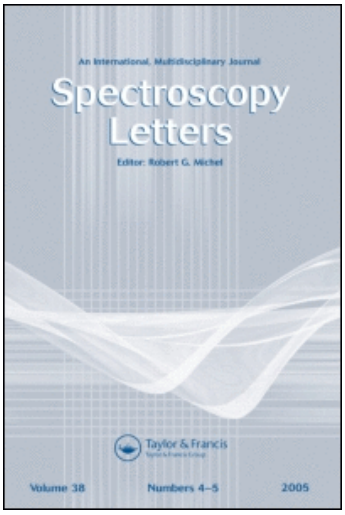

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SYNCHRONOUS FLUORESCENCE SPECTRA OF FIBRINOLYTIC PRINCIPLE FROM SNAKE VENOM OF *AGKISTRODON ACUTUS*

Shou-Ye Wang[a]; Qing-Liang Liu[a]; Xiao-Long Xu[a]; Yong-Shu Xie[a]
[a] Department of Chemistry, University of Science and Technology of China, Hefei, P.R. China

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SYNCHRONOUS FLUORESCENCE SPECTRA OF FIBRINOLYTIC PRINCIPLE FROM SNAKE VENOM OF *AGKISTRODON ACUTUS*

Shou-Ye Wang, Qing-Liang Liu,* Xiao-Long Xu, and Yong-Shu Xie

Department of Chemistry, University of Science and Technology of China, Hefei, 230026, P.R. China

ABSTRACT

The synchronous fluorescence spectra of fibrinolytic principle (FP) from snake venom of *Agkistodon acutus* have been studied. It is found that the synchronous fluorescence spectrum of FP for 70 nm wavelength intervals shows the characteristic spectra of tryptophan residues in FP molecules. The effects of solution pH and ethylenediaminetetraacetic acid (EDTA) on the synchronous fluorescence spectra and fibrinolytic activity of FP have been also investigated. The results reveal that the conformation of FP is stable in the pH range 4–7 and FP under acidic conditions is more stable than in alkaline conditions and, that calcium ion in FP plays a critical role in keeping its biological activity.

*Corresponding author. Fax: +86-551-3631760; E-mail: qliu@ustc.edu.cn

Key Words: Synchronous fluorescence spectra; Fibrinolytic principle; pH; Tryptophan

INTRODUCTION

Synchronous fluorescence spectroscopy is recorded by scanning simultaneously (or synchronously) both the excitation and emission wavelengths keeping a constant wavelength interval between them. This approach offers several advantages, such as the narrowing of spectral peaks, the improvement in the spectrum resolution, and the elimination of the Rayleigh scattering peak[1]. Especially, the peaks overlapped in the conventional fluorescence spectra can be or separated in the synchronous fluorescence spectra when the suitable wavelength intervals are chosen.

Although this technique has been quite successful with the analysis of polycyclic hydrocarbons and when used in forensic cases, its application in protein has been limited. Up to now, there are only few reports concerning synchronous fluorescence spectra of proteins[1–3].

Fibrinolytic principle (FP) purified from *Agkistrodon acutus* venom, exhibits direct fibrin(ogen)olytic activity. The enzyme, FP, possesses the arginine esterase activity, but not the proteolytic, hemorrhagic or lethal activity[4]. It has a molecular weight of 29675.5 and an isoelectric point of pH 5.5[4,5]. FP has received considerable attention in recent years for its potential application as an antithrombotic agent. The cDNA clones of FP (also named Agkisacutacin) were accomplished and sequenced. The amino acid sequence of FP reveals that FP contains 14 tryptophans(Trps) and 8 tyrosines(Tyrs)[6].

In this work, we report the synchronous fluorescence spectra of FP solution at 70 nm wavelengths intervals ($\Delta\lambda = 70$ nm) and the effects of the solution pH and EDTA on the synchronous fluorescence spectra and fibrinolytic activity of FP.

EXPERIMENTAL

Materials and Instruments

The crude snake venom was supplied by the Hospital for Treatment of Snakebites at Qimen, Southern Anhui, P.R. China. Tyr, Trp and human fibrinogen were purchased from Sigma Chemical Co. Other regents were of analytical grade.

