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Comparison of Quenching Kinetics and Mechanism of Tryptophan by Acrylamide and Genistein Studied by Fluorescence Spectroscopy

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ABSTRACT

Fluorescence quenching using acrylamide and genistein as quenchers has been used to investigate the quenching kinetics and mechanism of tryptophan (Trp) monomer. Fluorescence quenching experiments of Trp by acrylamide were revised as references in present work. The *in vitro* inhibitory potential against α-glucosidase of genistein has been reported in previous literature, suggesting that the contacts of Trp residue of enzyme and genistein are responsible for the inhibitory activity of genistein. Therefore, genistein was selected as a quencher to symmetrically investigate the quenching kinetics and mechanism of Trp monomer in phosphate buffer pH 6.9. Prior to scanning fluorescence intensity of Trp solutions with (F) and without (F_o) quenchers in a wavelength range from 300-450 nm, fluorophore was excited at 295 nm. Consequently, the bimolecular quenching constants (k_q) were graphically extracted from the Stern-Volmer plot of F_o/F versus quencher concentration [Q]. The values of k_q for bimolecular quenching of Trp by acrylamide and genistein are 2.2×10^9 $M^{-1}s^{-1}$ and 2.0×10^{12} $M^{-1}s^{-1}$, respectively. These experimental results indicated that Trp was quenched by acrylamide through a dynamic quenching mode. Compared with acrylamide, the ground state formation, i.e., a static quenching process of Trp was dominant in the presence of genistein.

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1. Introduction

Fluorescence is the phenomenon of absorption of light at a particular wavelength by a fluorophore (F) to promote to an excited state and subsequently, the excited fluorophore (F^*) relaxes to the ground state by photon emission. Fluorescence quenching refers to the decrease in fluorescence intensity via radiative and/or non-radiative processes. In the presence of a quencher (Q), the interactions between F^* and Q such as electron transfer [1], energy transfer [2] or the complex formation [3] of F and Q result in the nonfluorescent transitions. The quenching process occurs either through dynamic or static quenching and both mechanisms require a contact between fluorophore and quencher [4]. In the case of dynamic (collisional) quenching, during the lifetime of F^* the quencher must diffuse to F^* to form a complex $(FQ)^*$, which decays to the ground state without emission. The population of F* can be reduced by forming a ground state complex (FQ). This quenching mechanism is defined as static quenching. It is noted that both mechanisms can be observed in several experiments, i.e., the collisions and ground state complex are present during the lifetime of F^* (Figure 1) [5].

In order to interpret the bimolecular quenching constant, k_q (Figure 1), fluorescence intensities of fluorophore are measured in the absence (F_o) and presence of quencher (Q). The linear dependence of F_o/F on the quencher concentration [Q] is known as the Stern-Volmer equation: $F_o/F = 1 + K_{SV}[Q]$. Quenching data are described as a plot of F_o/F versus [Q]. The k_q value can be obtained from slope of the Stern-Volmer plot, $K_{SV} = k_q\tau_o$ (τ_o is the lifetime of F^* in the absence of Q). The k_q reflects the efficiency of quenching, i.e., the accessibility of the quencher to the fluorophore. Therefore, this parameter is considered in terms of the rate constant for bimolecular diffusion-controlled reactions, which result in values of k_d ($k_d = 4\pi N\times r_{AB}\times D_{AB}$: N is the Avogadro number. r_{AB} (4×10^{-10} m) is approximately the sum of molecular radii of solutes A (r_A) and B (r_B). D_{AB} (2×10^{-9} m^2s^{-1}) refers to the

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diffusion coefficient. Thus, k_d was calculated and often taken as ca. 1.0×10^{10} $M^{-1}s^{-1}$) [6]-[9]. The diffusion-controlled quenching can be distinguished from the static quenching by measuring K_{SV} at various temperatures [6], [8], [10], [11], or lifetimes with (τ) and without (τ_o) quenchers [5], [12], [13]. In addition, the experimental k_q values can be used to indicate the quenching mechanism. The values of k_q smaller than k_d refer to a quenching process by the dynamic quenching mechanism, while the larger values indicate a static quenching via ground state complex [6].

