pH 值和钙离子对尖吻蝮蛇抗血小板凝集素二级结构的影响

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摘要：尖吻蝮蛇毒中抗血小板凝集素是凝血因子 IX/凝血因子 X 结合蛋白,它具有抗凝血和抑制血小板凝集双重活性。用红外光谱、拉曼光谱和 CD 谱研究了抗血小板凝集素的二级结构以及 pH 值和钙离子对其二级结构的影响。用 CD 谱测得,在水溶液中,抗血小板凝集素的主要骨架构象为 β-折叠(26.3%)和 α-螺旋(19.6%)结构。拉曼光谱显示,在粉末状态,其 α-螺旋含量显著降低。CD 谱还表明,抗血小板凝集素在 pH 值 3.0~11.0 范围内保持稳定的天然结构,钙离子诱导的抗血小板凝集素结构变化是可逆的,钙离子在稳定抗血小板凝集素的天然结构中起重要作用。

关键词：抗血小板凝集素；二级结构；圆二色谱；拉曼光谱；红外光谱
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Effects of pH Value and Ca²⁺ Ion on the Secondary Structure of Agkisacutacin from Agkistrodon Acutus Venom

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Abstract: Agkisacutacin isolated from the venom of Agkistrodon acutus is a coagulation factor IX/coagulation factor X-binding protein with marked anticoagulant- and platelet-modulating activities. The effects of pH value and Ca^{2+} ion on the secondary structure of Agkisacutacin have been studied by FTIR, Raman and circular dichroism (CD) spectroscopy. The main backbone conformations of holo-Agkisacutacin are α-helix (19.6%) and β-sheet (26.3%) as determined by CD spectroscopy. According to the Raman measurements, the content of α-helix of holo-Agkisacutacin in solid powder is less than that in solution. The conformation of Agkisacutacin is stable in the pH range of 3.0~11.0 as shown by CD spectroscopy. Ca^{2+}-induced conformation change of Agkisacutacin is reversible. Ca^{2+} ion plays an important role in the stabilization of the structure of Agkisacutacin.

Key words: agkisacutacin; secondary structure; circular dichroism; Raman Resonance; FTIR

0　Introduction

　　Snake venoms contain a variety of proteins that affect thrombosis and hemostasis [1,2]. Because blood coagulation and platelet aggregation are important factors in cardiovascular and cerebrovascular diseases, snake venom proteins that interfere in these processes have received considerable attention in recent years[3]. Three anticoagulant proteins, anticoagulation factor Ⅰ (ACF Ⅰ), anticoagulation factor Ⅱ (ACF Ⅱ) and agkisacutacin have been purified from the venom of Agkistrodon acutus. They have very similar amino acid compositions and high homologous sequences, and form 1∶1 complexes with coagulation factor IX/

coagulation factor X and prolong the clotting time[4~6]. Agkisacutacin is a bifunctional protein[6]. It has been identified as both platelet membrane receptors-binding protein and coagulation factor IX/coagulation factor X-binding protein[7]. Agkisacutacin does not possess prot-eolytic, hemorrhagic, and lethal activities. Therefore, it can be used as a basis for designing anticoagulant and thrombolytic drugs, as well as a useful tool for elucidating the complex mechanisms involved in clot-ing process [8,9]. Currently the detailed three-dimensio-nal structure of agkisacutacin has remained undeter-mined. Agkisacutacin binds with one Ca^{2+} ion which is essential for the binding of agkisacutacin to FIX/FX[10]. However, the effect of Ca^{2+} ion on the conformational stabilization of agkisacutacin is still unclear.

In this work, the effects of pH value and Ca^{2+} ion on the secondary structure of Agkisacutacin have been studied by FTIR, Raman Resonance and CD spectros-copy.

1 Experimental

1.1 Materials

The *Agkistrodon acutus* venom powder was provided by Qimen Institute for Treatment of Snake Bites (Anhui, P. R. China). All other reagents were of analytical reagent grade. Milli-Q purified water was used throughout. Buffer solutions include Gly-HCl (pH value of 3.0), NaAC-HAC (pH value of 4.0~6.0), Tris-HCl (pH value of 7.0~9.0) and Gly-NaOH (pH value of 10.0 ~12.0). The concentration of all the buffer solution was $0.02\ mol \cdot L^{-1}$.

