Enhanced UV Photo-Stabilization of Tyrosine with Benzotriazole Structure Formed Through Chemical Modification

Zongqian Wang*, Yinchun Fang and Jun Li
School of Textile and Garment, Anhui Polytechnic University, Wuhu Anhui, 241000, P. R. China.
wzqkeyan@126.com*

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Summary: In this paper, tyrosine was modified by the benzotriazole structure through the coupling reaction with the diazonium salt to form the intermediate and the ring-closing reaction of the intermediate to form the benzotriazole structure. Structure of intermediate and modified tyrosine was characterized via high performance liquid chromatography (HPLC), fourier transform infrared spectroscopy (FTIR), hydrogen nuclear magnetic resonance (^{1}H-NMR) and mass spectra (MS) methods; then the photo-degradation curve under ultraviolet (UV) exposure of the modified tyrosine was measured and compared with the tyrosine; UV and fluorescence spectrum were used to analyze the photo-stabilization mechanism of the modified tyrosine. Results showed that the photo-degradation rate of modified tyrosine was less than one fifth of tyrosine after UV irradiation for 2 h and far less than that of tyrosine in the whole irradiation experiment in this work, proving that the photo-stability of the tyrosine was greatly improved by introducing the benzotriazole structure after the modification. From the analysis of UV and fluorescence spectrum, the photo-stabilization mechanism of the modified tyrosine was revealed.

Keywords：Tyrosine, Benzotriazole structure, Photo-degradation, Fluorescence spectra, Mechanism.

Introduction

Tyrosine is the main component of silk fibroin with the weight ratio of 10.84% [1], and considered to be an important role in the photo yellowing of silk, wool and other protein fibers especially under UV irradiation [2-4]. Tyrosine contains an aromatic ring structure and has characteristic absorption of UV light, and tyrosine was confirmed to be involved in photo oxidative reactions resulting in the formation of yellow products [5]. Furthermore, in our previous work, we confirmed that the tyrosine content in silk fibroin declined gradually with the prolonging of UV exposure time [1]. In silk fibroin, tyrosine presents as tyrosine residue and is mainly distributed on the surface and in the amorphous area of the silk fibroin which would easily attacked by the external reagents [6-8], indicating that tyrosine residues and tyrosine have the similar reaction characteristics. Due to the poor photostability of silk, improvement its UV photo-stabilization is alway a research topic, and modification on the tyrosine residues to cut off the UV energy transfer channel in silk fibroin molecules is a useful way to improve its photostabolity. Also in our previous work, tyrosine residues in silk fibroin were modified by the chemical synthesis to form the covalent bonding with UV characteristic absorption groups which was an effective method to improve its photostability [9]. Therefore, study on the photostability of tyrosine is meaningful and the research results will provide the basis for the research of the photostability of silk fibroin and other proteins.

Benzotriazole structure UV absorber possessing high molar adsorption coefficient in the wavelength band of 300~385 nm is a kind of high efficient photostabilizers with low toxicity which is widely used in the food packaging, textiles, paint and other material fields [10, 11]. The UV protection mechanism of the benzotriazole structure UV absorbers have been studied for a long time and obtained a lot of system theories, in which of them phototautomer theory is the widely accepted and recognized [12, 13]. The efficient of the tautomerization process is extremely high and almost repeating indefinitely. Thus the benzotriazole structure UV absorbers possess high photostability. In this paper, tyrosine was modified by the benzotriazole structure through the coupling reaction with the diazonium salt to form the intermediate and the ring-closing reaction of the intermediate to form the benzotriazole structure. The chemical structure of the modified tyrosine was characterized by Fourier transform infrared spectroscopy (FTIR), hydrogen nuclear magnetic resonance (^{1}H-NMR) and mass spectra (MS). The photo-degradation curve under ultraviolet (UV) exposure of the modified tyrosine was measured and compared with the tyrosine; UV and fluorescence spectrum were used to analyze the photo-stabilization mechanism of the modified

*To whom all correspondence should be addressed.

tyrosine. This research would provide the experimental basis of the chemical modification method to improve the photostability of the tyrosine and silk protein.