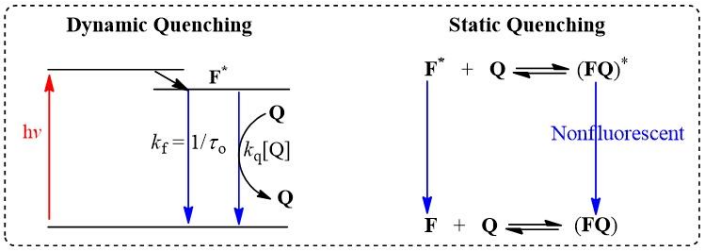

Figure 1. *Comparison of dynamic and static quenching. For a dynamic quenching process, fluorophore absorbs a photon (hv) to the excited state (F^*). The F^* state returns to the ground state either by emission with rate constant, $k_f = 1/\tau_o$ or a bimolecular quenching step in the presence of quencher Q. The formation of a ground state complex (FQ) refers to a static quenching. Figure was taken from Ref. [6].*

The kinetics of fluorescence quenching has been widely studied in both fundamental and biological processes. There are many substances acting as fluorophores and quenchers. The fluorophores of amino acids in biological systems including tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) are classified as intrinsic fluorophores (Figure 2). The fluorescence of enzyme/protein originates from these amino acids. The emission of Trp is due to the indole moiety and thus, the fluorescence quenching experiments of Trp can reveal the molecular interactions between inhibitors and enzymes/proteins [5], [8]-[13]. Quenchers like oxygen and acrylamide (Figure 2) demonstrate high quenching efficiencies (near unity) [14]-[18] toward Trp fluorescence. In addition, acrylamide is a small and neutral molecule and interacts with Trp via physical contact with the indole ring. Acrylamide quenching of Trp is a dynamic quenching with $k_q = 5.9\times10^9$ $M^{-1}s^{-1}$ [4], [19]. Therefore, the Trp fluorescence quenching experiments were revised by selecting acrylamide as an efficient quencher.

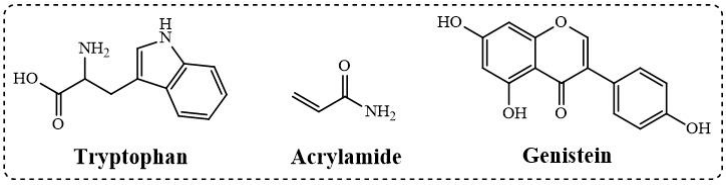

Figure 2. *Chemical structures of tryptophan, acrylamide and genistein.*

Genistein (Figure 2) is an isoflavone found in soybean and exhibits a promising inhibitory activity against α-glucosidase, an enzyme hydrolysing carbohydrate to release α-glucose in blood [20]-[22]. Inhibition kinetics and mechanism of genistein quenching of α-glucosidase have been reported in detail [21]-[23]. However, to the best of our knowledge, genistein kinetics and mechanism quenching of Trp monomer have not been fully reported so far. Currently, our group is focusing on studying the inhibition kinetics and mechanism of flavonoids against α-glucosidase. Therefore, in present work, we firstly select genistein as a quencher to throw light on the genistein kinetics and fluorescence quenching mechanism of tryptophan fluorescence, thereby guiding ongoing research on α-glucosidase. Furthermore, Trp fluorescence quenching by acrylamide has also been revised by monitoring the fluorescence intensity of Trp monomer at various concentrations of acrylamide.

2. Materials and Methods

2.1. Materials and solution preparations

Chemicals were purchased from suppliers without further purification. Acrylamide (99%, Acros Organics, Belgium), genistein (99%, Thermo Fisher Scientific, USA), tryptophan (Trp, 99%, Acros Organics, Belgium) were utilized as quenchers and fluorophore, respectively. Dimethylsulfoxide

(DMSO, 99%, Thermo Fisher Scientific, USA), K_2HPO_4 (99%, China) and KH_2PO_4 (99%, China) were used as solvent and chemicals for preparing the buffer solution. Quencher and fluorophore are dissolved completely in phosphate buffer solution (pH 6.9, 50 mM, DMSO 2%). Trp was prepared as 1.0 mM in buffer solution. Acrylamide was prepared as a 5.0 M stock solution, while the concentration of a stock solution containing genistein was 0.25 mM. For Trp fluorescence quenching by quenchers, acrylamide concentrations were varied in a range from 0.0 to 0.4 M, while genistein was added to Trp solution to produce various concentrations in the range 0.0 to 150 µM.

