Quantification the Recombinant SUMO-psTnI in *E.coli* by SPE-HPLC

Tu ZHANG, Qiu-Ling XIE*, Yong LUO, Cai-Kun WANG

[1]College of Life Science and Technology, Jinan University, Guangzhou 510632, China

[2]Guangdong Provincial Key Laboratory of Bioengineering Medicine, Guangzhou 510632, China

[3]National Engineering Research Center of Genetic Medicine, Guangzhou 510632, China

[a]email: x_qiuling@hotmail.com

*Corresponding author

Keywords: SUMO-psTnI, RP-HPLC, SPE/ HPLC, Quantification method, *E.coli.*

Abstract. A simple and rapid reversed-phase liquid chromatography (RP-HPLC) method has been developed and validated for the quantification of recombinant SUMO-psTnI in *E.coli* BL21/PET-3C. This method could be used to monitor the target protein during fermentation and purification process. After Sonication in 20mMTris buffer for 18 minutes, the supernatant was purified by solid-phase extraction using Ni-NTA column with His tag and then detected on C_{18} column (250×4.6 mm i.d.,5 μm) at 280 nm. The assay was linear over the concentration range 0.30–3.02mg/mL with the correlation coefficients at 0.9953. Standard addition recovery rates of solid-phase extraction from different sample batch were between 91.5% and 98.7%. Relative standard deviation was better than 3.1% .The result showed that the content of SUMO-psTnI inside the bacteria was calculated to be 10.88mg/g. The procedure was rapid and simple to determine the recombinant SUMO-psTnI in *E.coli*.

Introduction

This shark Troponin I (TnI), which was cloned in our lab, was found to inhibit angiogenesis as its human homolog. The polypeptide from (Lys91-Asp123) of shark TnI (psTnI) is thought to be the active site of TnI and also confirmed to be angiogenesis inhibitor [1]. In order to obtain the psTnI, the recombinant plasmid of SUMO-psTnI was constructed and expressed in *Escherichia coli*.

The content measurement methods for polypeptide drugs include classical chemical method, high performance liquid chromatography (HPLC), capillary electrophoresis, bioassay and immunoassay [2, 3]. However, for the analysis of complex protein mixtures (such as serum, bacterial proteins), some classical method seems to be insufficient. In general, there are two options available to quantify the target protein content inside the bacteria, SDS-PAGE grayscale scanning and HPLC[4].SDS-PAGE grayscale scanning was chosen by most laboratories for the simple operation and inexpensive instrument. Since the complexity of bacterial metabolite and low concentration of the target protein expressed in *E.coli*, this method proved to be subjective, large deviation and low sensitivity. Compared with SDS-PAGE grayscale scanning, HPLC was time-saving, better repeatability and more precise[5,6].

In recent years, HPLC either with ultraviolet or electrochemical detection has been extensively used to analysis biological samples. Nevertheless, the analysis of proteins expressed in bacteria by HPLC was rarely reported. Through rapid and effective HPLC detection methods, purification conditions of fusion protein could be real-time monitored.

Samples injected into chromatographic column required a pretreatment step to be enriched and cleaned-up [7,8]. Recently, solid-phase extraction (SPE) has become an attractive choice for simultaneous extraction and cleanup of biological matrices due to many favorable features such as simplicity, short processing time, and employs much less organic solvents [9,10].

In the present paper, we established a simple and rapid RP-HPLC method using C_{18} column to quantify the recombinant psTnI in *E.coli* BL21/PET-3C. The HPLC method described here used a rapid solid phase extraction (SPE) procedure, so that the extract could be direct inject into the chromatographic system. This method was fully validated with acceptable recovery.

Experiment

Materials and Reagents

The recombinant *E. coli* BL21/PET-3C/ SUMO-psTnI was constructed by Bioengineering Institute of Jinan University, Guangzhou, China.HPLC grade solvents, methanol (99.9%), trifluoroacetic acid, and acetonitrile (99.9%) were purchased from Shanghai ANPEL Scientific Instrument Co., Ltd. Deionized distilled water from a Hydro Reverse Osmosis system (EWELL Bio-Technology Co.,LTD, Guangzhou) in combination with a arium @ 611UF Plus filtration system(Sartorius, Germany) was used in this assay. Other chemicals and solvents used were of analytical grade, obtained from local suppliers.

