ratio, respectively. The solid smooth line in Fig. 1b₁ or b₂ represents the best fit of the experimental data using the independent binding sites model. The thermodynamic parameters for the interaction of α -tocopherol with trypsin/pepsin obtained from ITC are listed in Table 1.

Fig. 1 (a₁) Raw data for the injection of $2 \times 10^{-3} \text{ mol l}^{-1}$ α -tocopherol into $2 \times 10^{-4} \text{ mol l}^{-1}$ trypsin at 298 K and pH 7.4. (a₂) Raw data for the injection of $2 \times 10^{-3} \text{ mol l}^{-1}$ α -tocopherol into $2 \times 10^{-4} \text{ mol l}^{-1}$ pepsin at 298 K and pH 2.0. (b₁) Integrated heat profile of the calorimetric titration shown in panel a₁. (b₂) Integrated heat profile of the calorimetric titration shown in panel a₂

Table 1 Thermodynamic parameters and binding parameters for the interaction of α -tocopherol with trypsin/pepsin obtained from ITC at 298K.^a

System	$\Delta_r H_m^\theta$ (kJ mol ⁻¹)	K_a^θ (l mol ⁻¹)	n	$\Delta_r G_m^\theta$ (kJ mol ⁻¹)	$\Delta_r S_m^\theta$ (J mol ⁻¹ K ⁻¹)
α -Tocopherol-trypsin	-11.37 ± 0.21	$(1.009 \pm 0.141) \times 10^3$	1.12 ± 0.15	-17.11 ± 0.35	19.26 ± 0.47
α -Tocopherol-pepsin	-13.78 ± 0.15	$(1.121 \pm 0.107) \times 10^3$	1.08 ± 0.04	-17.39 ± 0.24	12.11 ± 0.30

^a The listed errors are the asymptotic 95% confidence intervals

The binding constants of the α -tocopherol-trypsin system and the α -tocopherol-pepsin system are $(1.009 \pm 0.141) \times 10^3$ l mol⁻¹ and $(1.121 \pm 0.107) \times 10^3$ l mol⁻¹, respectively. The value of the binding constant of the α -tocopherol-bovine serum albumin (BSA) system obtained by ITC is 6.906×10^5 l mol⁻¹ [6], and the binding constant is 4.142×10^3 l mol⁻¹ for the α -tocopherol-human serum albumin (HSA) system obtained from spectroscopic methods [7]. They are moderate compared to other strong protein-ligand complexes with binding constants ranging from 10^7 – 10^8 l mol⁻¹ [27]. The value of the stoichiometric binding number approximately equals 1, suggesting that one molecule of α -tocopherol combines with one molecule of trypsin/pepsin and no more α -tocopherol binding to trypsin/pepsin occurs at concentration ranges used in this study. The negative values of free energy change and enthalpy change support the conclusion that the binding of α -tocopherol to trypsin/pepsin is spontaneous and exothermic. There may be several types of weak interactions that simultaneously contribute to the negative enthalpy change for the α -tocopherol-trypsin/pepsin system. (1) The hydrophobic interaction between the α -tocopherol molecule and trypsin/pepsin would cause an exothermic process. (2) The expulsion of water molecules from the hydrophobic microenvironments to bulk solution is also exothermic, which leads to the negative heat effect being more evident, because energy of water molecules in hydrophobic microenvironments is more than that in the bulk aqueous solution. (3) Direct electrostatic interaction of dipole groups of the α -tocopherol molecule with peptide sections of trypsin/pepsin molecules also causes an exothermic effect. The positive entropy change and negative enthalpy change of the α -tocopherol-trypsin/pepsin system indicate that the binding of α -tocopherol to trypsin/pepsin is synergistically driven by enthalpy and entropy.