Synchronous fluorescence spectra were recorded on a RF-5000 spectrofluorometer with 150 W xenon lamp (Shimadzu). The scan speed was 60 nm/min. Both the excitation and the emission side slit widths are 10 nm. All measurement were carried out at room temperature. In all cases the pH's of the buffered enzyme solutions were determined with a pHS-2C pH meter.

Methods

FP was isolated and purified from snake venom of *Agkistrodon acutus* as previously described[7]. Fibrinolytic activity was determined as described by Guan *et al*[8].

RESULTS AND DISCUSSION

Synchronous Fluorescence Spectra of FP

Figure 1 shows the synchronous fluorescence spectra of FP at $\Delta\lambda = 70$ nm, the synchronous fluorescence spectra of free Trp and a 14:8 (molecular ratio) mixture of Trp and Tyr, the same ratio as found in FP. It can be observed in Figure 1 that at $\Delta\lambda = 70$ nm the spectrum of FP is similar to that of free Trp and the mixture of Trp and Tyr. Thus, at $\Delta\lambda = 70$ nm, the observed spectra of FP are attributed only to 14 Trp residues and not to the contribution of 8 Tyr residues. The fluorescence intensity of Trp residues in FP is less than that of an equimolar free Trp solution because of the quenching through the process of efficient energy transfer from the donor, Trp, to the acceptor, the charged carboxyl and/or amino group in the FP molecule[9]. The $\lambda_{s,em,max}$, the wavelength of the maximum emission in synchronous scan is 338 nm and it shows about 7 nm blue shift relative to that of free Trp. It is well known that the emission maxima of Trp and its derivative are highly sensitive to solvent polarity. As a result, one may expect the emission maxima of the Trp residues in proteins to be dependent upon those factors which affect the exposure of the Trp residues to the aqueous phase. Therefore, the blue-shifted emission maximum of FP is interpreted as being due to the shielding of the Trp residue from aqueous phase by the protein. This result is good agreement with our previous result obtained by conventional fluorescence spectra[10].

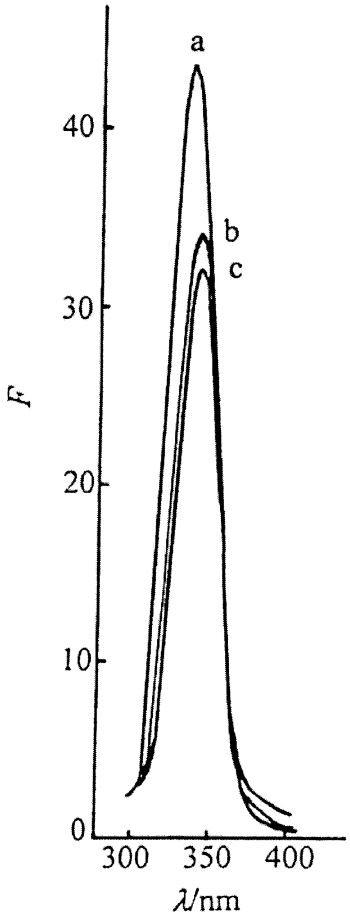

Figure 1. Synchronous fluorescence spectra of FP(a), free tryptophan(b), and a mixture of tyrosine and tryptophan(c) equal to that found in FP. Difference between the excitation and emission wavelength $\Delta\lambda = 70$ nm. Concentrations of FP, free Trp and Trp in the mixture of Tyr and Trp are all 1 μM in pH 7.6, 0.02 M Tris-HCl buffer (containing 0.15 M NaCl). The temperature was 25 °C.