Apparatus and Chromatographic Conditions

The HPLC system consisted of Waters 2695 Separations Module (Waters, USA), Autosampler (Waters, USA), Empower Workstation and Waters 2998 Photodiode array detector (Waters, USA). The column was Kromasil C_{18} column (250×4.6 mm i.d., 5μm; Sweden). The gradient profile, as shown in Table 1, consisted of ultra-pure water (0.1% trifluoroacetic acid added; solvent A) and acetonitrile (solvent B) and a flow rate of 1mL/min was utilized with a total run time of 35min. The injection volume was 50 μl. All measurements were performed at 30 ℃.

Preparation of Standards, Calibration Curves

In this study, purified SUMO-psTnI was used as standard protein. The recombinant protein was purified with ion-exchange chromatography by optimized isocratic elution. Then, the enriched fusion protein was attached to Ni-NTA column with His-tag. Recovery of SUMO-psTnI in the first steps was only 54.3%. At last, standard protein with 98.0% purity was obtained.. All standard proteins were lyophilized and stored at -80 ℃ until analysis.

For analysis, standard protein was precise weighed, dissolved in ultra-pure water and constanted volume in a 5mL volumetric flask. Finally, the standard protein was prepared at concentrations of 3.02mg/mL. The calibration curve was constructed by plotting the peak area vs the concentrations of SUMO-psTnI. To detect the linearity relationship between the protein content and peak area, the standard was diluted to

produce five different concentration levels (3.02, 1.51, 0.75, 0.60, 0.30 mg/mL). Each level tested three times and calculated the average.

Solid-phase Extraction for Sample Preparation

5.00g*E.coli* BL21/PET-3C/SUMO-psTnI (accurate to 0.01g) was homogenized by vortexing prior with 40mL 20mMTris buffer. Then sonicated and centrifuged (20000rpm,45min,4℃), 42mL supernatant was collected at last. The Solid phase extraction column using Ni-NTA with His tag was balanced with Ultra-pure water, then rinsed three column volumes with equilibration buffer for loading sample preparation. 10mL supernatant were removed and slowly placed onto solid-phase extraction affinity column. After inject supernatant, two column volumes wash buffer (20mM Imidazole, 300mMNacl, 50mMTris, pH 7.4) was syringed. The target protein was eluted by eluent buffer (300mM Imidazole, 300mMNacl, 50mMTris, pH7.4). Eluent obtained constanted volume to 5mL. The purified protein was analyzed directly on C_{18} column(250×4.6 mm i.d.,5μm).

Solid-phase Extraction Recovery Test

To evaluate the accuracy of this SPE method, standard addition was applied in recovery test. In the control group, 10mL supernatant was placed onto the solid-phase extraction affinity column and quantified, while standard protein, which half amount of the control group, was added to 5mL supernatant in the experimental group. According to the definition of recovery test, improved formula was derived based on the differences of SUMO-psTnI content before and after standard addition .All experiments above were repeated with four different sample batches.

Results

Established of HPLC Detection Methods

In present study, various properties of the column have been tested to establish a suitable HPLC detection method. It has been observed that SUMO-psTnI could not be retained on HIC, WCX-10, SCX-10 column. Although the fusion protein was adsorbed on WAX-10 anion exchange column, it eluted in all four-step elution gradient. Even after gradient elution optimized, the consequent was not acceptable. All columns were purchased from the Dionex Corporation of United States. Fig. 1 showed representative chromatograms on each column.