Experimental

Materials and reagents

L-tyrosine standard substance (≥99.5%), o-nitroaniline, hydrochloric acid (37%), sodium nitrite, thiourea dioxide (TD), sodium hydroxide, N,N-dimethyl formamide (DMF), methanol, tetrahydrofuran (THF), ethyl acetate (EA) and methylene chloride (CH_2Cl_2) were of analytical reagent grade and supplied by Aladdin Chemicals Co. Ltd, China. Ultra pure water was used in this work.

Instruments and Equipment

XPA-Ⅱ photochemical reactor (Nanjing Xujiang Equipment Co., Ltd. China), and which equipped with a 500W ultraviolet Hg lamp (Philips, USA), F-4600 Fluorimeter (Hitachi, Japan), Lambda 900 spectrolightmeter (PerkineElmer, USA), Agilent 1260 LC HPLC (Agilent Technologies company, USA), IR Prestige-21 Fourier transform infrared spectrometer (Shimadzu, Japan), pHS-3C pH meter (Shanghai Leici Equipment Co., Ltd. China), DKB-1915 Low temperature thermostat bath (Shanghai Jing Hong Experimental Equipment Co., Ltd. China).

Synthesis of modified tyrosine

The modification of tyrosine was conducted following the process shown in Fig. 1.

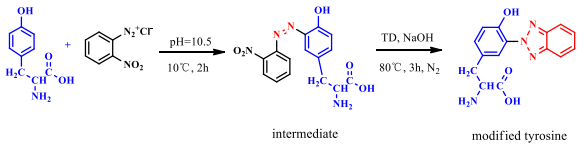


Fig. 1: The modify route of tyrosine by benzotriazole structure.

Stage 1: Preparation of diazonium salt

0.06 mol o-nitroaniline was added into 250 mL three-necked flask, and then 0.30 mol concentrated HCl and 30 mL distill water were added slowly. The mixture was heated to 70 ˚C slowly and maintained at this temperature till the complete dissolving of o-nitroaniline. And then the temperature of o-nitroaniline dissolving solution was controlled to below 5 ˚C. 0.072 mol sodium nitrite was added into the o-nitroaniline solution in three times and each time with an interval of 5 min. The diazotization reaction was strictly controlled below 5 ˚C for 30 min. The prepared o-nitroaniline diazotization solution was kept in the ice bath avoiding light.

Stage 2: Preparation of intermediate

0.03 mol tyrosine and 30 ml sodium carbonate solution with pH=11 were added into the 500 mL three-necked flask. The mixed solution was slowly heated to 40 ˚C and kept at this temperature till the tyrosine completely dissolved. The temperature of the tyrosine dissolved solution was reduced to 10 ˚C slowly in case the precipitation of tyrosine. The coupling reaction was conducted by adding dropwise the prepared o-nitroaniline diazotization solution into the tyrosine solution. The temperature of the coupling reaction mixture was precisely controlled below 10 ˚C, and the pH of which was adjusted by the sodium carbonate solution of pH =11 and maintained at 10.5 ± 0.5. After 3 h reaction, silica-gel plate chromatography method was used to determine the reaction end point. After the finishing of the reaction, the reaction solution was acid separated by hydrochloric acid (HCl) solution (pH=2). Then it was washed by HCl solution (pH=4) for 3 times, suction filtered and heated at 60 ˚C under vacuum to obtain the intermediate. The intermediate was purified by column separation (the separation column was lad-made, the fillers of the column was ODS-A-HG C18 reverse phase spherical silica gel, the particle and pore diameter of silica gel were 50 μm and 12 nm). The synthesized product was dissolved in methanol aqueous solution (the volume ratio of methanol and water was 1:1) to prepare saturated solution. The mobile phase of the column separation was methanol aqueous solution with the volume ratio of 7:3 which flushed the separation column with the flow rate of 0.5 ml/min controlling by the the high pressure infusion pump. The separated solution with the purity of higher than 99% was collected and rotary evaporated to remo ve the solvent. The purified intermediate was obtained after drying at 60 ˚C on vacuum oven. The structure of intermediate compound was characterized and confirmed by FTIR, ^{1}H-NMR, and MS. The ^{1}H-NMR spectrum of intermediate was shown in Fig. 2. ^{1}H-NMR(DMSO)/ppm: δ 10.52 (s, 1H, -OH), 8.03(s, 2H, Ar-H), 7.76 (d, 1H, Ar-H), 7.55(d, 2H, Ar-H), 7.30(d, 1H, Ar-H), 7.07(d, 1H, Ar-H), 3.71(d, 1H, -CH), 2..85~3.11 (d, 2H, -CH_2); MS (m/z, %): 331.12 (M+H, 100).