3.2 Steady-state fluorescence spectroscopic studies

3.2.1 Effect of α -tocopherol on trypsin/pepsin fluorescence

Proteins can exhibit strong endogenous fluorescence due to the presence of tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues. In this study, the excitation wavelength was set at 280 nm where the emission fluorescence from phenylalanine can be ignored [28]. In this case, the fluorescence of trypsin/pepsin is mainly from the emission of Trp and Tyr. Figure 2a and b show the fluorescence emission spectra obtained for trypsin/pepsin with the addition of α -tocopherol at $\lambda_{exc} = 280$ nm. It can be seen that the fluorescence intensity of trypsin/pepsin decreases in the presence of α -tocopherol, indicating that the binding of α -tocopherol to trypsin/pepsin can quench their intrinsic fluorescence [29]. Furthermore, a small blue shift is observed with increasing α -tocopherol concentrations, which suggests a reduction in the polarity of the microenvironment around the fluorophore after the addition of α -tocopherol [30].

Fig. 2 **a** Emission spectra of trypsin in the presence of different concentrations of α -tocopherol at 298 K, pH 7.4, and $\lambda_{\text{exc}} = 280$ nm. $c(\text{trypsin}) = 1.0 \times 10^{-5} \text{ mol L}^{-1}$; the *inset* corresponds to the molecular structure of α -tocopherol. **b** Emission spectra of pepsin in the presence of different concentrations of α -tocopherol at 298 K, pH 2.0, and $\lambda_{\text{exc}} = 280$ nm. $c(\text{pepsin}) = 2.0 \times 10^{-5} \text{ mol L}^{-1}$

3.2.2 Fluorescence-quenching mechanisms

The different mechanisms of quenching are usually classified as either dynamic (a collisional process) or static (the formation of a complex between quencher and fluorophore). For fluorescence quenching, the decrease in intensity is usually described by the Stern–Volmer equation [31]:

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (5)$$

where F_0 and F represent the steady-state fluorescence intensities in the absence and presence of quencher, respectively, k_q is the bimolecular quenching constant and τ_0 is the lifetime of the fluorescence in absence of quencher. The value of τ_0 is generally taken to be 10^{-8} s [28]. $[Q]$ is

the concentration of the quencher, and K_{SV} is the Stern–Volmer quenching constant. Figure 3a and b show the Stern–Volmer plots for the trypsin/pepsin fluorescence quenching by α -tocopherol. The values of K_{SV} and k_q for the interaction of α -tocopherol with trypsin/pepsin at four different temperatures are shown in Table 2. In the present case, a linear Stern–Volmer plot is observed for α -tocopherol, which means that only one type of quenching mechanism occurs (dynamic or static). Dynamic and static quenching can be distinguished by their different dependence on temperature. For dynamic quenching, higher temperatures result in faster diffusion and greater quenching. The quenching constant increases with increasing temperature, but the reverse effect will be observed for static quenching [32]. The results show that the K_{SV} values decrease with increasing temperature, and the values of k_q are greater

Fig. 3 Stern–Volmer plots of trypsin (a) and pepsin (b) fluorescence quenched by α -tocopherol at four different temperatures

Table 2 The quenching constants (K_{SV}) and bimolecular quenching constants (k_q) of the α -tocopherol-trypsin and α -tocopherol-pepsin systems at different temperatures^a

System	<i>T</i> (K)	K_{SV} (l mol ⁻¹)	k_q (l mol ⁻¹ s ⁻¹)	R^b	SD ^c
α -Tocopherol-trypsin	293	$(3.347 \pm 0.024) \times 10^3$	$(3.347 \pm 0.024) \times 10^{11}$	0.9954	0.0202
	298	$(3.276 \pm 0.017) \times 10^3$	$(3.276 \pm 0.017) \times 10^{11}$	0.9955	0.0194
	303	$(3.237 \pm 0.011) \times 10^3$	$(3.237 \pm 0.011) \times 10^{11}$	0.9952	0.0200
	313	$(3.161 \pm 0.021) \times 10^3$	$(3.161 \pm 0.021) \times 10^{11}$	0.9914	0.0262
α -Tocopherol-pepsin	293	$(3.593 \pm 0.023) \times 10^3$	$(3.593 \pm 0.023) \times 10^{11}$	0.9934	0.0266
	298	$(3.199 \pm 0.014) \times 10^3$	$(3.199 \pm 0.014) \times 10^{11}$	0.9936	0.0235
	303	$(3.055 \pm 0.017) \times 10^3$	$(3.055 \pm 0.017) \times 10^{11}$	0.9975	0.0139
	310	$(2.833 \pm 0.012) \times 10^3$	$(2.833 \pm 0.012) \times 10^{11}$	0.9933	0.0212