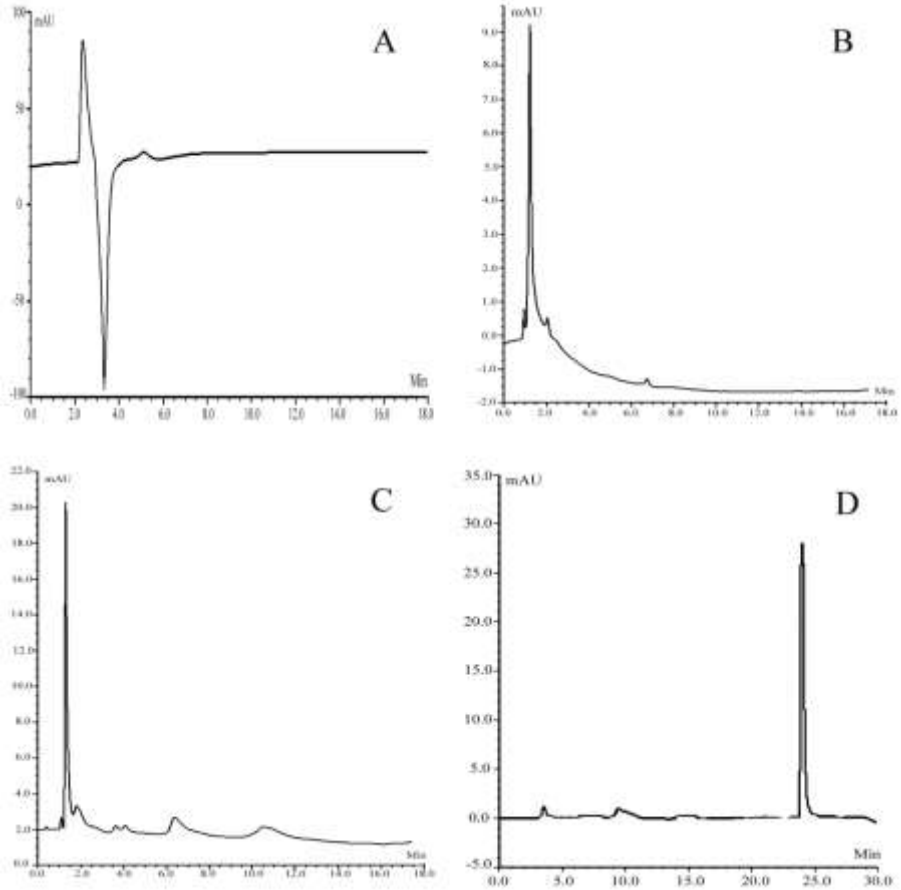

Figure 1. The representative chromatograms of SUMO-psTnI on HIC (A), WCX-10 (B), SCX-10 (C) and WAX-10 (D). SUMO-psTnI could not be retained on HIC, WCX-10, SCX-10 column. Although the fusion protein was adsorbed on WAX-10 anion exchange column, it eluted in all four-step elution gradient.

However, using reversed-phase chromatography, sharp and well-resolved peaks were obtained. Gradient profile for SUMO-psTnI HPLC assay was showed in Table 1. The retention time of SUMO-psTnI was about from 12 min to 14 min. The retention time of chromatographic peaks of the highest value was about 12.402 min.

Table 1.Gradient profile for SUMO-psTnI HPLC assay

Time (min)	Mobile phase A Ultra-pure water (0.1% trifluoroacetic acid added)	Mobile phase B acetonitrile	Flow rate (mL/min)
0	95%	5%	1.0
10	60%	40%	1.0
25	0%	100%	1.0
30	0%	100%	1.0
35	95%	5%	1.0

Preparation of Standards and Calibration Curves

In order to verify the linearity of the SUMO-psTnI, standard protein solutions with the working concentration were prepared and injected into the HPLC. The image of standard protein detected by SDS-PAGE and C_{18} HPLC were showed in Fig. 2.