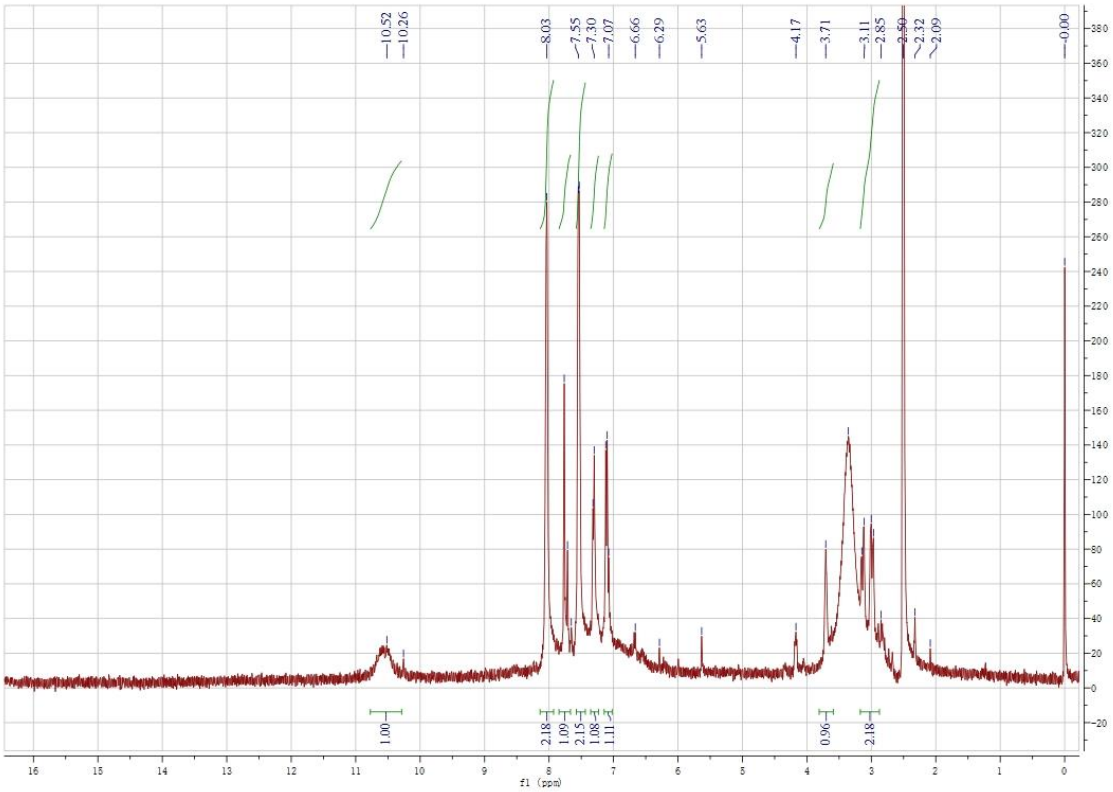


Fig. 2: ^{1}H-NMR spectrum of intermediate.

