Acta Biochim Biophys Sin (2009): 948–954 | © The Author 2009. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmp089.
Advance Access Publication 13 October 2009

Expression and characterization of Kunitz domain 3 and C-terminal of human tissue factor pathway inhibitor-2

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Human tissue factor pathway inhibitor-2 (hTFPI-2) is a serine protease inhibitor and its inhibitory activity is enhanced by heparin. The Kunitz domain 3 and C-terminal of hTFPI-2 (hTFPI-2/KD3C), which has the activity toward heparin calcium, have been successfully expressed in *Pichia pastoris* and purified by SP-Sepharose and heparin-Sepharose chromatography. The Fourier transformed infrared spectroscopy (FTIR), Raman spectroscopy, and circular dichroism (CD) experiment results implied that hTFPI-2/KD3C contained small contents of α-helix and β-strand, but large amounts of random coil and two kinds of disulfide bonds, gauche-gauche-gauche (ggg) and trans-gauche-trans (tgt). The interaction of hTFPI-2/KD3C with heparin calcium was investigated by CD. It was found that heparin calcium induced β-strands in hTFPI-2/KD3C to different extents depending on the ratio of hTFPI-2/KD3C and heparin calcium.

Keywords Kunitz domain 3 and C-terminal of hTFPI-2; heparin; secondary structure; *Pichia pastoris*

Received: April 28, 2009 Accepted: July 13, 2009

Introduction

Human tissue factor pathway inhibitor-2 (hTFPI-2) is a 32-kDa Kunitz-type serine proteinase inhibitor that strongly inhibits trypsin, plasmin, chymotrypsin, plasma kallikrein, and factor XIa amidolytic activity, but only weakly inhibits the activities of factor VIIa, tissue factor, factor IXa, cathepsin G, matrix metalloproteinase-2 (MMP-2) and -9 [1,2], and fails to inhibit MMP-1 [3]. hTFPI-2 is synthesized by endothelial cells [4,5], keratinocytes and fibroblasts [6], and secreted into extracellular matrix [7].

hTFPI-2 has a similar overall domain organization and considerable primary amino acid sequence homology with hTFPI-1 [8,9]. The structures and functions of hTFPI-1's Kunitz domains 1, 2, 3, and C-terminal, which can inhibit tissue factor/VIIa complex, Xa and bind the heparin, and so on, have been well known [10–12]. The first Kunitz domain of hTFPI-2 has been studied [13,14]. Kong *et al*. [2] found that both hTFPI-2 and hTFPI-2/KD1 could inhibit matrigel invasion by HT1080 and inhibit the plasmin, trypsin, MMP-2, and -9, but the inhibitive ability of hTFPI-2/KD1 was quite weaker than that of hTFPI-2. It means that the Kunitz domains 2, 3, and C-terminal may play an important role in the inhibition of hTFPI-2.

It is necessary to produce a certain amount of hTFPI-2's Kunitz domain 3 and C-terminal with high purity in order to do further investigations. In this work, hTFPI-2/KD3C has been successfully expressed by *Pichia pastoris*, and its secondary structure has been studied by Fourier transformed infrared spectroscopy (FTIR), circular dichroism (CD), and Raman spectroscopy for the first time. The interaction of hTFPI-2/KD3C with heparin calcium was studied by CD. It was found that the content of β-strand in hTFPI-2/KD3C was increased with the amount of heparin calcium gradually.