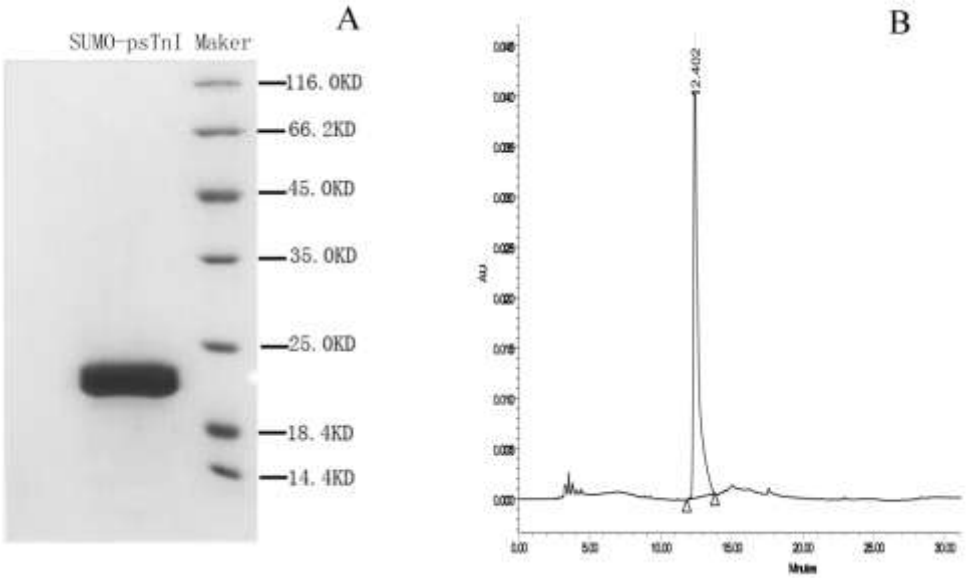

Figure2.The SUMO-psTnI detected by SDS-PAGE (A) and C18 HPLC (B) .The results showed that the purity tested byHPLC result to be over 98.0%.

Calibration curve was found to be linear over the calibration range0.30-3.02mg/mL for SUMO-psTnI (As shown in Fig. 3). The linear regression equation for calibration curve in recombinant *E.coli* BL21/PET-3C/SUMO-psTnI was Y= 98.48X+610.34. Correlation coefficient of this curve was 0.9953.

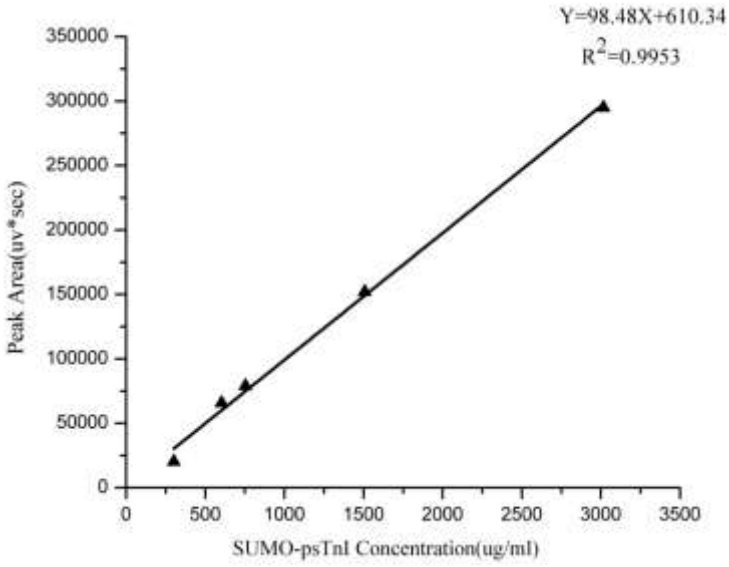

Figure 3.Calibration curve for SUMO-psTnI.

Solid-phase Extraction

To select one appropriate extraction method, we compared several common solid-phase extraction methods in biological sample preparation, including weak anion exchange SPE, reversed phase SPE and affinity SPE. However, because of the serious protein losses in the first two methods, we got a low recovery. As SUMO-psTnI was a tagged fusion protein, it's possible to extract fusion protein from recombinant *E. coli* byaffinity column. This affinity SPE method was simple, quick andhighly reproducible. Thus, the samples were prepared by affinitycolumn SPE. This procedure

provided sufficient sample clean-up,quick preparation, high recovery and little interference on the chromatograms. The peak of supernatant before and after the treatment of SPE was compared in Fig. 4.