Effect of pH on the Synchronous Fluorescence Spectra and the Stability of FP

The relative fluorescence intensity of FP as a function of pH is shown in Figure 2. It is obvious that there are three different regions where the

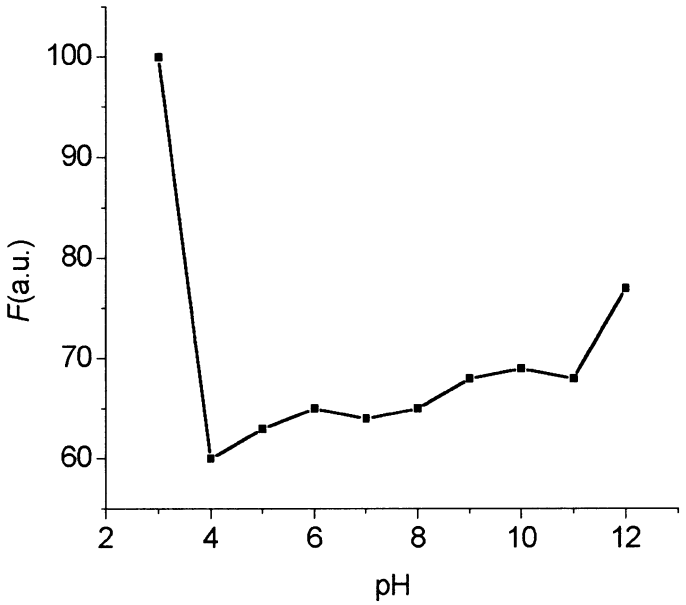

Figure 2. The dependence of the intensity of the synchronous fluorescence spectra for FP on the solution pH, 338 nm peak, $\Delta\lambda = 70$ nm. The concentration of FP is 4 μM. The temperature was maintained at 25 °C.

synchronous fluorescence changes markedly: acidic region (pH < 4.0); neutral region (pH = 4.0–11.0) and alkaline region (pH > 11.0).

Acidic region—The large increase in quantum yield is observed. The synchronous fluorescence emission spectrum undergoes red shift of 3 nm (Figure 3), suggesting that at pH 3.0 the Trps in FP should be in somewhat more polar environments than at pH 4.0. This probably represents a greater exposure of the Trps to the solvent, although exposure is still not complete because the $\lambda_{s,em.max}$, of FP at pH 3.0 (341 nm) is still less than that observed for free Trp in water(345 nm). In the acid region, FP retains approximately 85% of its activity after 6 h incubation at pH 3.0 (Figure 4) and this result together with the above one indicates that FP undergoes a conformation change in the acid region.

Neutral region—There is not any significant change for the fluorescent intensity of FP in the pH range 4–11 and not any appreciable shift for the peak position of FP fluorescence. The pH stability of FP seems to be consistent with this result. In fact, nearly equal activity of FP in the pH range 4–7 can be observed in Figure 4. So it is probably that the conformation of

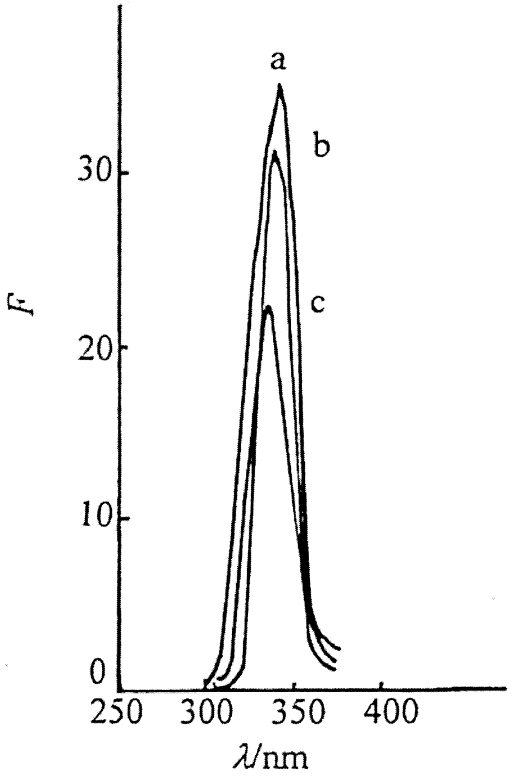

Figure 3. The Synchronous fluorescence spectra of FP at different pH at $\Delta\lambda = 70$ nm. pH (a) 12.0 (b)3.0 (c)7.6. Conditions were as described in the caption for Figure 2.

FP is stable at the pH range 4–7. However, in the range of pH 8–11, FP losses its activity partly or completely. This occurs possibly due to its denaturation to some extent without a significant change in conformation of FP in solution at this pH range.

