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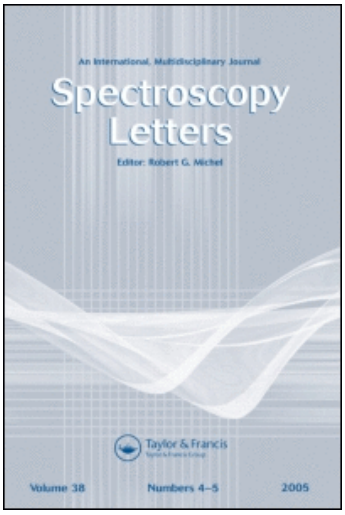

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Investigation of the Interaction Between Rutin and Trypsin in Solution by Multi-Spectroscopic Method

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ABSTRACT The interactions between rutin and trypsin were investigated by UV-Vis absorption, CD, fluorescence, resonance light-scattering spectra, synchronous fluorescence, and three-dimensional fluorescence spectra techniques under physiological pH 7.40. Rutin effectively quenched the intrinsic fluorescence of trypsin via static quenching. The enthalpy change and entropy change were estimated to be $-8.23\,\mathrm{kJ\cdot mol^{-1}}$ and $53.66\,\mathrm{J\cdot mol^{-1}\cdot K^{-1}}$ according to the van't Hoff equation. The process of binding rutin to trypsin was a spontaneous molecular interaction procedure. This result indicates that hydrophobic and electrostatic interactions played a major role in stabilizing the complex. The conformation of trypsin was discussed by CD, synchronous, and three-dimensional fluorescence techniques.

KEYWORDS binding constant, fluorescence spectroscopy, rutin, trypsin

INTRODUCTION

Dietary flavonoids are main polyphenols that display anticancer, antiviral, anti-allergic, antispasmodic, anticarcinogenic, and antiinflammatory bioactivities.[1–3] Rutin (Fig. 1) is one of the most abundant dietary flavonoids and the main active component of many medicinal plants.[4] Like other flavonoids, rutin exhibits many of the above activities.[2,3] A nontoxic and nonoxidizable molecule, rutin offers an advantage over myricetin, quercetagenin, and other flavonoids.[5,6] Proteinases play an important role in the regulation of many biologically relevant processes in the human body.[1] Trypsin is a serine proteinase that is excreted by the pancreas in the small intestine and takes part in the digestive deconstruction of food proteins.[7] Flavonoids were considered as building blocks for the inhibitors of trypsin family enzymes. Therefore, it would be interesting to obtain more detailed information on the rutin–trypsin interaction at the molecular level.

It is well known that rutin often binds to biomolecules, such as serum albumins, hemoglobin, and DNA.[8–10] Pastukhov et al. have studied the binding of rutin to human serum albumin (HSA) and found that the binding site for rutin is situated within the subdomain IIA of HSA.[8] Guo and

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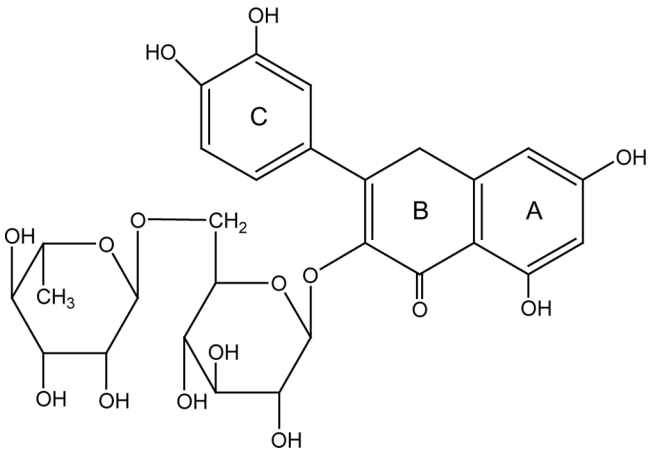

FIGURE 1 **The molecular structure of rutin.**

coworkers have studied the interactions between rutin and hemoglobin in lecithin liposomes.[9] Tian et al. have studied the binding of rutin with DNA by electrochemical and spectroscopic methods.[10] Maliar et al. have reported the computer-assisted drug design of flavonoids, which has been found to be a perspective trypsin/trypsin-like enzyme inhibitor.[1] The thermodynamics parameters including such as mode of interaction, association constant, and number of binding sites are important to make predictions about enzyme–ligand complexes from a pharmacological point of view. In this report, we investigated the interaction of rutin with trypsin by UV-Vis absorption, fluorescence, resonance light-scattering spectra, circular dichroism (CD), synchronous fluorescence, and three-dimensional fluorescence spectra techniques. The work could benefit the understanding of a new inhibitor with more favorable interactions with the enzyme. This investigation may open up new avenues for design of the most suitable flavonoids derivatives that have better plasma stability and fewer side effects.