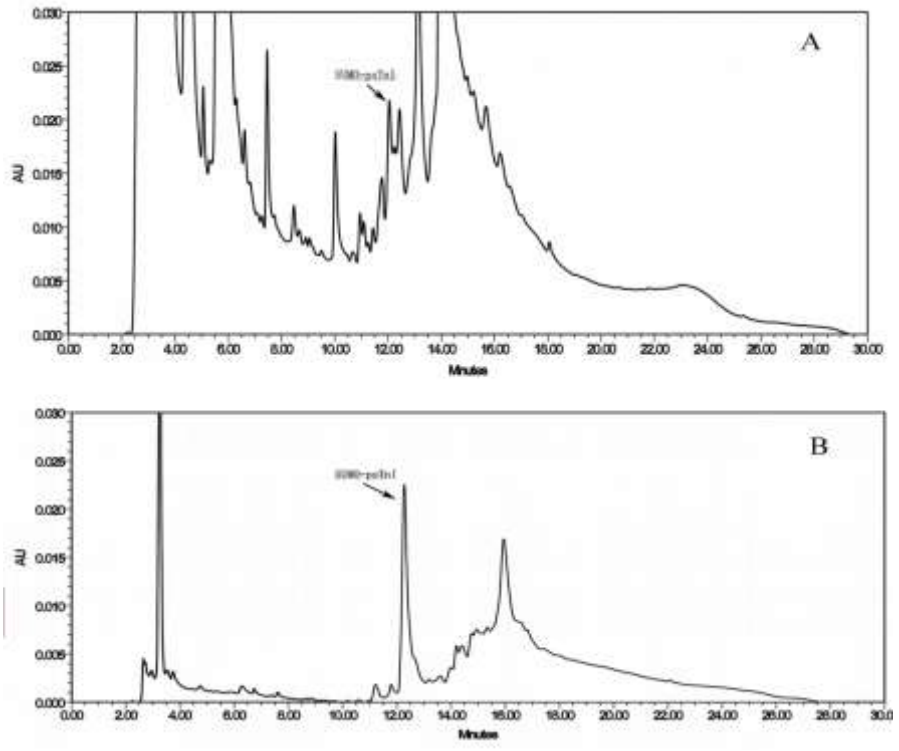

Figure 4.The peak of supernatant of the BL21/PET-3C/ SUMO-psTnI (A) and the peak after the treatment of SPE (B).Gradient HPLC analysis using a Kromasil C18 column(250×4.6 mm i.d.,5μm), flow rate =1mL/min.

Quantification of SUMO-psTnI in Recombinant E. coli

Purified by solid-phase extraction, most impurities and other interfering components could remove from the supernatantof recombinant *E. coli*. Eluent obtained constant volume to 5mL.Then analyzed directly using C_{18} column (250×4.6 mm**,**i.d.,5μm) and quantified by ultraviolet detection at 280 nm. The experiments were repeated four times. Peak area average of SUMO-psTnI in this eluent was 255604uv×sec. According to standard curve, the concentration of eluent was 2.60mg/mL.Content of SUMO-psTnI in Supernatant was calculated to be1.30mg/mL. Thus, fusion protein SUMO-psTnI inside the bacteria was calculated to be 10.88mg/g

Solid-phase Extraction Recovery Test

In the experimental group, 6.50g standard protein was added, which according to the the standard curve. The absolute extraction recoveries of SUMO-psTnI from supernatant ranged from 91.5% to 98.7%, and the relative standard deviation was 3.1%. Results of recovery ratios were shown in Table 2. The recoveries were acceptable.