^a Values are the average of three independent experiments^b R is the correlation coefficient^c SD is standard deviation

than the limiting diffusion rate constant of the biomolecule (2×10^{10} l mol⁻¹ s⁻¹) [33] (Table 2), which suggests that the mechanism is static quenching [29].

3.2.3 Binding parameters

When small molecules bind independently to a set of equivalent sites on a macromolecule and the equilibrium between the free and the bound molecules has been reached, the fluorescence intensities obey the following equation [34, 35]:

$$\log \frac{F_0 - F}{F} = n \log K_a - n \log \frac{1}{[Q] - \frac{(F_0 - F)[P]}{F_0}} \quad (6)$$

F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. K_a is the apparent binding constant to a set of sites, and n is the average number of binding sites per trypsin/pepsin. $[Q]$ and $[P]$ are the total quencher concentration and the total protein concentration, respectively. From the plot of $\log(F_0 - F)/F$ vs. $\log(1/([Q] - (F_0 - F)[P]/F_0))$, the number of binding sites n and binding constant K_a can be obtained (Fig. 4 and Table 3). At 298K, the binding constants K_a of the α -tocopherol-trypsin system ($(3.038 \pm 0.019) \times 10^3$) and the α -tocopherol-pepsin system ($(3.397 \pm 0.027) \times 10^3$) obtained by the spectroscopic method are of the same order of magnitude (10^3 l mol⁻¹). K_d is the equilibrium dissociation constant and $K_d = 1/K_a$. Therefore, the equilibrium dissociation constants K_d of the α -tocopherol-trypsin system and the α -tocopherol-pepsin system are $(3.292 \pm 0.020) \times 10^{-4}$ and $(2.944 \pm 0.023) \times 10^{-4}$, respectively. The value of the stoichiometric binding number n of the two systems obtained by the spectroscopic method is approximately equal to 1. These results are consistent with the results obtained from ITC. The decreasing trend of K_a with increasing temperature is in accordance with K_{SV} 's dependence on temperature, which coincides with the static quenching mechanism. The value of K_a decreases with the rise of temperature, which also indicates that temperature rising decreases the stability of the α -tocopherol-trypsin complex and the α -tocopherol-pepsin complex. That is, the binding reactions between α -tocopherol and trypsin/pepsin are both exothermic [7]. These results are validated by the enthalpy change, as shown in Table 1.

Fig. 4 The plots of $\log(F_0-F)/F$ vs. $\log(1/([Q]-(F_0-F)[P]/F_0))$ for α -tocopherol-trypsin (a) and α -tocopherol-pepsin (b) systems at four different temperatures

3.3 Time-resolved fluorescence studies

Time-resolved fluorescence is a very precise technique to distinguish static and dynamic quenching [36]. Therefore, the quenching mechanisms for the interaction of α -tocopherol with trypsin/pepsin were further confirmed through lifetime measurements. The time-resolved decay curves of trypsin and pepsin under different concentrations of α -tocopherol are shown in Fig. 5. The data fits well to the sum of a single exponential decay with a χ^2 value close to 1.00. The decay parameters for all the systems are summarized in Table 4. As illustrated in Fig. 5, there is no obvious difference in the decay curves of trypsin and pepsin with increasing amounts of α -tocopherol. α -Tocopherol has little effect on the fluorescence lifetime (τ) of

Table 3 The binding constants (K_a) and the number of binding sites (n) of the α -tocopherol-trypsin and α -tocopherol-pepsin systems at different temperatures^a