Materials and Methods

Strains, vectors, and reagents

The *Escherichia coil* DH5α was stored in the Key Laboratory of Molecular Medicine, Ministry of

Education, Fudan University (Shanghai, China). *Pichia pastoris* host strain GS115, expression vector pPIC9K, yeast nitrogen base (peptone, yeast extract), and D-biotin were purchased from Invitrogen (Carlsbad, USA) and G418 was from Promega (Madison, USA). dNTP and *Pfu* DNA polymerase were obtained from Shanghai Shenergy Biocolor Bioscience (Shanghai, China). The restriction endonuclease and T4 DNA ligase were the products of NEB (Beijing, China). Plasmid extract kit was obtained from Boda Company (Shanghai, China). Protein concentration assay kit was purchased from Bio-Rad (Hercules, USA). SP-Sepharose was obtained from Amersham Biosciences (Buckinghamshire, UK); heparin-Sepharose CL-6B was purchased from Pharmacia (San Francisco, USA). Low molecular weight (LMW) heparin calcium was from Saibaoer Company (Shenzhen, China). The other reagents were all of analytic purity. PCR System 2400 PCR was from Perkin-Elmer (Waltham, USA). SmartSpec 3000 spectrophotometer, GenePulser II, and SDS–PAGE electrophoresis system were the products of Bio-Rad. Image Master VDS was purchased from Pharmacia.

Construction of the expression vector

The hTFPI-2/KD3C gene was optimized with the *P. pastoris* preferred codons. cDNA was obtained from human placenta total RNA by RT–PCR. The hTFPI-2/KD3C was amplified by PCR with oligonucleotides: 5′-AGAGAATTCGCTCCAAAGAAGATCCCAT-3′ and 5′-AGAGCGGCCGCCTATTAGAACTGCTTCTTACGAAT-3′.

The PCR product was digested with *Eco*RI and *Not*I, and inserted between the *Eco*RI and *Not*I sites of the *P. pastoris* expression vector pPIC9K. The resulting plasmid was named as pPIC9K-hTFPI-2/KD3C.

Transformation of *P. pastoris*

Pichia pastoris GS115 was electrotransformed with linear-fragmented pPIC9K-hTFPI-2/KD3C by digestion with *Sal*I. A total of 1 ml of 1 M sorbitol on ice was added into the cuvette immediately. The 600 μl of cell suspension were spread on MD plates (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose, and 1.5% agar) and incubated at 30°C for 48 h.

Screen for multicopy transformants by G418 selection

Single colonies were inoculated into 96-well plates by selecting from MD plates and incubated at 30°C. His^+ transformant yeast clones were grown on YPD medium (2% peptone, 1% yeast extract, 2% dextrose, and 2% agar) containing G418 (0.5–2.0 mg/ml) for selection of multiple insertions according to the manufacturer's instructions (Invitrogen).

Expression evaluation of pPIC9K-hTFPI-2/KD3C

The selected clone was cultured with shaking in 5 ml YPD for 12 h at 30°C until the OD_{600} reached 5–6. Then, 5 ml of YPD was inoculated into 200 ml BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol) and cultured with shaking for ~24 h until the OD_{600} reached 15–20. Cells were harvested by centrifuging at 5000 *g* for 15 min at room temperature and then the cell pellet was resuspended into 200 ml BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% YNB, 4×10^{-5}% biotin, and 1% methyl) to induce expression. Methanol was added to a final concentration of 0.5% every 12 h to maintain induction. The culture was maintained for 60 h and the supernatant was harvested by centrifugation to analyze by Tricine–SDS–PAGE.

Purification

The supernatant was diluted with 9 volumes of PB buffer (20 mM sodium phosphate buffer, pH 7.0, and 1 mM EDTA). SP-Sepharose equilibrated with PB buffer was loaded with the culture supernatant and washed with a linear gradient of 0–1 M NaCl in equilibrated buffer. Fractions were collected and dialyzed against PB buffer for 4 h, and then loaded on the heparin-Sepharose column equilibrated with PB buffer. The column was eluted with a linear gradient of 0–1 M NaCl in PB buffer. Fractions were collected and analyzed by 10% Tricine–SDS–PAGE. The concentration of hTFPI-2/KD3C was measured by the Bradford method.