MATERIALS AND METHODS

Materials

Trypsin (MW = 23,500 Da) from bovine pancreas was purchased from Sigma Aldrich (Foster, USA) and used without further purification. Rutin was obtained from Fluka (Buchs, Switzerland). The buffer Tris was purchased from Acros (Geel, Belgium), and NaCl, HCl, etc., were all of analytical purity. For the CD experiments, a 0.02 M phosphate buffer of pH 7.40 was exclusively prepared in deionized water.

Equipments and Spectral Measurements

The UV-Vis spectrum was recorded at 298 K on a SPECORD S 50 (Jena, Germany) equipped with 1.0-cm quartz cells. All fluorescence spectra were recorded on a Perkin-Elmer LS-50B Spectrofluorometer (Foster, USA) equipped with 1.0-cm quartz cells and a thermostat bath. The widths of both the excitation slit and the emission slit were set to 10.0 nm/3.0 nm for trypsin, respectively. The CD spectra were measured by a Jasco J-810 spectropolarimeter (Tokyo, Japan) using a 0.1-cm quartz cell at 0.1-nm intervals, the bandwidth was 2.0 nm and the san speed was $20\,\mathrm{nm}\cdot\mathrm{min}^{-1}$.

Procedures

Trypsin solution (2.5 mL) was titrated with successive rutin solution. Titrations were performed manually by using trace syringes. The fluorescence spectra were then measured (excitation at 280 nm and emission wavelengths of 290–500 nm) at two temperatures (298, 308 K). The fluorescence intensity was corrected using the relationship[11]

$$F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2} \quad (1)$$

where F_{cor} and F_{obs} are the fluorescence intensity corrected and observed, respectively; A_{ex} ($\lambda_{\mathrm{ex}} = 280$ nm) and A_{em} ($\lambda_{\mathrm{em}} = 339$ nm) are the absorbance of system at excitation and emission wavelength, respectively. Synchronous fluorescence spectra of trypsin in the presence of rutin were recorded at room temperature. The D value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 15 or 60 nm. The three-dimensional fluorescence spectrum was performed under the following conditions: the emission wavelengths at 270–600 nm, the excitation at 200 nm, scanning number 15, and increment 10 nm with other parameters the same as those of the fluorescence quenching spectra. Resonance light-scattering (RLS) spectra were obtained by synchronous scanning ($\Delta\lambda = 0$ nm) with the wavelength range of 200–600 nm on the spectrofluorophotometer at 298 K. The UV-Vis absorbance spectrum of rutin solution was recorded at 298 K. The CD spectra of trypsin solutions containing rutin were recorded from 190 to 300 nm with two scans averaged for each CD spectrum.

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RESULTS AND DISCUSSION

Interaction Between Rutin and Trypsin

There are two main absorption bands in the UV spectra of rutin, which can be assigned to band I (250–280 nm) and band II (325–400 nm), respectively.[12] Figure 2 shows the UV absorption spectra of rutin from 250 to 500 nm in methanol, Tris-HCl, and trypsin solutions. The maximum UV absorption wavelengths (λ_{max}) of band I and band II in methanol were situated at 260 and 360 nm, respectively, and they shifted to 270 and 366 nm. The red shift is related to the stronger interaction between the water and the phenolic hydroxyl on the rutin molecule. Addition of the trypsin to rutin solution resulted in the little red shift of the band I and band II maximum in the absorption spectrum of rutin. The change of maximum UV absorption wavelength indicates the ground state complex formation between rutin and trypsin.

Fluorescence spectroscopy is essentially a probe technique sensing changes in the local environment of the fluorophore of protein. There are four tryptophans (Trp 50, Trp 141, Trp 215, and Trp 237) in trypsin that can be used as intrinsic fluorophores.[7] As shown in Fig. 3, the emission maximum of trypsin in solution was observed at 339 nm, which indicates that specific tryptophans of trypsin are partly exposed to the solvent. The effect of rutin on trypsin