Table 2.Solid-phase extraction recovery ratios

NO.	10 mL supernatant (mg)	5 mL supernatant added 6.50 mg standard protein （mg）	Recovery (%)	RSD (%)
1	12.75	12.79	98.7	3.1
2	12.08	11.99	91.5	
3	12.02	12.20	95.2	
4	11.83	12.05	94.4	

Conclusions

An easy bio-analytical method was developed and validatedfor the quantification of SUMO-psTnI in recombinant *E.coli* BL21/PET-3C/SUMO-psTnI. Several steps were involved in this method, including sample preparation by SPE, followed detection by RP-HPLC, which could be easy repeat in conventional equipments available in most laboratories. According to the validation parameters, the present method proved to be simple, time-saving and high repeatability. This method could be used to real-time monitor the target proteinconducive to identify problems early and minimize losses.However, this study just provided a test method that available for reference. The method was not applicable to all proteins. For different proteins, different SPE strategies and HPLC detection methods might be selected.

Acknowledgment

This study was supported by Major National Science and Technology Project for New Medicine (2012ZX09202-301-001), Project for Key Technology of Strategic Emerging Industries of Guangdong Province (2012A080800008), Innovation project of Jinan University (21612110).

References

[1] QiulingXie, Sheng Yao, Xiaojia Chen, LihuiXu, WendanPeng, Ling Zhang, Qihao Zhang, Xu-Fang Liang, An Hong. A polypeptide from shark troponin I can inhibit angiogenesis and tumor growth. MolBiol Rep, 2012, (39):1493–1501.

[2] Catarina Marques Fernandes, Francisco Jose BaptistaVeiga.A simple method for nicardipine hydrochloride quantification in plasma using solid-phase extraction and reversed-phase high-performance liquid chromatography.Biomed.Chromatogr, 2003, (17): 33–38.

[3] Fei Liu, Yu Xu, Jinchang Huang, ShuGao and QingxiangGuo. Sensitive liquid chromatography/mass spectrometry assay for the quantification of azithromycin in human plasma.Biomed.Chromatogr, 2007,(21): 1272–1278.

[4] Hassan Y. Aboul-Enein, Suhair Abu-Zaid.High performance liquid chromatography analysis of 9-(2’,3’-dideoxy-2’β-fluoro-D-threo-pentafuranosyl) adenine and its metabolite in human plasma using solid-phase extraction on a polyfluorinated reversed stationary phase. Biomed.Chromatogr, 2001, (15): 267–270.

[5] Bahrami BG, Mirzaeei S and Kiani.A high performance liquid chromatographic determination of azithromycin in serum using fluorescence detection and its

application in human pharmacokinetic studies. Journal of Chromatography B, 2005, (820): 277–281.

[6] Fei Li, Xiuwei Yang .Quantification of myrislignan in rat plasma by solid-phase extraction and reversed-phase high-performance liquid chromatography.Biomed.Chromatogr, 2008, (22): 601-605.

[7] D.Jornet, O. Busto, J. Guasch. Solid-phase extraction applied to the determination of ochratoxinin wines by reversed-phase high-performance liquid chromatography. Journal of Chromatography A, 2000, (882): 29–35.

[8] Young G. Shin, Steve A. Jones, Stan C. Murakami, NageshwarBudha, Joseph Ware, Harvey Wong, Michael H. Buonarati, Brian Dean , Cornelis E. C. A. Hop. Validation and application of a liquid chromatography-tandem mass spectrometric method for the determination of GDC-0152 in human plasma using solid-phase extraction. Biomed.Chromatogr, 2013, (27): 102–110.

[9] Nirogi RVS, Kandikere VN, Shukla M, Mudigonda K, Maurya S, Boosi R and Yerramilli.ASensitive and selective liquid chromatography–tandem mass spectrometry method for the quantification of azithromycin in human plasma.AnalyticaChimicaActa, 2005,(553): 1–8.

[10] Haoying Yu, Robert M. Straubinger, Jin Cao, HaoWang, Jun Qu. Ultra-sensitive quantification of paclitaxel using selective solid-phase extraction in conjunction with reversed-phase capillary liquid chromatography/tandem mass spectrometry. Journal of Chromatography A, 2008,(1210):160–167.