Western blot analysis

hTFPI-2/KD3C was separated by 10% Tricine–SDS–PAGE and transferred electrophoretically onto polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat-dried milk in TTBS (25 mM Tris–HCl, 150 mM NaCl, and 0.02% Tween-20) for 3 h at room temperature, then washed and incubated with rabbit anti-TFPI-2 antibody (1:2000 in TTBS) as the first antibody [2,8]. HRP-conjugated goat anti-rabbit IgG (1:500) was used as the secondary antibody. The immunoreactive proteins were visualized using ECL kit.

FTIR measurements

FTIR spectra were measured by Nicolet NEXUS 470 FTIR spectrometer (Nicolet Instrument, Madison, USA)

with a thermostatically controlled Perkin-Elmer solution cell (Spectra-Tech, Shelton, USA) and CaF_2 windows. FTIR spectra were collected from 400 to 4000 cm^{-1} at a resolution of 2 cm^{-1}. A total of 32 scans were accumulated to generate each spectrum. Absorbance spectra were obtained against a background spectrum collected from empty sample cells. The resultant spectra were smoothed with a seven-point Savitzky–Golay smoothing function to remove the white noise. The data were processed by the computer program of Origin 7.5.

Raman measurements

The lyophilized hTFPI-2/KD3C was detected at room temperature by a Dilor-Labram 1B Raman spectrometer (Jobin Yvon, Villeneuve d'Ascq, France), equipped with a He–Ne laser at wavelength of 632.8 nm and 6 mW of power. The recorded resolution of the spectrum was 1 cm^{-1}.

CD measurements

The lyophilized hTFPI-2/KD3C was dissolved in H_2O. The protein concentration was 0.16 mg/ml. CD spectra were acquired on a 0.1-cm path length cell of a Jasco-715 spectrometer (Jasco, Tokyo, Japan) equipped with an RTE-111 bath/circulator (NESLAB, Tokyo, Japan). After purging with N_2 for 25 min, the spectra were recorded from 190 to 250 nm with a resolution of 0.2 nm after triple scans. The samples were hTFPI-2/KD3C with various concentrations of LMW heparin calcium.

Results

Expression and detection of hTFPI-2/KD3C in *P. pastoris*

hTFPI-2/KD3C gene with the *P. pastoris* preferred codons was optimized, constructed as the plasmid of pPIC9K-hTFPI-2/KD3C, and electrotransformed into GS115. A strain of high yield was selected from the multiple clones. Tricine–SDS–PAGE (10%) detected the hTFPI-2/KD3C expression strain. Western blot analysis showed that the protein was recognized by specific rabbit anti-hTFPI-2 antibody and certificated that the expressed protein was the desired protein.

Purification of hTFPI-2/KD3C

SP-Sepharose was used to purify the hTFPI-2/KD3C. The suitable ionic strength and pH value were explored. As a result, the supernatant was diluted to decrease the ionic strength, and the pH value of equilibrated buffer was 7.0. The heparin-Sepharose was employed as the second progress in order to purify the target protein further and to determine the interaction of the hTFPI-2/KD3C with heparin. Eluting with the 0–1 M NaCl in PB buffer, the distinct peaks were obtained and analyzed by 10% Tricine–SDS–PAGE (**Fig. 1**). The recovery of the recombinant protein in the purification procedure was 97% and the yield was 15 mg from one liter of culture supernatants (**Fig. 1**).

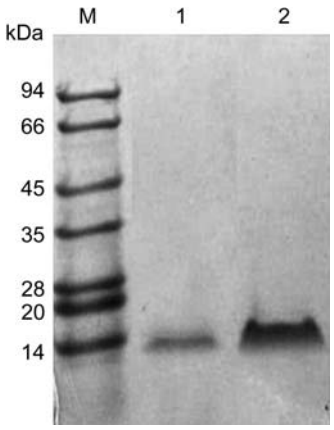

Figure 1 hTFPI-2/KD3C was purified by SP-Sepharose and heparin-Sepharose Lane M, protein marker; lane 1, purified by SP-Sepharose; lane 2, purified by heparin-Sepharose.