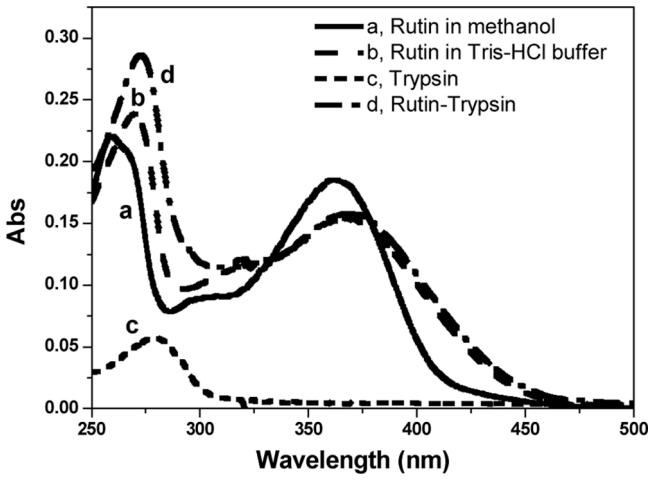

FIGURE 2 **Absorption spectra of rutin, trypsin, and rutin–trypsin system. (a) The absorption spectrum of rutin in methanol; (b) the absorption spectrum of rutin in Tris-HCl solution; (c) the absorption spectrum of trypsin only; (d) the absorption spectrum of rutin–trypsin system). c (trypsin) = c (rutin) = 10.0 μM.**

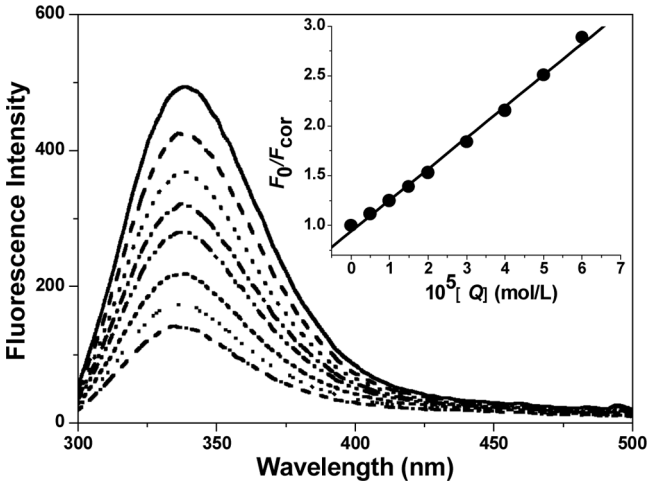

FIGURE 3 **Effect of rutin on fluorescence spectra of trypsin ($T = 298$ K, pH = 7.40, $\lambda_{ex} = 280$ nm). The insert shows the Stern-Volmer plots for the quenching of trypsin by rutin. Total concentration of rutin: c (rutin)/(10^{-5} M), curve (from top to bottom): 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0, respectively. Trypsin concentration was at 10.0 μM.**

fluorescence intensity is shown in Fig. 3. As the data show, the addition of rutin to trypsin leads to a significant reduction in the fluorescence intensity with a little shift of emission to a shorter wavelength. The results showed that the binding of rutin with trypsin quenches the intrinsic fluorescence of trypsin and the conformational changes are induced in trypsin by rutin.

Generally, quenching types often include static and dynamic quenching. It is necessary to know quenching procedure and type for researching the mechanism of quenching. In this article, we have used the binding constants' dependence on the temperature to elucidate the quenching mechanism. It is known that the dynamic quenching constants are expected to increase when there is a rise in temperature. In order to confirm the quenching mechanism, the fluorescence quenching data are analyzed by the Stern-Volmer equation:[13]

$$\frac{F_0}{F_{cor}} = 1 + k_q\tau_0[Q] = 1 + K_{sv}[Q] \qquad (2)$$

where F_0 is the fluorescence intensities before the addition of the quencher, F_{cor} is the fluorescence intensity corrected, k_q is the bimolecular quenching constant, τ_0 is the average lifetime of the biomolecule without quencher ($\tau_0 = 10^{-8}$ s), K_{sv} is the Stern-Volmer dynamic quenching constant, and [Q] is the concentration of the quencher. The insert in Fig. 3

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displays the Stern-Volmer plots of the quenching of trypsin tryptophan residues fluorescence by rutin at 298 K. Linear Stern-Volmer plots may either indicate the existence of a single type of quenching or reveal the occurrence of just a binding site for quencher in the proximity of the fluorophore.[14] In Table 1, the rise in temperature does not increase the K_{sv} values and the k_q was the order of $10^{12}\,\mathrm{L\cdot moL^{-1}\cdot s^{-1}}$. Obviously, this indicates that the quenching was not initiated from dynamic collision but from the formation of a complex.[15]

Consequently, the quenching process was also analyzed according to the modified Stern-Volmer equation:[16]

$$\frac{F_0}{\Delta F}=\frac{F_0}{F_0-F_{cor}}=\frac{1}{f_0}+\frac{1}{K_a f_0}\cdot\frac{1}{[Q]} \qquad (3)$$