Stage 3: Preparation of the modified tyrosine by benzotriazole structure

The intermediate and sodium hydroxide with the molar ratio of 1:11 and 300 mL distilled water were added into the 500 mL three-necked flask which was slowly heated to 80 ℃. Then a certain amount of thiourea dioxide (the molar ratio of the intermediate and thiourea dioxide was 1:5) was added into the mixture slowly under protection of N_2 atmosphere. When the reaction conducted for 3 h, silica-gel plate chromatography method was used to determine the reaction end point. After the finishing of the reaction, the reaction solution was acid separated by HCl solution (pH=2). Then it was washed by HCl solution (pH=4) for 3 times, suction filtered and heated at 60 ℃ on vacuum oven to obtain the benzotriazole modified tyrosine product. The purified modified tyrosine product was obtained through the same purifying process of the intermediate. The structure of intermediate compound was characterized and confirmed by FTIR, ^{1}H-NMR, and MS. The ^{1}H-NMR spectrum of modified tyrosine was shown in Fig. 3. ^{1}H-NMR(DMSO)/ppm: δ 11.60 (s, 1H, -OH), 8.85 (s, 1H, Ar-H), 8.45(d, 2H, Ar-H, Ar-H), 8.10(d, 2H, Ar-H, Ar-H), 7.90(d, 1H, Ar-H), 7.82(d, 1H, Ar-H), 5.25(s, 1H, -CH), 3.92~4.16(d, 2H, -CH_2); MS (m/z, %): 299.10 (M+H, 100).

Spectrum test

The tyrosine and modified tyrosine were dissolved in different solvents and measured by Lambda 900 spectrolightmeter (PerkineElmer, USA) to determine their UV spectrum. The corresponding pure solvent was as the reference solution with the scanning wavelength scope from 250 to 500 nm.

The non-modified and modified tyrosine were dissolved in different solvents and measured by F-4600 Fluorimeter (Hitachi, Japan) to determine their fluorescence spectrum. The fluorescence spectrum influencing by different solvents was analyzed. The excitation wavelength was set as 200~700 nm. In order to avoid the influence of first order Rayleigh scattering, the scanning wavelength scope was selected from 215 to 700 nm per nanometre under voltage of 220 v.

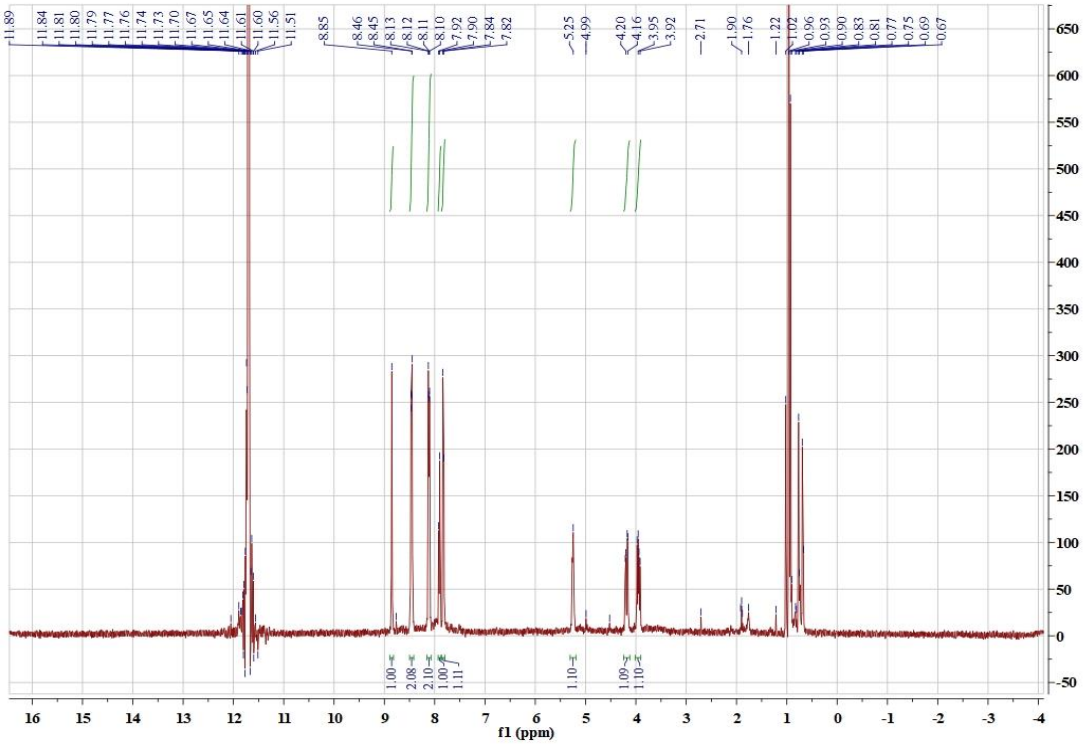


Fig. 3: ^{1}H-NMR spectrum of modified tyrosine.