Conformation analysis of hTFPI-2/KD3C by FTIR

Spectroscopy has been widely used in the structural studies of biomolecules [15–17]. Absorbance difference spectrum and the second-derivative spectrum of FTIR for hTFPI-2/KD3C in D_2O are shown in **Fig. 2(A)** and **(B)**, respectively. In **Fig. 2(A)**, there are three apparent peaks at 1681, 1652, and 1644 cm^{-1} and shoulders at 1673, 1667, 1635, and 1618 cm^{-1} which indicate that the amide I′ mode consists of various overlapping components. These component bands can be better observed by reference to the second-derivative spectrum in **Fig. 2(B)**. The percentage composition of the individual amide I′ component bands (**Table 1**) was determined by band fitting of the absorbance spectrum of the amide I′ region as shown in **Fig. 2(A)**. From **Table 1**, the sum of individual amide I′ intensities and the percentage of intensity of each peak for hTFPI-2/KD3C can easily be calculated. According to the above peak assignment, the relative contents of secondary structure in hTFPI-2/KD3C are shown in **Table 2**.

Conformation of hTFPI-2/KD3C from Raman spectroscopy

The Raman amide III and I regions are sensitive to secondary structures of protein [18] and the secondary

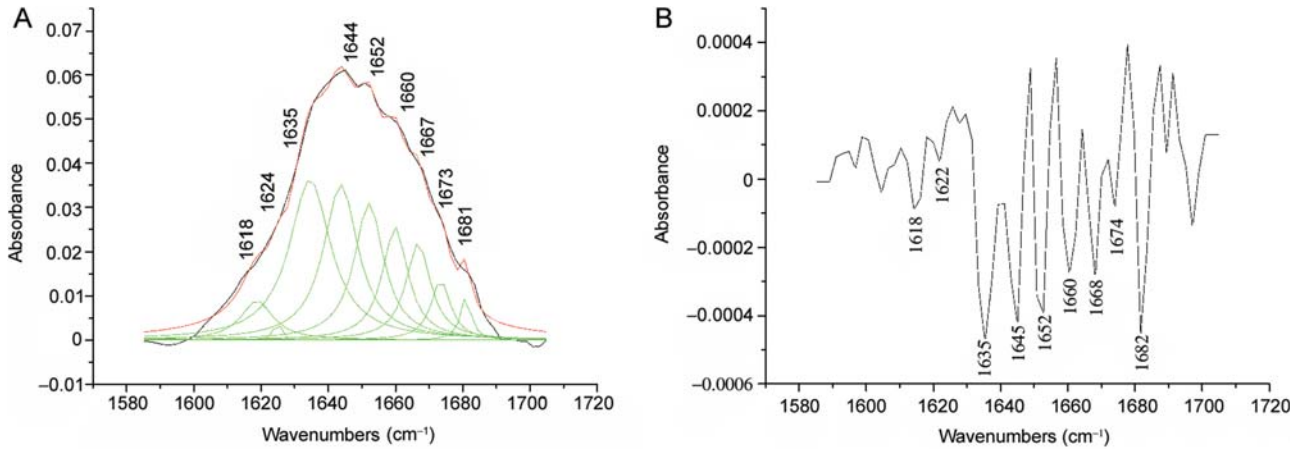

Figure 2 Amide I′ region (1690–1620 cm^{-1}) of FTIR spectrum for hTFPI-2/KD3C at 25°C (A) Amide I′ infrared band of hTFPI-2/KD3C in D_2O with the best-fitted individual component band. Spectrum exhibits the individual Lorentzen component. (B) Second-derivative spectrum of hTFPI-2/KD3C amide I′ band in D_2O.