In this case, f_a and K_a are the fraction of accessible fluorescence and the effected quenching constant for the accessible fluorophores, respectively. Within certain concentrations, the curve of $F_0/(F_0-F_{cor})$ versus $1/[Q]$ (modified Stern-Volmer curve) would be linear for static quenching.[16] From Table 1, it is known that under certain rutin concentrations, the value of correlation coefficient (R) is more than 99.9% and the curves of $F_0/(F_0-F_{cor})$ versus $1/[Q]$ were linear. All these results indicate that there were obviously characters of static quenching. In this article, the effected quenching constants obtained from the modified Stern-Volmer curves were applied to analyze the thermodynamic parameter and the nature of the binding forces.

The RLS spectra of trypsin and trypsin–rutin complex recorded with synchronous scanning from 200 to 600 nm are shown in Fig. 4. Upon addition of rutin to trypsin solution, an obviously decreased RLS was observed (Figs. 4a, 4b). The RLS intensity is dominated primarily by the particle dimension of the formed aggregate in solution,[17] so it is inferred from the results that the added rutin may interact with trypsin in solution, forming a new trypsin–rutin complex. The size of new complex particles may be smaller than that of trypsin, because light-scattering signal decreased under the given conditions.

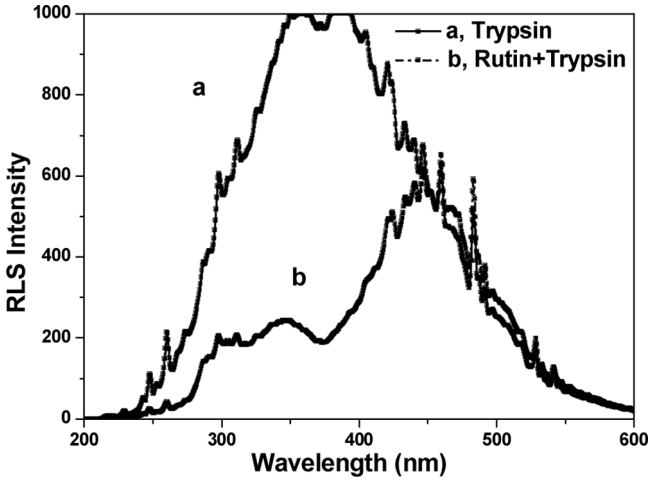

FIGURE 4 RLS spectra of trypsin (a), trypsin–rutin (b). c (trypsin) = 10.0 μM; c (rutin) = 20.0 μM.

Thermodynamic Parameters and Nature of the Binding Forces

Van der Waals forces, electrostatic, hydrogen bonds, and hydrophobic interactions can play an important role in ligand binding to protein.[18] To obtain this information, the thermodynamic parameters of rutin binding with trypsin were calculated from the van't Hoff equation.[19,20] From Table 2, it can be seen that the negative sign for ΔG° indicates the spontaneity of the binding of rutin with trypsin. ΔH° is the negative value, and ΔS° is a positive value. The main source of ΔG° value was derived from a large contribution of ΔS° term with little contribution from the ΔH° factor. A positive ΔS° value is frequently

TABLE 1 Stern-Volmer Quenching Constant and Modified Stern-Volmer Association Constant of the Interaction of Rutin–Trypsin at Different Temperatures

	Eq. (2)				Eq. (3)		
T(K)	K_{sv} ($\mathrm{L\cdot mol^{-1}}$)	K_q ($\mathrm{L\cdot mol^{-1}}$)	R^a	SD^b	K_a ($\mathrm{L\cdot mol^{-1}}$)	R^a	SD^b
298	3.14×10^4	3.14×10^{12}	0.9980	0.0446	1.76×10^4	0.9999	0.0455
308	3.12×10^4	3.12×10^{12}	0.9980	0.0432	1.58×10^4	0.9995	0.0959

[a]Correlation coefficient.
[b]Standard deviation.