The separation, purification of Agkisacutacin and the measurement of the enzymic activity were descri-bed previously[10]. All utensils used during the experi-ments were made metal-free by soaking in $2\ mol \cdot L^{-1}$ HNO_3 for 24 h and then by extensively rinsing with Milli-Q super pure water.

1.2 FTIR spectroscopy

Infrared spectra were obtained on a Fourier-transform instrument with BaF_2 pellets at room tempe-rature. $30\ mg \cdot mL^{-1}$ Agkisacutacin solutions (D_2O) were dried by blowing nitrogen, and then mixed with KBr pellet. The mix was pressed into a disk. Each spectrum was an average of 128 scans at a resolution of $4\ cm^{-1}$, and corrected for baseline.

1.3 Raman spectroscopy

Because the Raman spectroscopy of Agkisacuta-cin in solution showed high fluorescence background, we used solid powder of Agkisacutacin for Raman spectrum measurement. The Raman spectra were excited at 632.8 nm. All spectra were then corrected for the fluorescence background. Each spectrum was the average of eight scans.

1.4 Circular dichroism spectroscopy

CD measurements were carried out with a JASCO J-715 spectropolarimeter equipped with a cell holder thermostatically controlled by circulating water from a bath. The spectra were recorded over a wavelength range of 195~250 nm using a curette of 1 mm and at a scan speed of $10\ nm \cdot s^{-1}$ and a response time of 0.25 s. Each spectrum was the average of three scans. The data were expressed in mean residue ellipticity $[\theta]$ in $° \cdot cm^2 \cdot dmol^{-1}$, which is defined as $[\theta]=100\theta_{obs}\ (lc)^{-1}$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeter[11].

2 Results and discussion

2.1 FTIR

Infrared spectroscopy is one of well-recognized techniques for studying protein secondary structure. The infrared amide I is sensitive to the secondary structure, such as α-helix, β-sheet and random coil[12]. Fourier self-deconvolution and second derivative methods were used to analyze the data of infrared spectrum of holo-Agkisacutacin [13]. Fig.1 shows the original fourier self-deconvoluted and second deriv-ative spectra of holo-Agkisacutacin between $1\ 700\ cm^{-1}$ and $1\ 620\ cm^{-1}$. For both spectra, the peaks of the amide I at the ranges of $1\ 615 \sim 1\ 638 cm^{-1}$, $1\ 638 \sim 1\ 645\ cm^{-1}$, $1\ 645 \sim 1\ 662 cm^{-1}$ and $1\ 662 \sim 1\ 695 cm^{-1}$ correspond to β-sheet, random coil, α-helix and β-turn components, respectively. The peak assignments are shown in Table 1, which indicates that the main backbone conformations of Agkisacutacin in D_2O solu-tion are α-helix and β-sheet.
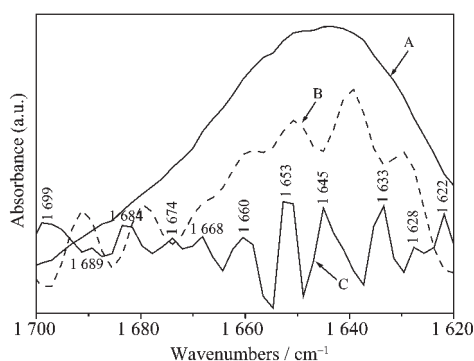
A: Original undeconvolved spectrum of holo-Agkisacutacin in D$_2$O; B: Spectrum was obtained after band narrowing by self-deconvolution method with resolution enhancement parameter 2.5 and full width at half height 18 cm^{-1}; C: Second derivative spectrum of holo-Agkisacutacin. The concentration of holo-Agkisacutacin is 30 mg·mL^{-1}

Fig.1　FTIR spectra of holo-Agkisacutacin

Table 1　Assignments of the FTIR frequencies of Agkisacutacin for amide Ⅰ bands

Peaks / cm^{-1}	Assignment	Peaks / cm^{-1}	Assignment
1 699	β-turn	1 653	α-helix
1 689	β-turn	1 645	Random
1 684	β-turn	1 633	β-sheet
1 674	β-turn	1 628	β-sheet
1 668	β-turn	1 622	β-sheet
1 660	α-helix		