System	T (K)	K_a (l mol^{-1})	n	R^b	SD ^c
α -Tocopherol-trypsin	293	$(3.115 \pm 0.016) \times 10^3$	0.89 ± 0.02	0.9945	0.0417
	298	$(3.038 \pm 0.019) \times 10^3$	0.97 ± 0.03	0.9912	0.0569
	303	$(3.013 \pm 0.021) \times 10^3$	1.03 ± 0.02	0.9956	0.0424
	313	$(2.909 \pm 0.018) \times 10^3$	1.08 ± 0.04	0.9950	0.0475
α -Tocopherol-pepsin	293	$(3.470 \pm 0.023) \times 10^3$	0.82 ± 0.03	0.9950	0.0286
	298	$(3.397 \pm 0.027) \times 10^3$	0.90 ± 0.02	0.9951	0.0313
	303	$(3.244 \pm 0.017) \times 10^3$	1.14 ± 0.02	0.9961	0.0343
	310	$(3.171 \pm 0.011) \times 10^3$	1.30 ± 0.01	0.9958	0.0402

^a Values are the average of three independent experiments^b R is the correlation coefficient^c SD is standard deviation

trypsin and pepsin (Table 4). As Lakowicz reported [37], for static quenching the fluorescence lifetime of fluorophore will not be disturbed during the complex formation. Thus, this observation shows that quenching follows static mechanism. These results are in good agreement with the results of steady-state fluorescence.

3.4 CD spectroscopic studies

Circular dichroism (CD) is usually employed to monitor the conformational changes of proteins because of its accuracy and sensitivity. Far-UV CD spectra (260–200 nm) are used to determine the secondary structure of a protein. The characteristic CD bands reflect the content of the secondary structure, such as α -helices, β -sheets, β -turns and random coils [38].

The CD spectra of trypsin/pepsin in the absence and presence of α -tocopherol are shown in Fig. 6. As shown in Fig. 6, the spectrum of free trypsin/pepsin exhibits a negative band in the ultraviolet region at about 209 nm and there are no positive peaks. After α -tocopherol was added, the intensity of the negative peak increased without any significant shift of the peaks. The calculations for the secondary structural elements obtained from the CDPro software are listed in Table 5. As shown in Table 5, with the addition of α -tocopherol into the trypsin/pepsin solution, the contents of secondary structures fluctuated in different trends. The β -sheet content decreased accompanied by a slight increase of α -helix and β -turn content, while the random coil content remained virtually unchanged. These results indicate that the addition of α -tocopherol induces changes in the natural secondary structure of trypsin/pepsin, which may lead to changes in the physiological function of trypsin/pepsin, such as enzyme activity [38–40].

3.5 Inhibition of trypsin/pepsin activity by α -tocopherol

In order to explore the effects of these binding interactions on the activity of trypsin/pepsin, we measured the trypsin/pepsin activity changes induced by α -tocopherol. As shown in Fig. 7, the α -tocopherol activity inhibition values are, respectively, 10.3% and 42.1% for α -tocopherol:trypsin and α -tocopherol:pepsin at a mass ratio of 1.2:1. These results indicate that α -tocopherol does not

Fig. 5 Time-resolved fluorescence decay profile of trypsin and pepsin in the absence and presence of α -tocopherol. $c(\text{trypsin/pepsin}) = 1 \times 10^{-4} \text{ mol l}^{-1}$; $c(\alpha\text{-tocopherol}) = 1 \times 10^{-4} \text{ mol l}^{-1}$

Table 4 Fitting parameters of fluorescence lifetime measurements of trypsin and pepsin at different concentrations of α -tocopherol

System	τ (ns)	χ^2 ^a
Free trypsin	0.179 ± 0.017	0.911
α -Tocopherol-trypsin	0.175 ± 0.017	0.921
Free pepsin	0.227 ± 0.016	0.955
α -Tocopherol-pepsin	0.223 ± 0.017	0.975

^a The magnitude of χ^2 denotes the goodness of the fit

Fig. 6 **a** Circular dichroism spectra of Trypsin ($1 \times 10^{-5} \text{ mol l}^{-1}$) in the absence and presence of α -tocopherol ($1 \times 10^{-4} \text{ mol l}^{-1}$). **b** Circular dichroism spectra of Pepsin ($1 \times 10^{-5} \text{ mol l}^{-1}$) in the absence and presence of α -tocopherol ($1 \times 10^{-4} \text{ mol l}^{-1}$)