2.2. Fluorescence quenching measurements

Solutions containing Trp and quenchers were stirred for 10 min prior to transferring to a septa-sealed quartz cuvette. In order to remove dissolved oxygen, the solutions were sparged with nitrogen gas for 10 min. The emission spectra of Trp in the absence and presence at different concentrations of quenchers were scanned in three separate measurements using a Horiba spectrofluorometer (FluoroMax-4, Horiba, Japan). The fluorophore was excited at 295 nm and its emission was monitored in a wavelength range of 300-450 nm. Slit widths for both excitation and emission are 2.0 nm. The fluorescence data were plotted and analyzed using OriginPro 9.0 software.

3. Results and Discussion

3.1. Quenching of Trp by acrylamide

Quenching of Trp emission in phosphate buffer solutions (pH 6.9) was monitored with an addition of acrylamide at various concentrations. As depicted in Figure 3 (left panel), Trp exhibited an emission in the wavelength range of 300-450 nm with an intensity maximum at 357 nm upon excitation at 295 nm. The addition of increasing acrylamide amounts resulted in a progressive decrease in the fluorescence intensity of Trp. The fluorophore emission was quenched about 90% at 357 nm by acrylamide (0.4 M), indicating that acrylamide is an efficient quencher of the fluorescence of Trp [16]. It should be noted that no photochemical products were detected using reverse-phase high-performance liquid chromatography (RP-HPLC) when Trp was irradiated with acrylamide. In other words, the reduction of Trp fluorescence intensity in the presence of acrylamide was attributed to the physical quenching mechanism [16].

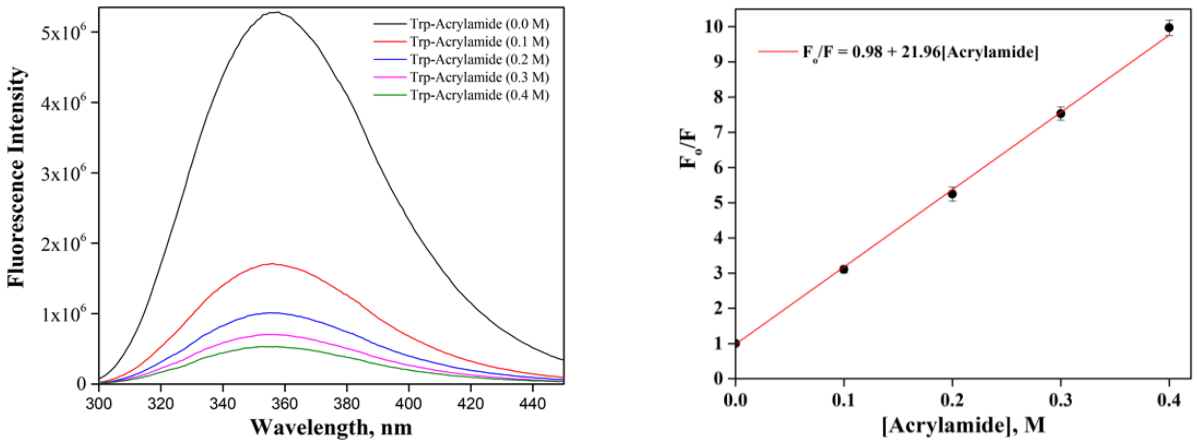

Figure 3. *Left panel: Fluorescence spectra of Trp (1.0 M) obtained in the absence and presence of increasing acrylamide concentrations (0-0.4 M) in phosphate buffer pH 6.9. Right panel: Trp quenching by acrylamide as observed by the ratios of the Trp fluorescence intensities at 357 nm in the absence and presence of acrylamide. The red line refers to a fitting line of experimental points using OriginPro 9.0 software.*