HPLC test

The high performance liquid chromatography (HPLC) was Agilent 1260 LC (Agilent Technologies company, USA) with the ZORBAX Eclipse XDB-C18 chromatography column (150mm×4.6mm, 3.5μm particle diameter). The gradient mobile phase with the flow rate of 1.0 ml/min was used in this research. The volume ratio of 8 mmol/L formic acid aqueous solution and acetonitrile of the mobile phase changed from 95:5 to 5:95 during 0~9 minutes. And from 9 to 12 minutes, the volume ratio of them was kept at 5:95. The injected amount of sample was 20 μL under the column temperature of 30 ℃ and maximum pressure of 260 bar. Using G1315B DAD as the detector, the detection wavelength was set according to the characteristic adsorption peak of the samples.

Determination of photodegradation rate

The photodegradation experiment was conducted on XPA-Ⅱ photochemical reactor (Nanjing Xujiang Equipment Co.,Ltd., China) which equipped with a 500 W ultraviolet Hg lamp (Philips, USA). The HPLC peak area of samples which were UV irradiated for different time was determined, and the photodegradation rate was calculated from equation (1).

$$A\% = (1 - \frac{A_1}{A_0}) \times 100\% \quad (1)$$

where A_i was the HPLC peak area of samples which were UV irradiated for different time, A_0 was the HPLC peak area of samples before irradiation.

Results and Discussion

Characterization of the modified tyrosine

The tyrosine was modified by the benzotriazole structure through the coupling reaction with the diazonium salt to form the intermediate and the ring-closing reaction of the intermediate to form the benzotriazole structure, then the chemical structure of the intermediate and modified tyrosine were characterized in this paper. The chemical structure the intermediate and the modified tyrosine was characterized and the results of ^{1}H-NMR and MS were as shown in the experimental section. The result of FTIR was as shown in Fig 4, the purity of modified tyrosine was determined by HPLC as shown in Fig 5.