Table 5 The influence of α -tocopherol on the secondary structure of trypsin and pepsin

System	α -Helix (%)	β -Sheet (%)	β -Turn (%)	Random coil (%)
Free trypsin	6.1	44.5	10.3	39.1
α -Tocopherol-trypsin	6.5	43.7	10.9	38.9
Free pepsin	9.4	41.3	18.5	30.8
α -Tocopherol-pepsin	10.1	39.9	19.1	30.9

Fig. 7 Inhibition rate of trypsin and pepsin by α -tocopherol.
 $c(\text{trypsin/pepsin}) = 4.0 \times 10^{-5} \text{ mol l}^{-1}$

remarkably influence the activity of trypsin, but α -tocopherol can influence the microenvironment of the pepsin activity site, which results in reduced pepsin activity [41].

3.6 Molecular docking studies

Molecular docking of the ligand with macromolecules provides insight into the preferred binding location and can be exploited to corroborate experimental observations to a large extent. The most likely binding mode between α -tocopherol and trypsin is showed in Fig. 8. As shown in Fig. 8c, α -tocopherol is surrounded by Tyr-39, 151, His-40, Thr-149, Trp-141, Pro-152, Asp-153, Ile-73, and Asn-74. In addition, the electrostatic interactions between α -tocopherol and His-40, Asp-153 and the hydrophobic interactions between α -tocopherol and the hydrophobic side chains, such as Trp-141, Pro-152, and Ile-73, are also involved in the association. The electrostatic interactions and the hydrophobic interactions simultaneously contribute to the negative enthalpy values for the α -tocopherol-trypsin system. In addition, the expulsion of water molecules from the hydrophobic microenvironments to bulk solution can cause positive entropy. These results justify the speculation obtained from ITC. As shown in Fig. 8d, the distances between α -tocopherol and Trp-141 and Tyr-151 are 7.0 Å and 5.7 Å, respectively, which suggests that Trp-141 and Tyr-151 are in close proximity to α -tocopherol, and α -tocopherol approaches the Tyr more than the Trp. Furthermore, only one hydrogen bond exists between α -tocopherol and Tyr-151. The length of the hydrogen bond is 2.979 Å (Fig. 8c). These results provide a good structural basis to explain the efficient fluorescence quenching of trypsin emission in the presence of α -tocopherol. There are two fundamental characteristics of the active site region of trypsin. The first is that Ser-195, His-57, and Asp-102 are three catalytic residues in the region; the second is that there is a “pocket” near Ser-195 defining the cut position of the peptidic bond [42, 43]. The binding site is far away from the active site, which can provide a good explanation for why trypsin activity is not affected significantly by α -tocopherol, which agrees well with the activity test of trypsin.

The most likely binding mode between α -tocopherol and pepsin is given in Fig. 9. α -Tocopherol enters into the hydrophobic cavity of pepsin via hydrophobic interactions between α -tocopherol and pepsin (Fig. 9a and b). As shown in Fig. 9c, α -tocopherol is surrounded by Thr-74, 77, 218, Tyr-75, 189, Gly-76, 34, Ala-130, Asn-37, Ser-36, and Ile-128. There is only one hydrogen bond between α -tocopherol and residue Ser-36 (distance, 2.194 Å), which