In order to verify the kinetics and quenching mechanism of Trp by acrylamide, the fluorescence data were treated according to the Stern-Volmer equation by plotting F_o/F against acrylamide concentration. As presented in Figure 3 (right panel), it was found that the Stern-Volmer plot conformed to the linear form. The slope of the plot yielded a Stern-Volmer quenching constant of K_{SV} = 21.96±0.33 M^{-1}, as shown in Table 1. This value is consistent with previous results reported in literatures, in which the

fluorescence quenching of Trp in the presence of acrylamide (0-0.5 M) resulted in a Stern-Volmer quenching constant of K_{SV} = 21±3 M^{-1} [17] and the value of K_{SV} = 20±2 M^{-1} [18]. The lifetime (τ_o) of excited Trp in the absence of quencher was 10^{-8} s [6], [9] and thus, the bimolecular quenching constant, k_q was calculated from K_{SV} ($K_{SV} = k_q \tau_o$) as 2.2×10^9 $M^{-1}s^{-1}$ (Table 1). The value of k_q is smaller than the diffusion-controlled limit ($k_d \sim 1.0\times10^{10}$ $M^{-1}s^{-1}$), indicating a collision (dynamic) quenching mechanism of acrylamide with Trp, which was in agreement with the observations of similar previous work (k_q = 5.9×10^9 $M^{-1}s^{-1}$). As mentioned above, k_q reflects the accessibility of the quencher to the fluorophore. Acrylamide is an efficient quencher toward Trp fluorescence (near unity). However, acrylamide is larger than oxygen and thus, it has a smaller diffusion coefficient. Therefore, the smaller value of k_q compared to oxygen ($k_q = 1.2\times10^{10}$ $M^{-1}s^{-1}$) was expected, which resulted from the steric shielding [6], [15].

Table 1. *Fluorescence quenching kinetics and mechanism of Trp by acrylamide and genistein.*

Parameter/Mechanism	Trp/Acrylamide	Trp/Genistein
K_{SV} (M^{-1})	21.96± 0.33	2.0×10^4±0.0004
k_q ($M^{-1}s^{-1}$)	2.2×10^9±0.33	2.0×10^{12}±0.0004
τ_o (s)	10^{-8}	10^{-8}
Quenching mechanism	Dynamic quenching	Static quenching

3.2. Quenching of Trp by genistein

Genistein, an isoflavone isolated from soybean, displayed a remarkable inhibitory activity against α-glucosidase with an in vitro IC_{50} ~ 8.0 µM [21], [22], [24]. A recent report has demonstrated the inhibition mechanism of genistein against α-glucosidase using enzyme fluorescence quenching measurements. The intrinsic fluorescence of α-glucosidase indicated that α-glucosidase was quenched by genistein through the static quenching mode [25]. However, a study on genistein quenching kinetics and mechanism of Trp monomer has not been fully investigated so far. In present work, we elucidated the quenching kinetics and mechanism of Trp monomer using genistein as quencher by fluorescence spectroscopy.