Alkaline region—Like the acid region, the fluorescence intensity increases again and is accompanied by a red shift (Figure 3). In comparison with the 3 nm red shift at pH 3.0, a 5 nm red shift at pH 12.0 indicates that some Trps in the FP molecule are more exposed to solvent. At the same time, at pH 12.0, FP completely loses its fibrinolytic activity while only about 15% loss of fibrinolytic activity at pH 3.0 can be

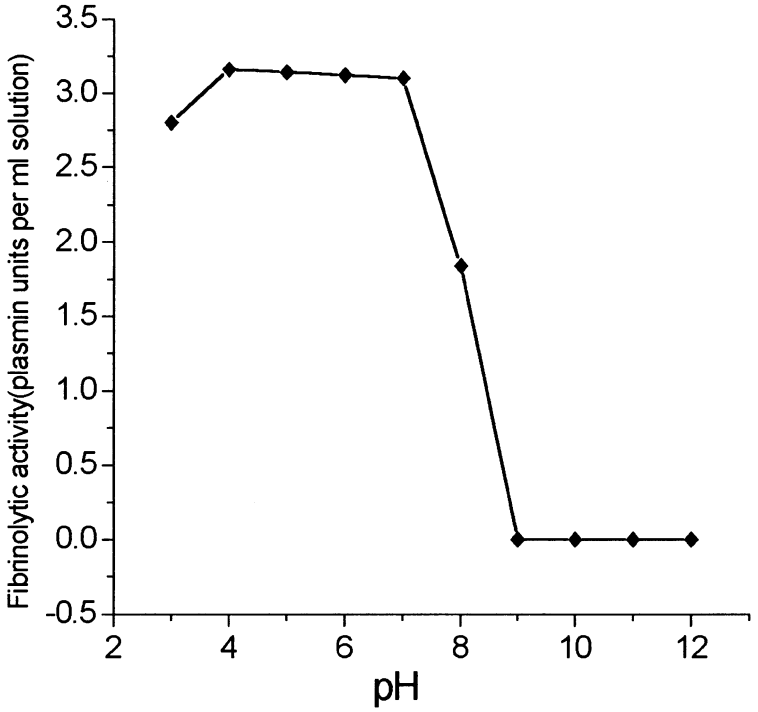

Figure 4. Solution pH effect on the fibrinolytic activity of FP. FP(in dilute Tris buffer, pH 7.6) was incubated with equal volumes of buffers at various pH values at room temperature for 6 h.

observed (Figure 4). This demonstrates that denaturation of FP is quite extensive in the alkaline region, and FP undergoes an intensive three-dimensional structure change.

By comparing information on the above synchronous fluorescence and activities of FP under different pH conditions, it seems that the conformation of FP is stable in the pH range 4–7 and FP under acidic conditions is more stable than in alkaline conditions. It appears that denaturation of FP occurs to some extent at acidic and alkaline conditions.

Effect of EDTA on the Synchronous Fluorescence of FP

Our previous reports have revealed that each FP molecule contains only one calcium ion[5], however the effects of calcium on the structure and

function are still not clear. For this, we investigated the effects of EDTA, which can remove calcium ion in the FP molecule, on the synchronous fluorescence and fibrinolytic activity of FP. One can not observe the appreciable changes in the shape of the synchronous fluorescence emission band ($\lambda_{s,em,max}$) and the emission intensity of apo-FP after the removal of calcium in FP (data not shown). This suggests that there should be no significant conformation change when removing the calcium. In addition, the fibrinolytic activity is inhibited by EDTA (data not shown). This result demonstrates that the calcium ion in FP plays a critical role in its activity. Therefore it is reasonable from the above results that the calcium ion in FP is essential only for activity of the native protein, not for its conformation.

In summary, the synchronous scan approach appears to be useful for the studies on proteins. The changes in peak position and the intensity in the synchronous fluorescence spectra reflect the conformation changes of the FP molecule. Thus, it can be concluded that the synchronous fluorescence spectroscopy can be used as a probe of conformational states of proteins.

ACKNOWLEDGMENT

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