2.2　Raman spectrum

Fig.2 shows the Raman spectrum of holo-Agkisacutacin in solid power between 700 cm^{-1} and 1 700 cm^{-1} and Table 2 shows the assignments for the peaks in its Raman spectrum. The analysis of the amine Ⅰ band permits to estimate the secondary structure of protein. The main contribution to the amine Ⅰ band is from the carbonyl stretching vibration of peptide, and a small contribution is from the N-H in-plane bending vibration (1 550~1 680 cm^{-1}). The main contribution to the amine Ⅲ band is from the N-H in-plane bending vibration and the C-N stretching vibration (1 230~1 310 cm^{-1})[14]. The amine Ⅰ band of protein usually appears at 1 650 cm^{-1} for α-helix and 1 665 cm^{-1} for β-sheet. The amine Ⅲ band of protein usually appears at 1 274 cm^{-1} for α-helix and 1 239 cm^{-1} for β-sheet. As shown in Fig.2, the amine Ⅰ band of holo-Agkisacutacin at 1 665 cm^{-1} and its amide Ⅲ band at 1 239 cm^{-1} indicate that holo-Agkisacutacin has β-sheet structure. No obvious peaks at 1 274 cm^{-1} and 1 650 cm^{-1} for α-helix were detected, suggesting that the α-helix content of holo-Agkisacutacin in solid powder is much less than that in D$_2$O solution. holo-Agkisacutacin has 12 Phe residues[6], so that it gives strong peak at 1 003 cm^{-1}.
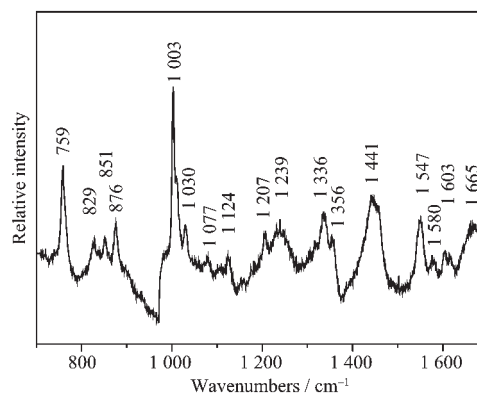

Fig.2　Raman spectrum of holo-Agkisacutacin in solid powder excited at 632.8 nm at 25 ℃

Table 2　Assignments of the Raman frequencies of Agkisacutacin

Peaks / cm^{-1}	Assignment	Peaks / cm^{-1}	Assignment
1 665	Amide Ⅰ	1 207	Phe
1 603	Phe	1 077	C-N bend
1 580	Trp	1 030	Phe
1 547	Trp	1 003	Phe
1 441	C-H bend	876	Trp
1 356	Trp	851	Tyr
1 336	Trp	829	Tyr
1 239	Amide Ⅲ	759	Trp

2.3　Effects of pH value on CD spectrum of holo-Agkisacutacin

Far-UV CD spectra of holo-Agkisacutacin at different pH values are shown in Fig.3. The CD spectrum of holo-Agkisacutacin is not a typical spectrum of α-helix, β-sheet or random coil, indicating that holo-Agkisacutacin contains different conformations. The secondary structural content was estimated from the CD spectrum using the method of Chen et al.[11]. The main backbone conformation of holo-Agkisacutacin at pH value of 7.4 are β-sheet (26.3%) and α-helix (19.6%). Agkisacutacin consists of two chains with an amino acid sequence similar to habu IX/X-bp, a coagulation factor IX/coagulation factor X-binding protein purified from the habu snake: a 78% identity

for the A-chain, and a 91% identity for the B-chain[6,15]. The secondary structure contents of habu IX/X-bp on the basis of the X-ray structure are 19.0% for α-helix and 24.6% for β-sheet[15]. The similarity of secondary structure between habu IX/X-bp and Agkisacutacin suggests that Agkisacutacin probably has a similar three-dimensional structure to habu IX/X-bp.
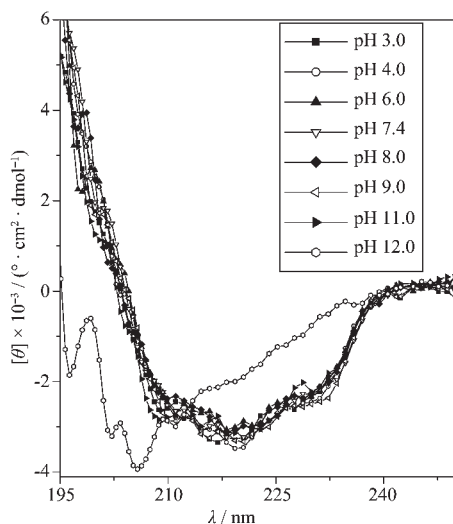

Concentration of holo-Agkisacutacin is 0.2 mg·mL⁻¹; Concentration of buffer is 0.02 mol·L⁻¹

Fig.3　Far-UV CD spectra of holo-Agkisacutacin in different pH buffer solution

As shown in Fig.3, no apparent effects of pH values on the CD spectrum of holo-Agkisacutacin have been observed in the pH range of 3.0 ~11.0, suggesting that the conformation of holo-Agkisacutacin is stable in the large pH range. Agkisacutacin contains seven disulfide bonds[6], which may be one of the reasons why holo-Agkisacutacin is stable in so large pH range.