Table 1 Component band positions, relative integrated intensities, and secondary structure assignment for hTFPI-2/KD3C

FTIR			Raman	Assignment
ν (cm^{-1})	Area	A_F^*		
1681	0.059	0.018	1683	Aggregated β-strand
1673	0.150	0.045	1670, 1232	β-strand
1667	0.310	0.094	1663	β-turn
1660	0.373	0.113	1650, 1266	α-helix
1652	0.521	0.157	–	Random coil
1644	0.689	0.208	1645, 1247	Random coil
1635	0.853	0.257	–	β-strand
1624	0.195	0.059	–	Random coil
1618	0.164	0.049	–	Random coil
–	–	–	506	ggg S-S bond
–	–	–	534	tgt S-S bond

*A_F: fractional area; –, not available.

Table 2 Fractional composition of secondary structure for hTFPI-2/KD3C as estimated by infrared spectroscopy

Secondary structure	Proportion (%)
Random coil	49.1
α-helix	11.3
β-strand	30.2
β-turn	9.4

*Proportion (%) is based on infrared amide I′band.

structure of hTFPI-2/KD3C is reflected in the Raman spectra in **Fig. 3**, as one might expect. On the basis of the published Raman data of proteins, the Raman bands of hTFPI-2/KD3C were assigned and are shown in **Table 1**. The bands at 1670 and 1232 cm^{-1} were assigned as β-strand; the band at 1663 cm^{-1} was assigned to β-turn; the bands at 1650 and 1266 cm^{-1} were assigned to α-helix; and the bands at 1645 and 1247 cm^{-1} were assigned to random coil. It indicates that there are α-helix, β-strand, β-turn, and random coil structures in hTFPI-2/KD3C, which is identical with the result of FTIR.

Interestingly, there are two obvious peaks at 506 and 534 cm^{-1} in **Fig. 3(C)**, which are the characteristic peaks of gauche-gauche-gauche (ggg) disulfide bonds and trans-gauche-trans (tgt) disulfide bonds, respectively. It is known that Kunitz families have three disulfide bonds. As to KD3C, the Raman intensity of the signal at 506 cm^{-1} is about twice of that at 534 cm^{-1}, which suggests that there are two ggg disulfide bonds and one tgt disulfide bond in hTFPI-2/KD3C.

Structural changes of hTFPI-2/KD3C monitored by CD

The CD spectrum of hTFPI-2/KD3C is shown in **Fig. 4**. The negative minimum ellipticity at ~200 nm is characteristic of a random coil structure, and a slight shoulder at ~222 nm stemmed from α-helix structure [19,20]. Computer program K2D2 has been employed to analyze the CD spectrum [21,22]. It is found that hTFPI-2/KD3C contains ~8.0% α-helix, 22.1% β-strand, and 69.9% random coil, which is similar to the result obtained by FTIR.

In this work, CD has been employed to study the interaction of hTFPI-2/KD3C with heparin calcium [23]. **Figure 5** is the CD spectra of hTFPI-2/KD3C in the