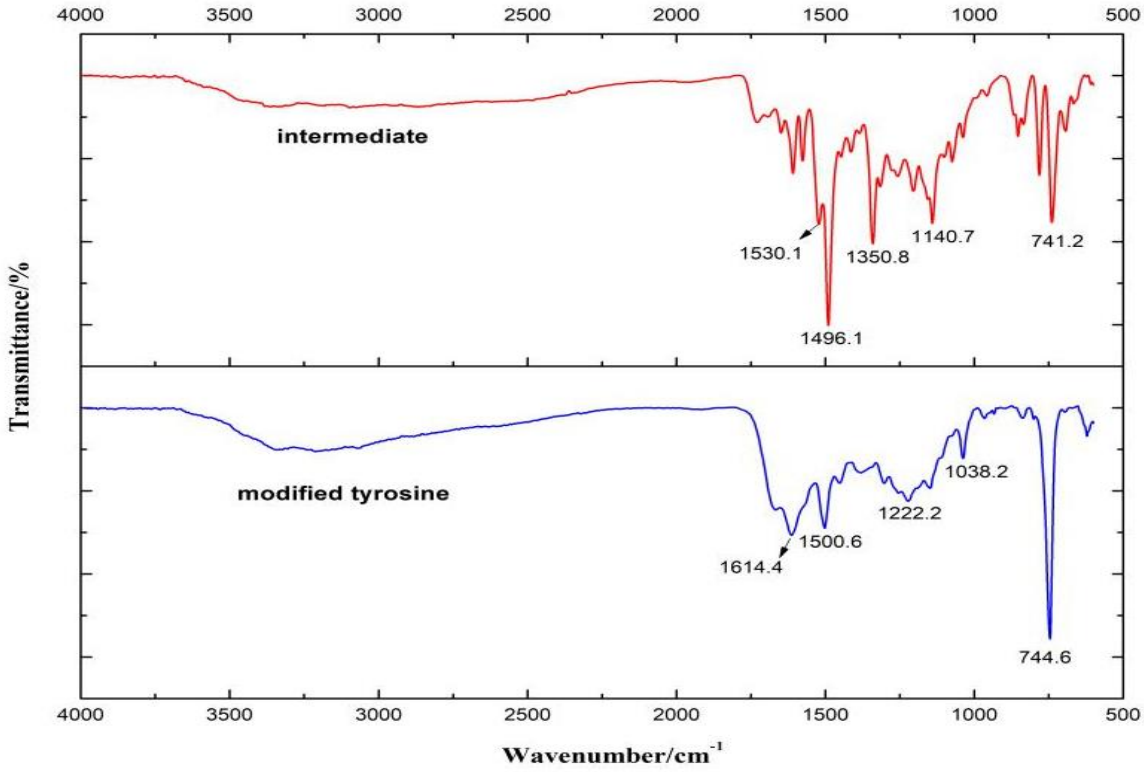


Fig. 4: FTIR spectra of the intermediate and modified tyrosine.

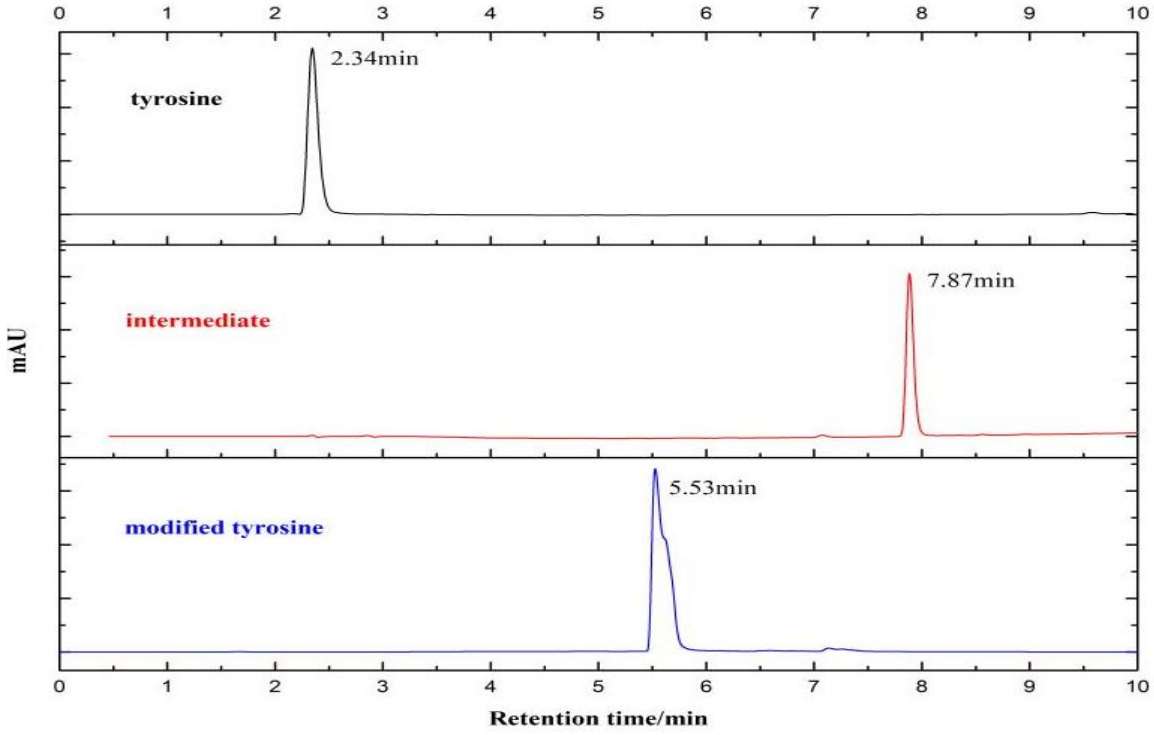


Fig. 5: HPLC spectrum of tyrosine and modified tyrosine.