The changes of mean residue ellipticity (MRE) at 222 nm reflect the rearrangements of the secondary structure of the protein, especially for α-helix[16]. The dependence of MRE at 222 nm of holo-Agkisacutacin on pH value is shown in Fig.4. MRE_{222} of holo-Agkisacutacin is around $-3\,000°\cdot cm^2\cdot dmol^{-1}$ in the pH range of 3.0~11.0, and decreases to $-1\,659°\cdot cm^2\cdot dmol^{-1}$ at pH value of 12.0. The content of α-helix decrease from 18%~19% at pH range of 3.0~11.0 to 3% at pH value of 12.0. The result indicates that holo-Agkisacutacin has been denatured at pH value of

12.0, which is in agreement with the results of its synchronous fluorescence spectrum[10].
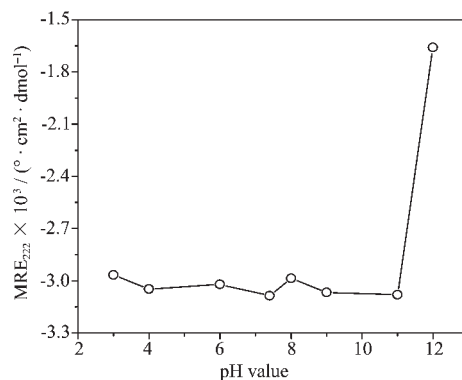

Condition is the same as in Fig.3

Fig.4　pH-induced conformational transition of holo-Agkisacutacin as followed by MRE measurements at 222 nm

2.4　Effects of Ca²⁺ on the secondary structure of holo-Agkisacutacin

As shown in Fig.5, the CD spectrum of holo-Agkisacutacin decreases significantly after removal of Ca²⁺ from the protein. The content of α-helix structure greatly decreases with a significant increase of random coil content after decalcification. The CD spectrum of apo-Agkisacutacin essentially recovers after addition of Ca²⁺ to apo-Agkisacutacin solution, suggesting that Ca²⁺-induced conformation change of Agkisacutacin is reversible. This result reveals that Ca²⁺ ion plays an important role in the stabilization of the structure of
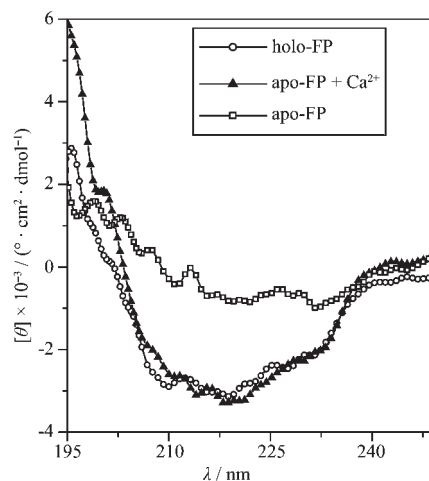
Concentration of Agkisacutacin is 0.15 mg·mL⁻¹

Fig.5　Far-UV CD spectra of holo-Agkisacutacin and apo-Agkisacutacin in the absence or presence of 1 mmol·L⁻¹ Ca²⁺ in This-HCl (pH value of 7.4)

Agkisacutacin. This may be the reason why Agkisacutacin binds to the factor IX/factor X in a calcium-dependent fashion[7].

3   Conclusions

The conformation of Agkisacutacin is stable in the pH range of 3.0~11.0 determined by CD spectroscopy. The main backbone conformations of holo-Agkisacutacin in D_2O solution are α-helix (19.6%) and β-sheet (26.3%) as determined by CD spectroscopy. According to the Raman measurements, the content of α-helix of holo-Agkisacutacin in solid powder is less than that in D_2O solution. Ca^{2+}-induced conformation change of Agkisacutacin is reversible. Ca^{2+} ion in the Agkisacutacin possibly plays a dominant role in keeping the secondary structure of Agkisacutacin.

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