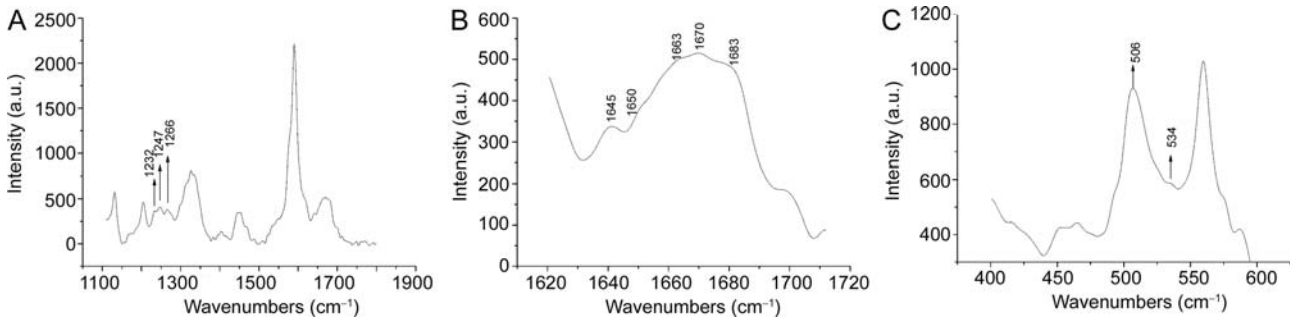

Figure 3 Raman spectra of freeze-dried hTFPI-2/KD3C (A) Raman spectrum of hTFPI-2/KD3C at room temperature. The bands at 1232 cm^{-1} were assigned as β-strand; the bands at 1247 cm^{-1} were assigned as random coil, and 1266 cm^{-1} was α-helix. (B) Raman amide I regions. There are five well-defined peaks. The band at 1645 cm^{-1} was assigned as random coil, 1650 cm^{-1} was α-helix, 1663 cm^{-1} was β-turn, 1670 cm^{-1} was β-strand, and 1683 cm^{-1} was aggregated β-strand. (C) The characteristic peaks of gauche-gauche-gauche disulfide bonds and trans-gauche-trans disulfide bonds. The intensity of the signal at 506 cm^{-1} is about twice as much as one at 534 cm^{-1} which suggests that there are two gauche-gauche-gauche disulfide bonds and one trans-gauche-trans disulfide bond in hTFPI-2/KD3C.

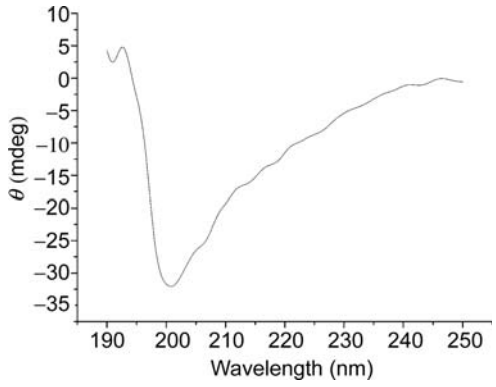

Figure 4 CD spectra for the Kunitz domain 3 and C-terminal of human tissue factor pathway inhibitor-2

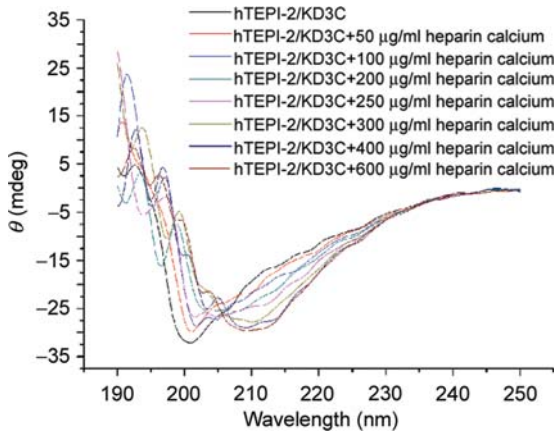

Figure 5 CD spectra of hTFPI-2/KD3C in the different concentrations of LMW heparin calcium (from 50 to 600 μg/ml)

different concentrations of LMW heparin calcium (MW 4000–6000 Da). From **Fig. 5**, it is found that the 200 nm peak is shifted to a new minimum near 212 nm when the concentration of heparin calcium is increased from 50 to 600 μg/ml gradually. When KD3C is in the 600 μg/ml heparin calcium, β-strand is 47%; α-helix is 4.3%; random coil is 48.7%. It implies that the β-strand in hTFPI-2/KD3C is increased after binding.

Discussion

Escherichia coli and yeast system are two kinds of the most widely used expression systems in the production of heterologous proteins for research and therapeutic purposes. *Escherichia coli* system has many advantages such as high yield, easy operation, low cost, and so on. However, *E. coli* can neither express post-translational modified proteins (e.g. glycosylation) nor help to form disulfide bond correctly. Compared with *E. coli*, yeast can overcome those shortcomings [24,25] and used to express our target protein.

Pichia pastoris has been an important host organism for the production of recombinant proteins for both commercial and academic organizations [19]. Because transcription of the system is a limiting factor in the expression of most foreign genes, even when they are under the control of the highly efficient methanol-inducible AOX1 promoter, the methods to increase the number of copies of recombinant protein expression cassettes have been developed and proven effective [20,26,27].