Fig. 8 Molecular docking of trypsin with α -tocopherol. **a** The hydrophobic cavity of trypsin with α -tocopherol. **b** The cartoon ribbons of trypsin with α -tocopherol. **c** The zoom on the binding site of trypsin with α -tocopherol. The hydrogen bond between α -tocopherol and trypsin is represented using *black dashed lines*. **d** The distances between α -tocopherol and Trp/Tyr of trypsin

suggests that Ser-36 may be contributing to the binding of α -tocopherol to pepsin. Moreover, due to the hydrophilic side chains, such as Thr-74, 77, 218, Tyr-75, 189, Gly-76, 34, Asn-37, and Ser-36 near the α -tocopherol molecule, there are also considerable electrostatic forces between α -tocopherol and pepsin. Therefore, the major driving forces are hydrophobic interactions, electrostatic interactions, and hydrogen bond interactions between α -tocopherol and pepsin. These results also justify the speculation about the reasons for the negative enthalpy values and the positive entropy obtained from ITC. Furthermore, it is important to note that the Trp-39, Tyr-75, and Tyr-189 of pepsin are not far from α -tocopherol (as shown in Fig. 9d, the distances between α -tocopherol and Trp-39, Tyr-75, and Tyr-189 are 9.7 Å, 3.1 Å, and 7.5 Å, respectively). Therefore, α -tocopherol can quench the intrinsic fluorescence of pepsin, which is in accordance with the results from the fluorescence spectroscopic experiment. Pepsin is composed of two structurally homologous domains, an N-terminal domain (residues 1–172) and a C-terminal domain (residues 173–326). Both of the domains consist almost entirely of β -sheets. The active binding site of pepsin is located in the cleft between the domains [44, 45]. As shown in Fig. 9a, α -tocopherol binds directly into the pepsin cavity site. The binding of α -tocopherol into the pepsin cavity influences the microenvironment of the pepsin activity site, which results in reduced pepsin activity.

Fig. 9 Molecular docking of pepsin with α -tocopherol. **a** The hydrophobic cavity of pepsin with α -tocopherol. **b** The cartoon ribbons of pepsin with α -tocopherol. **c** The zoom on the binding site of pepsin with α -tocopherol. The hydrogen bond between α -tocopherol and pepsin is represented using black dashed lines. **d** The distances between α -tocopherol and Trp/Tyr of pepsin

4 Conclusions

The binding mechanisms of α -tocopherol interacting with trypsin and pepsin were investigated using isothermal titration calorimetry, spectroscopic, and molecular modeling methods. Experimental results suggest that the binding of α -tocopherol to trypsin/pepsin is synergistically driven by enthalpy and entropy. α -Tocopherol can bind to trypsin/pepsin and quench the fluorescence of trypsin/pepsin. The quenching mechanism is of the static type and due to the formation of a ground state complex. The binding ability of α -tocopherol with trypsin/pepsin is in the intermediate range. The values of binding constants K_a increase in the order, K_a (α -tocopherol-trypsin) < K_a (α -tocopherol-pepsin) < K_a (α -tocopherol-HSA) < K_a (α -tocopherol-BSA). The stoichiometric binding number n approximately equals 1, suggesting that one molecule of α -tocopherol combines with one molecule of trypsin/pepsin and no more α -tocopherol binding to trypsin/pepsin occurs at concentration ranges used in this study. The results of CD suggest that α -tocopherol induces changes in the natural secondary structure of trypsin and pepsin by increasing the α -helix content and decreasing the β -sheet content. Molecular modeling method shows that the major driving forces are hydrophobic interactions, electrostatic interactions, and hydrogen bond interactions between α -tocopherol and trypsin/pepsin. In addition, α -tocopherol does not obviously influence the activity of trypsin because the binding site is far away from the active site. On the contrary, the binding of α -tocopherol

influences the microenvironment of the pepsin activity site, which results in reduced pepsin activity.

The conclusions are important in the field of pharmacology and biochemistry and are helpful for understanding α -tocopherol's effect on protease function and its biological activity in vivo. α -Tocopherol is the most biologically active form of vitamin E. People generally think that α -tocopherol has little side effects and can build up their resistance to disease by taking α -tocopherol as a dietary supplement. However, the conclusions of the current study suggest that α -tocopherol can influence the activity of pepsin and reduce absorption of other nutrients. α -Tocopherol does not remarkably influence the activity of trypsin; therefore, for the α -tocopherol-trypsin system, α -tocopherol is considered to be a safe natural product. This work focuses on the interactions of α -tocopherol with proteases, which are significant to ensure human health and safety in daily life.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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