The intermediate was prepared from the tyrosine which was modified by diazonium salt containing "-NO_2" in its chemical structure. As it can be seen from Fig. 4, the absorption peaks at 1530.1 cm^{-1} and 1350.8 cm^{-1} were attributed to the stretching vibration peaks of "-NO_2" existing in FTIR spectra of the intermediate [14]. While these two absorption peaks were disappeared in the FTIR spectra of modified tyrosine due to the "-NO_2" was reduced. The results of FTIR spectra combining with ^{1}H-NMR and MS indicated that the target modified tyrosine was successfully synthesized.

As Fig.5 shows, the peak of tyrosine appeared at 2.34 min under the setting chromatography condition in this research, while the peak of modified tyrosine appeared at 5.53 min. The different peak position of the non-modified and modified tyrosine indicated the changing of molecular polarity after the modification. Also from Fig. 5, the purity of the modified tyrosine after the column separation was higher than 98.85%. The modified tyrosine for subsequent experiments was the same product with the HPLC testing.

Photodegradation of the modified tyrosine

The modified tyrosine and tyrosine were dissolved in 10 mmol/L formic acid aqueous solution, respectively. The same molar concentration of these two solutions were prepared and irradiated by 300 w mercury lamp. The peak area of tyrosine and modified tyrosine after irradiating different times was determined by HPLC. The photodegradation rates of different irradiation time were calculated according to equation 1 and shown in Fig. 6.

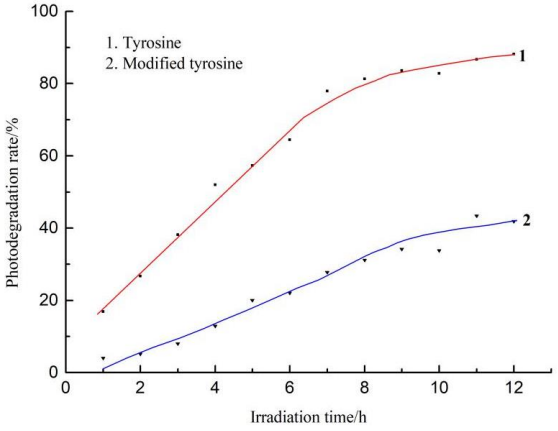


Fig. 6: Photodegradation curves of tyrosine and the modified tyrosine

As it can be seen from Fig. 6, the photodegradation rate of modified tyrosine was greatly reduced comparing with the tyrosine. The photodegradation rate of modified tyrosine was less than one fifth of the tyrosine after exposure for 2 h about 5.1% and 26.7%, respectively. After exposure for 12 h, the photodegradation rate of tyrosine was almost 85%, whereas the modified tyrosine was less than 40%. During the whole irradiation experiment, the photodegradation rate of modified tyrosine was far less than that of tyrosine. This result demonstrated that the photostability of tyrosine was greatly improved by introducing the benzotriazole structure after the modification.

UV spectrum of modified tyrosine analysis

The modified tyrosine was dissolved in several solvents with different polarity, and the UV spectrum was measured and shown in Fig. 7.

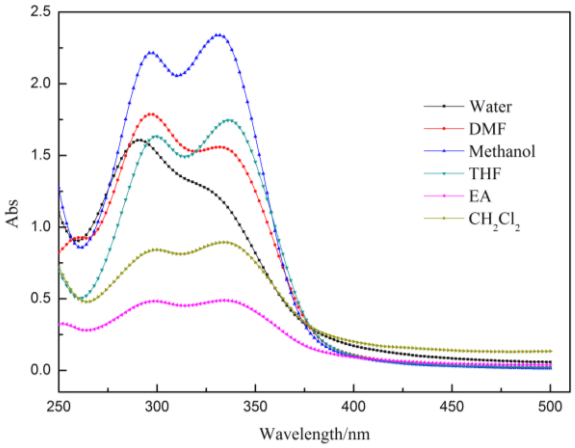


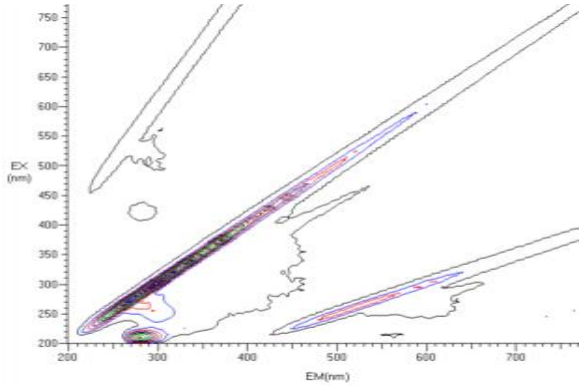
Fig. 7: UV spectrum of the modified tyrosine in different solvents.