Our laboratory had already expressed and purified the first Kunitz domain of hTFPI-2 and revealed codon optimization of target gene according to the *P. pastoris* preference and proved expression effectively [2]. From this work, we got high yield with optimized codons of the target gene.

This study described the cloning, expression, purification, identification, and characterization of hTFPI2/KD3C, which has not been reported yet. The expression results demonstrate some advantages of *P. pastoris* as an alternative expression. Its expression vector, pPIC9K-hTFPI2/KD3C, allows methanol-inducible production at a yield of 15 mg/l. The collected protein solution was further purified by two columns chromatography. The purity of protein was 97%.

In this work, the condition of the shake flask evaluation was very strict. First, the volume of culture was $<10\%$ of the volume of shake flask in order to ensure the oxygen concentration. Second, it is very critical for the right time to change the culture until the OD_{600} reached 15–20. If it was too earlier, no enough cells were in the culture, which would result in lower yield of target protein. If it is too late, cells were too dense in the culture, which would result in many dead cells.

In order to improve purification yield, the SP-Sepharose and heparin-Sepharose were employed. The supernatants were diluted to an appropriate concentration with the best ionic strength to which the SP-Sepharose was sensitive. Meanwhile, the flow rate should not be too fast and 5 ml/min was found to be suitable. For heparin-Sepharose, the ionic strength was more sensitive and dialyzing the sample after SP-Sepharose was acceptable.

The secondary structure of hTFPI-2/KD3C has been studied by FTIR, CD, and Raman spectroscopy. The combined results showed that hTFPI-2/KD3C contained $\sim$11.3% α-helix, 30.2% β-strand, 9.4% β-turn, and 49.1% random coil. As reported by Yuan and Song [28], the Kunitz family proteins always have α-helix in the C-terminal that is relatively conserved. It means that there are about 10 residues in hTFPI-2/KD3C, which may play an important role in the purification experiment of hTFPI-2/KD3C with heparin-Sepharose. These residues in the vicinity of α-helix may be the activated sites of hTFPI-2/KD3C interacting with heparin, which is similar to the result of hTFPI-1/KD3C [11].

With the help of Raman spectroscopy, it was found that six cysteines in hTFPI-2/KD3C, which form two ggg disulfide bonds and one tgt disulfide bond. These disulfides play an important role in keeping the stability of three-dimensional structures of the protein.

In the process of purification, the collected protein solution of hTFPI-2/KD3C is purified by heparin-Sepharose chromatography, which shows that hTFPI-2/KD3C can bind the heparin very well. By means of CD, it was found that heparin calcium induced β-strands in hTFPI-2/KD3C to different extents depending on the ratio of hTFPI-2/KD3C to heparin calcium. It indicated that there are heparin binding sites in hTFPI-2/KD3C. We suppose that the inhibitory activity of hTFPI-2 toward Factor Xa would be markedly enhanced by heparin. The heparin binding sites in hTFPI-2/KD3C may be pivotal not only in regulating the inhibitory specificity of Serpin but also in directing Serpin to its target tissues to perform its function. [11,29,30].

The results of hTFPI-2/KD1 and plasmin showed that hTFPI-2/KD1 could inhibit plasmin [2], but hTFPI-2/KD3C has little inhibition of plasmin. It is possible that Asp-19, together with Glu-48 forms an acidic patch in hTFPI-2/KD1, which interacted with the basic patch of plasmin [14]. Asp-163 and Gly-192 are the corresponding sites in hTFPI-2/KD3C. The neutral residue Gly-192 may influence the ionic interaction with the plasmin. If the residue Gly-192 is mutated, the new function of hTFPI-2/KD3C itself may be discovered.

Funding

This work was supported partly by the grants from the National Natural Science Foundation of China (No. 30670687) and the National Key Scientific Program (S973, No. 2009CB941704).

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