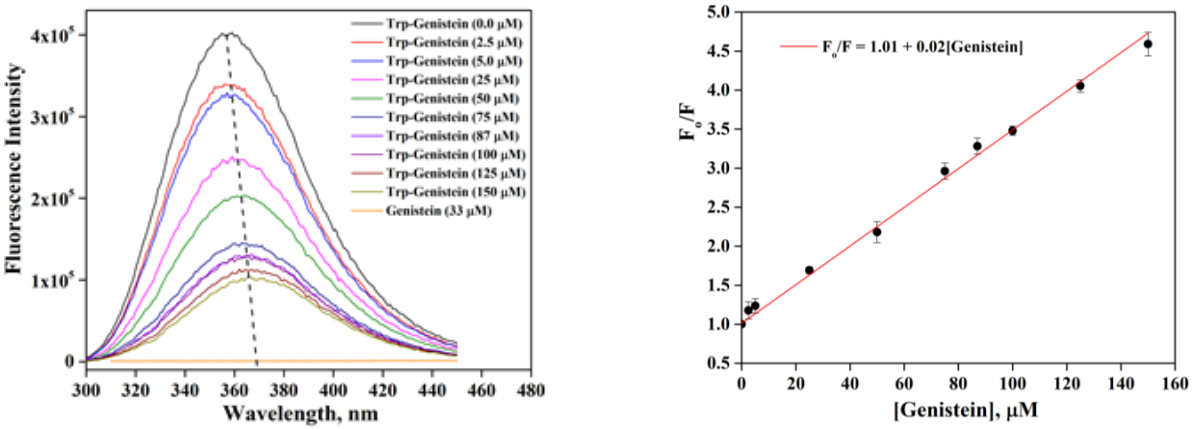

Figure 4. *Left panel: Fluorescence spectra of Trp (1.0 M) in the absence and presence of genistein at various concentrations (0-150 µM) in phosphate buffer pH 6.9. The dashed line depicts the positions of maximum peaks of Trp with increasing genistein concentrations. Right panel: The Stern-Volmer plot of fluorescence quenching of Trp by genistein. The concentrations of genistein were varied in a range from 0 to 150 µM. The red line illustrates a fitting line of experimental points using OriginPro 9.0 software.*

As shown in Figure 4 (left panel), when excited at 295 nm, Trp exhibited an emission signal in a wavelength range from 300-450 nm with a maximum fluorescence peak at 359 nm, while the emission of genistein was negligible. The Trp fluorescence intensity was quenched about 74% at the genistein concentration of 150 µM, indicating a physical contact between Trp and genistein. In addition, the emission of Trp was shifted in the presence of genistein. The red-shift spectrum of Trp at 366 nm (Δλ = 7 nm) was observed and a genistein concentration of 150 µM was added to the fluorophore solution.

This effect is attributed to the accessibility of Trp exposed in the aqueous phase to genistein [6], [25]. Alternatively, Trp molecules emitting at shorter wavelengths are quenched more readily than the longer wavelength Trp monomers.

The Stern-Volmer equation was utilized to explore the kinetics and mechanism of the photophysical intermolecular deactivation process of Trp by genistein. The linear Stern-Volmer graph was observed by plotting F_o/F versus genistein concentration (Figure 4-right panel). As it is evident from fluorescence data, the slope of the Stern-Volmer plot was denoted as the quenching constant $K_{SV} = 2.0\times10^4$ M^{-1}. As a result, the bimolecular quenching constant k_q of 2.0×10^{12} $M^{-1}s^{-1}$ was obtained from $K_{SV} = k_q\tau_o$. The resulting k_q value is two orders of magnitude greater than $k_d \sim 1.0\times10^{10}$ $M^{-1}s^{-1}$, suggesting that fluorescence quenching of Trp by genistein occurred by static quenching resulting from the Trp-genistein ground state complex formation.

4. Conclusions

Overall, the kinetics and mechanism of Trp fluorescence quenching by quenchers such as acrylamide and genistein were investigated using fluorescence experiments. The fluorescence quenching experiments of Trp monomer by acrylamide were revised by adding quenchers at various concentrations (0-0.4 M). The experimental results are in agreement with the previous reports. The Trp excited states were depopulated with increasing acrylamide amounts with a bimolecular quenching constant, $k_q = 2.2\times10^9$ $M^{-1}s^{-1}$ resulting from the dynamic quenching mechanism. Furthermore, genistein was selected as a quencher for Trp monomer fluorescence. The emission of Trp was gradually quenched with increasing genistein concentrations in a range from 0 to 150 µM. The fluorescence quenching of Trp monomer was attributed to the static quenching process with a bimolecular rate constant $k_q = 2.0\times10^{12}$ $M^{-1}s^{-1}$, i.e., the formation of a ground state complex was dominant. These findings further support the published results obtained by fluorescence quenching of α-glucosidase containing Trp residue, which is responsible for the interactions with genistein inhibitor.

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Conceptualization, H. Phuong and H. M. Hao; preparations of samples and fluorescence experiments, H. Phuong, N. P. Dat; writing-original draft, D. D. Khoi, H. M. Hao; writing-review and editing, H. Phuong, H. M. Hao. “This work belongs to the project grant No: T2024-169 funded by the Ho Chi Minh City University of Technology and Education, Vietnam”

Conflict of Interest

The authors declare no conflict of interest.

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