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TABLE 2 Thermodynamic Parameters of the System of Rutin–Trypsin System

T(K)	ΔH° (kJ · mol^{-1})	ΔG° (kJ · mol^{-1})	ΔS° (J · mol^{-1} · K^{-1})	K_A (L · mol^{-1})	n	R^a	SD^b
298	−8.23	−24.22	53.66	3.46×10^4	0.92	0.9976	0.0308
308		−24.76		3.44×10^4	0.93	0.9983	0.0265

[a]Correlation coefficient.
[b]Standard deviation.

taken as evidence for hydrophobic interaction.[18] The interaction of rutin with trypsin included the hydrophobic forces between the aromatic ring and the hydrophobic amino acid residues. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS° and a negative ΔH°. At pH = 7.40, trypsin (isoelectric point pI = 10.5)[7] bears a positive charge, and rutin ($pK_{a1} = 7.1$)[21] bears a negative charge because the most acidic phenolic OH groups of rutin are dissociated at physiological pH, though this process is far from being complete and results in a mixture of neutral and anionic species.[21] Therefore, the electrostatic could not be excluded in the interaction between rutin and trypsin. Accordingly, it is not possible to account for the thermodynamic parameters of the trypsin–rutin coordination complex on the basis of a single intermolecular force model. It is more likely the hydrophobic, electrostatic interactions are involved in its binding process.

Binding Constant and Binding Capacity

The apparent binding constant K_A and binding sites n can be found from the equation:[22]

$$\log\frac{F_0 - F_{cor}}{F_{cor}} = n\log K_A - n\log\left(\frac{1}{[Q_t] - (F_0 - F_{cor})[P_t]/F_0}\right) \quad (4)$$

where F_0 is the fluorescence intensities before the addition of the quencher, F_{cor} is the fluorescence intensity corrected, and $[P_t]$ and $[Q]$ are the total quencher concentration and the total protein concentration, respectively. By the plot of log $(F_0 - F_{cor})/F_{cor}$ versus log $[1/([Q_t] - (F_0 - F_{cor})\ [P_t]/F_0)]$, the number of binding sites n and the association constant K_A can be obtained. In Table 2, the binding constants K_A and binding sites n are listed for rutin associated with trypsin. The results showed that the binding constants K_A cannot change obviously with the temperature rising, which may indicate forming a stable compound. According to the literature,[8,9] it is evident that rutin could bind with human serum albumin and hemoglobin in a strong way as compared to trypsin. The observed results can be attributed to the difference in protien structure, because they may differ in affinities. The values of n approximately equal to 1 indicates the existence of just a single binding site in trypsin for rutin. According to the quenching data, some Trp residues are involved in the binding of rutin to trypsin and locate in the binding site.

Energy Transfer from Trypsin to Rutin

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between donor and acceptor.[23] Here the donor and acceptor were trypsin and rutin, respectively. There was a spectral overlap between the fluorescence emission spectrum of free trypsin (Fig. 5a) and absorption UV-Vis spectra of rutin (Fig. 5b).

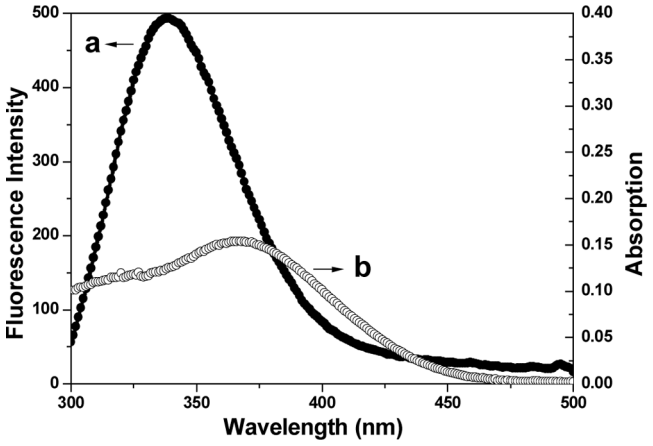

FIGURE 5 Overlap of the fluorescence emission of trypsin (a) with the absorption spectra of rutin (b). c (trypsin) = c (rutin) = 10.0 μM.