As it can be seen from Fig. 7, the modified tyrosine exhibited a strong UV adsorption in the range of 270~380 nm, and each spectrum curve was saddle-shaped, with two peaks around 290 and 330 nm, respectively. And this saddle-shaped spectrum was considered to be the spectral characteristic of the typical ultraviolet adsorber containing benzotriazole structure. This absorption characteristic was arisen from the electron transition from the π orbit of the phenolic ring in the chemical structure of the modified tyrosine to the π orbit of benzotriazole structure, namely the absorption arisen from the electron transition of $\pi \rightarrow \pi^*$. The electron transition was the basis of intramolecular charge transition in the phototautomer structure which was formed from the

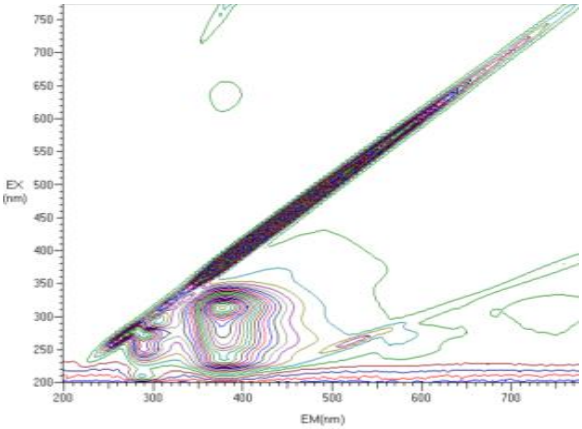
modified tyrosine molecule by the UV irradiation [14-16]. However, under different solvents, the relative heights of the modified tyrosine peaks at 290 and 330 nm were different as well as the highest absorption peaks were slightly shifted. The relative height peak at 330 nm was lower than that at 290 nm in the strong polarity solvents such as water and DMF; further, the relative height peak at 330 nm gradually enhanced with the polarity of solvent declining. Phototautomer of benzotriazole structure was related to polarity and protonation degree of solvents [18, 19], so the effect of the solvent property on the UV photo-stabilization of the modified tyrosine is worth to further study.

Fluorescence spectrum of modified tyrosine analysis

Tyrosine and the modified tyrosine were dissolved in ether respectively, then the fluorescence spectrum was measured and shown in Fig. 8.



a. Tyrosine



b. Modified tyrosine

Fig. 8: Fluorescence spectrum of tyrosine and the modified tyrosine.

As Fig. 8 shows, the fluorescence spectrum of modified tyrosine was obviously different from that of tyrosine, the modified tyrosine showed a characteristic fluorescence performance and could be excited by 310 nm UV light while emitting 380 nm visible light, indicating that it can convert the high-energy UV light into the low-energy visible light. This energy conversion is inferred to be caused by the phototautomerism of the modified tyrosine. The ground state Enol structure of the modified tyrosine will be transferred to the excited state Keto structure when excited by 310 nm UV light, leading to a large stokes's shift between the adsorption and emission spectra of the benzotriazole structure in modified tyrosine. All in all, the photo-stabilization mechanism of the modified tyrosine is due to the phototautomerism of the benzotriazole structure formed by the chemical modification.

Conclusions

Tyrosine was modified by benzotriazole structure through chemical modification of coupling and ring-closing reactions in this work, and the structure of the modified tyrosine was characterized. The photo stability of modified tyrosine was significantly improved, and the photo-stabilization mechanism of the modified tyrosine is the phototautomerism of the benzotriazole structure formed by this chemical modification. This research provides the experimental basis for the improvement of the photo stability of the tyrosine and silk protein using benzotriazole structure by chemical modification method.

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