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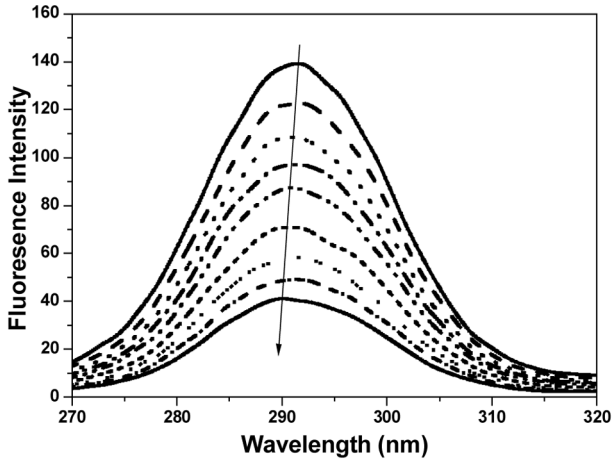
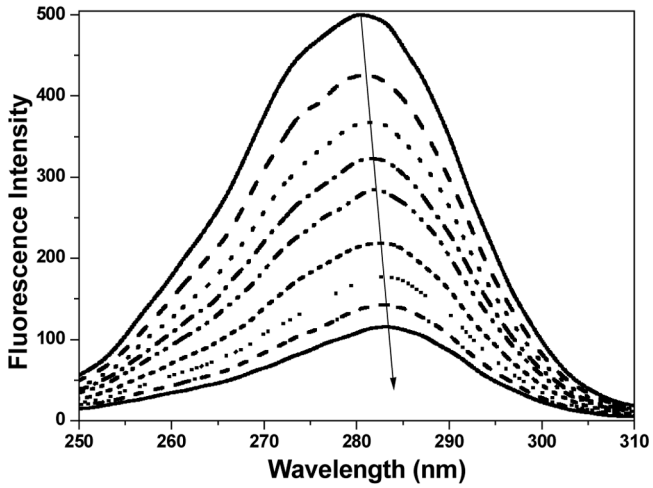


(a)



(b)

FIGURE 6 Synchronous fluorescence spectrum of trypsin in the presence of rutin at 298 K. Total concentration of rutin: c (rutin)/(10^{-5} M), curve (from top to bottom): 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0, respectively. Trypsin concentration was at 10.0 μM. (a) $\Delta\lambda = 15$ nm and (b) $\Delta\lambda = 60$ nm.

According to Förster's theory, the energy transfer efficiency E is defined as follows:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{5}$$

where r is the distance from the ligand to the tryptophan residue of the protein, and R_0 is the Förster critical distance at which 50% of the excitation energy is transferred to the acceptor.[23] It can be calculated from donor emission and acceptor absorption spectra using the Förster formula:

$$R_0^6 = 8.79 \times 10^{-25} k^2 n^{-4} \Phi J \tag{6}$$

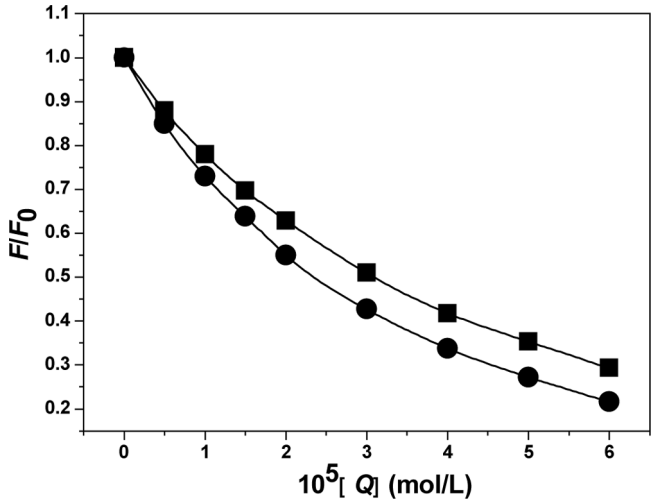


FIGURE 7 The quenching of trypsin synchronous fluorescence by rutin. c (trypsin) = 10.0 μM. (■) $\Delta\lambda = 15$ nm, (●) $\Delta\lambda = 60$ nm.

In Eq. (6), k^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles and $k^2 = 2/3$ for random orientation as in fluid solution; n is the average refractive index of medium in the wavelength range where spectral overlap is significant; Φ is the fluorescence quantum yield of the donor; J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor (Fig. 4), which can be calculated by Eq. (7):

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda) d\lambda} \tag{7}$$

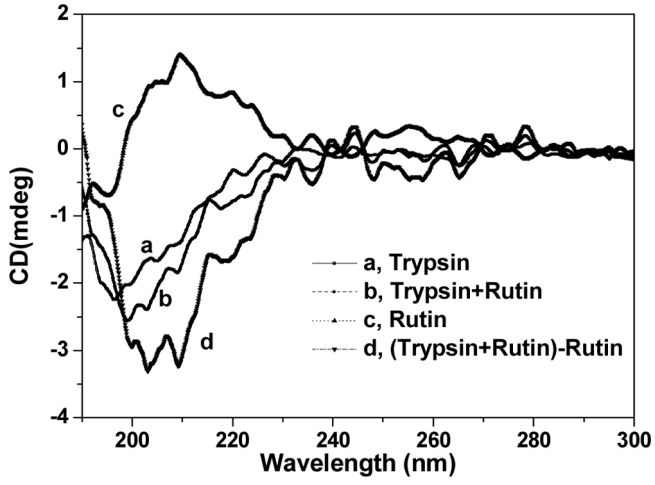


FIGURE 8 Far-UV CD spectra of trypsin and rutin. (The spectrum of rutin cannot be subtracted from that of the complex; i.e., [trypsin + rutin] − rutin ≠ trypsin). c (trypsin) = c (rutin) = 10.0 μM.

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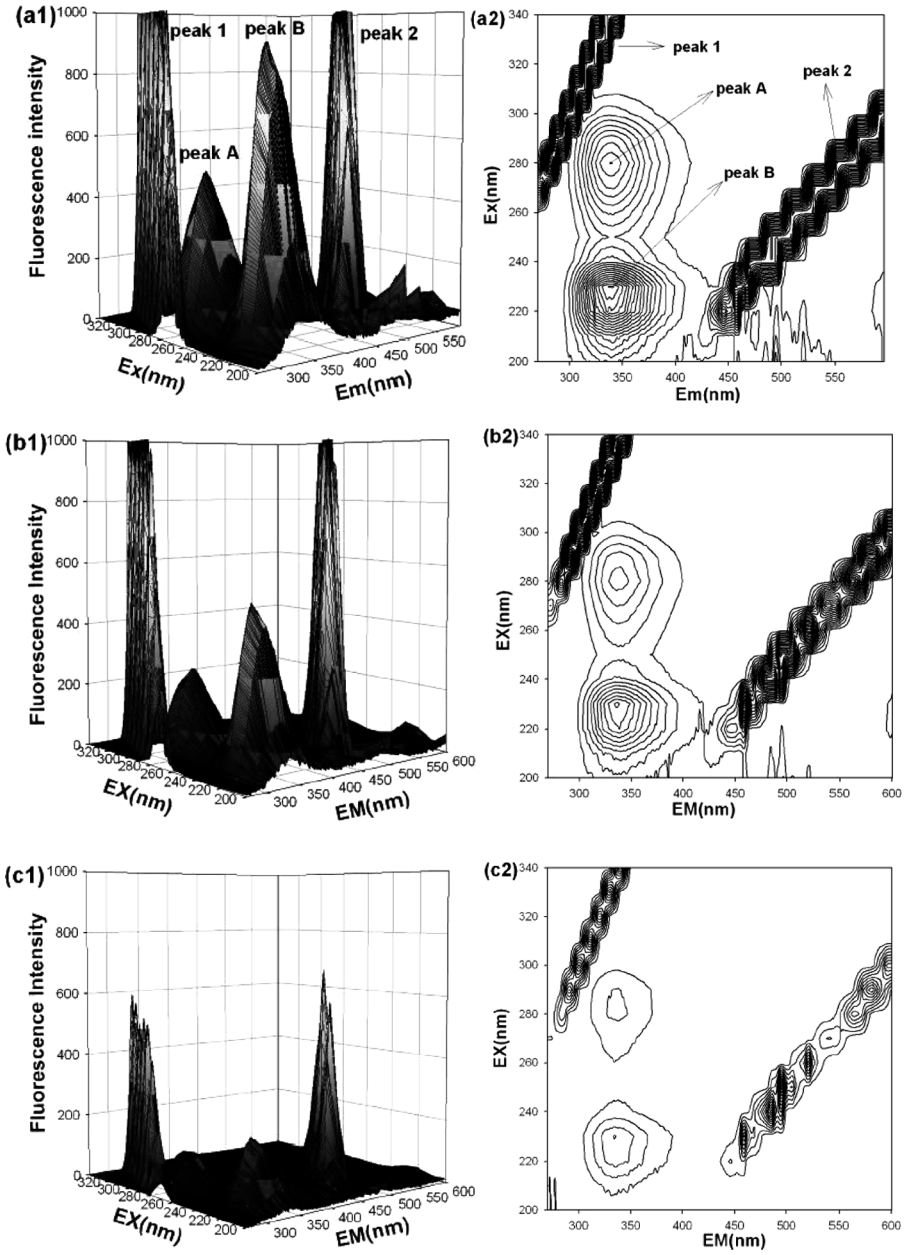

FIGURE 9 **The three-dimensional projections and the corresponding contour spectra of trypsin (a), rutin–trypsin (b, c). The concentration of trypsin: (a) 10.0 μM, (b) 10.0 μM, (c) 10.0 μM; the concentration of rutin: (a) 0 μM, (b) 20.0 μM, (c) 60.0 μM.**

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to $\lambda + \Delta\lambda$; $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ. In the present case, $n = 1.36$, $\Phi = 0.146$.[24] According to the above equations, we could calculate that $J = 9.36 \times 10^{-15}\,\mathrm{cm^3 \cdot L \cdot mol^{-1}}$, $E = 0.25$, $R_0 = 3.09\,\mathrm{nm}$,

TABLE 3 **Three-Dimensional Fluorescence Spectral Characteristics of Trypsin, Rutin–Trypsin System**

System		Peak A ($\lambda_{ex}/\lambda_{em}$)	$\Delta\lambda$ (nm)	Intensity	Intensity ratio	Peak B ($\lambda_{ex}/\lambda_{em}$)	$\Delta\lambda$ (nm)	Intensity
Trypsin		280/339	59	502.77	0.56:1	230/339	119	903.24
Rutin–trypsin [n(rutin):n(trypsin)]	2:1	280/334	54	280.18	0.55:1	230/334	104	511.28
	6:1	280/334	54	111.66	0.55:1	230/334	104	203.13

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$r = 3.70$ nm, $r < 7$ nm, which indicates that the energy transfer from trypsin to rutin occurred with high probability.

Conformation Investigation

To explore the structural change of trypsin by addition of rutin, we measured synchronous fluorescence spectra of trypsin (Fig. 6) with various amounts of rutin.

The synchronous fluorescence spectra give information about the molecular environment in the vicinity of the chromosphere molecules. When the D value ($\Delta\lambda$) between excitation wavelength and emission wavelength was stabilized at 15 or 60 nm, the synchronous fluorescence gave the characteristic information of tyrosine residues or tryptophan residues.[25] The effect of rutin on trypsin synchronous fluorescence spectroscopy is shown in Fig. 6. It is apparent from Fig. 6 that the emission maximums of tryptophan residues do red shift, which indicates that the polarity around the tryptophan residues was increased; however, the emission maximums of tyrosine residues do blue shift, which indicates that the hydrophobicity around the tyrosine residues was increased.[26] The conformations of trypsin were changed while binding rutin. It is also shown in Fig. 7 that the slope was higher when $\Delta\lambda$ was 60 nm, indicating that a significant contribution of tryptophan residues in the fluorescence of trypsin, rutin was closer to trytophan residues compared to tyrosine residues.

The CD spectrum provides information of the fractional content of different elements of secondary structure and changes in the tertiary structure of trypsin.[27] In the presence of rutin, the spectrum of trypsin is changed near 208 and 220 nm, which indicates that the random coil may be less with the increase in α-helicity of trypsin (Fig. 8). The residual CD spectrum following subtraction of the rutin spectrum from the trypsin–rutin complex could be due to any combination of conformational changes in protein.[28]

Total luminescence spectroscopy (TLS) techniques permit specific fluorescence signatures to be represented in a three-dimensional matrix or excitation–emission matrix. The three-dimensional fluorescence projections and the corresponding contour spectra are shown in Fig. 9.

Figure 9 presents the three-dimensional fluorescence spectrum of trypsin (Fig. 9a), and trypsin–rutin (Figs. 9b, 9c). As shown in Fig. 9, peak 1 and peak 2 are the Raleigh scattering peak ($\lambda_{ex} = \lambda_{em}$) and the second-ordered scattering peak ($\lambda_{em} = 2\lambda_{ex}$), respectively.[29] In Fig. 9a, two other typical fluorescence peaks can be easily observed in the three-dimensional fluorescence contour map of trypsin. Peak A mainly reveals the intrinsic fluorescence of tryptophan and tyrosine residues. Besides peak A, there is another new strong fluorescence peak B, which can provide some clues for us to investigate the characteristic of trypsin's characteristic polypeptide backbone structure C=O.[30] Analyzing from the intensity changes of peak A and peak B, they decreased obviously in the same degree in the trypsin–rutin system (Table 3). We can conclude that the interaction of rutin with trypsin disturbed the environment of the polypeptide of trypsin.

CONCLUSIONS

This article presents spectroscopic studies on the interaction of rutin with trypsin by different optical techniques. It was shown that the fluorescence of trypsin has been quenched for reacting with rutin and forming a new complex. The quenching belonged to static fluorescence quenching, with nonradiation energy transfer occurring within a single molecule. The results revealed the presence of a single class of binding site in the surrounding Trp and Tyr residues at the interface of trypsin; hydrophobic and electrostatic interactions played a major role in stabilizing the complex. The results of CD, synchronous fluorescence spectroscopy, and three-dimensional fluorescence spectra indicated that the structure of these tyrosine and tryptophan residue environments was altered and the secondary structure contents changed. The binding study of drugs with proteins is of great importance in pharmacy and pharmacology. This study is expected to provide important insight into the interactions of new trypsin inhibitors with proteinases.

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