



Purification and characterization of a new component of trichosanthin

Cuili Ma, Ping Li and Liqi Bi*

Department of Rheumatology and Immunology, China-Japan Union Hospital, Jilin University, Changchun, People's Republic of China

ABSTRACT

*Trichosanthin (TCS) whose molecular weight is 26000.0 (hereafter called injectable TCS) is a type I ribosome-inactivating protein possessing various pharmacological properties including anticancer effect, inducing mid-term abortion, immunomodulation and anti-HIV activities. Especially, its anticancer effect is more important. In clinic, injectable TCS has been used to treat chorioblastoma as an effective anticancer agent highly specific to choriocarcinoma cells. But injectable TCS also showed immunogenicity and was prone to cause allergic reaction, the application in clinic is restricted thereby. In this study, we initially purified new components of trichosanthin from fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type and artificial planting type respectively. Then we determined their molecular weights and N-terminal amino acid sequences. Finally, we compared the allergic reaction of guinea pigs to new component of trichosanthin and injectable TCS. In conclusion, the new component of trichosanthin can be used as a new drug with wide spectrum anticancer effect and without allergic reaction.*

Key words: trichosanthin, purification, molecular weight, N-terminal amino acid sequence, allergic reaction, anticancer effect

INTRODUCTION

Trichosanthin (TCS) is a type I ribosome-inactivating protein (RIP) possessing N-glycosidase activity and is isolated from the root tuber of *Trichosanthes kirilowii* Maxim. From 1960s, a lot of research in basic theory and clinic application have been performed on TCS whose molecular weight is 26000.0 (hereafter called injectable TCS) [1]. Presently its molecular structure, nucleotide sequence coding protein, physical and chemical characters has been known. Its gene can be expressed in *E.coli* and transgenic tobacco successfully [2]. In recent years, injectable TCS has also been found to possess various pharmacological properties including anticancer effect, inducing mid-term abortion, immunomodulation and anti-HIV activities [3-6]. Especially, its anticancer effect is more important. Many studies have shown that trichosanthin can induce apoptosis of human choriocarcinoma cells [7-9], cervical carcinoma HeLa cells [10,11], mouse melanoma cells [12], gastric cancer cells [13-15], hepatoma cells [16], Leukemia cells [17-19] and also can kill lung cancer cells, colon carcinoma cells, myeloma cells. In clinic, injectable TCS has been used to treat chorioblastoma as an effective anticancer agent highly specific to choriocarcinoma cells [20]. But injectable TCS also showed immunogenicity and was prone to cause allergic reaction [21-23], the application in clinic is restricted thereby. Therefore, in our research, we initially purified new components of trichosanthin from fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type and artificial planting type respectively. Then we determined their molecular weights and N-terminal amino acid sequences. Finally, we compared the allergic reaction of guinea pigs to new component of trichosanthin and injectable trichosanthin. The purpose of this study is to find a new drug with wide spectrum anticancer effect and without allergic reaction.

EXPERIMENTAL SECTION

Materials

Fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type and artificial planting type were gathered from

Henan Province of China. White guinea pigs were purchased from the animal laboratory of Jilin University.

Main reagents and apparatus

Injectable TCS (1.2mg/mL) was purchased from Jinshan Pharmacy Ltd, Shanghai. CM-52 cellulose was purchased from Waterman Company. G-75 gel was purchased from Pharmacia Company. Protein marker, TEMED, 2-ME, and SDS were purchased from Dingguo Biological Products Ltd, Beijing. Acrylamide was purchased from Fluka Company. Other reagents all were analytical reagents made in China. PVDF membrane and Transblot SD Cell apparatus were the products of Bio-Rad Company. Electrophoresis apparatus was made by Liuyi Apparatus Corporation, Beijing. Extra-violet detecting apparatus was made by Beijing New Technology Ltd. LDI-1700 MALDI-TOF MS was the product of linear Scientific Inc, U.S.A. ABI PROCISE™ 492 cLC protein sequencer apparatus was the product of Applied Biosystems Company.

Purification of new components of trichosanthin

All purification procedures were performed at 4°C. 750 g fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type were washed, decorticated, pulverized, and filtrated. Then, solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 40% saturation, the liquid was agitated for 30 minutes, and sedimentated for 4 hours. After centrifugation of the suspension at 3000r/min for 30 minutes, solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 75% saturation. After being left for 12 hours, the precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed against distilled water for 12 hours, then dialyzed against 0.05 mol·L⁻¹ pH 7.0 Tris-HCl buffer solution (Buffer A) for 48 hours. After dialysis, the clear supernatant (crude extract) was applied to a CM-52 cellulose column (6.0cm×20.0cm) pre-equilibrated with Buffer A. The column was eluted with 0.1, 0.2, 0.3 mol·L⁻¹ NaCl-Buffer A respectively. The flow rate was 30ml/h, and the flow-through fractions were collected accordingly.

Electrophoresis

The flow-through fractions and crude extract were analysed by SDS-PAGE [15% (w/v) gel], as described by Laemmli [24]. The protein bands were revealed by being stained with Coomassie Brilliant Blue R-250.

G-75 gel filtration

The flow-through fraction that had been eluted with 0.1 mol·L⁻¹ NaCl-Buffer A through CM-52 cellulose column was concentrated with PEG (20000) and applied to a G-75 gel filtration column (1.0cm×100.0cm) pre-equilibrated with Buffer A. The column was eluted with Buffer A with a flow rate of 12ml/h. The first flow-through fraction was collected.

The purifying procedure of new component of trichosanthin from fresh root tubers of *Trichosanthes kirilowii* Maxim of artificial planting type was as the same as that from wild type, except that there was no G-75 gel filtration.

Then all fractions were dialyzed extensively against distilled water and freeze-dried.

Determination of molecular weight

The molecular weights of new components of trichosanthin were determined by MALDI-TOF MS analysis with LDI-1700 MALDI-TOF MS. The wave length was 337 nm and the pulse width was 3 ns.

N-terminal amino acid sequence analysis

The N-terminal amino acid sequences of new components of trichosanthin were determined by using the automated Edman degradation method with ABI PROCISE™ 492 cLC protein sequencer apparatus.

Study on the allergic reaction of guinea pigs

Eighteen white guinea pigs were divided into 6 groups randomly; each group consisted of 3 guinea pigs. The guinea pigs of the first and second group were sensitized and attacked with egg albumin (0.05mg/ml); The guinea pigs of the third and fourth group were sensitized and attacked with injectable TCS (0.05mg/ml); The guinea pigs of the fifth and sixth group were sensitized and attacked with new component of trichosanthin (0.05mg/ml).

The guinea pigs of the first, third and fifth groups were attacked on the 14th day after the first administration TCS. The guinea pigs of the second, fourth and sixth groups were attacked on the 21st day after the first TCS administration. The grades of allergic reaction in guinea pigs [25] were observed and recorded.

RESULTS

Isolation and purification of new components of trichosanthin

The new component of trichosanthin was purified from the fresh root tubers of *Trichosanthes kirilowii* Maxim of

wild type by two simple steps. One major peak was obtained in the first step of CM-52 cellulose chromatography. In this fraction, a major protein and a minor protein were present, as revealed by SDS-PAGE analysis. Then the minor protein was removed in the second step by G-75 gel filtration (molecular sieve chromatography). The new component of trichosanthin was purified from the fresh root tubers of *Trichosanthes kirilowii* Maxim of artificial planting type by only one step. A major protein was obtained in the second step of CM-52 cellulose chromatography.

Protein electrophoresis

The flow-through fractions and crude extract were analysed by SDS-PAGE [15% (w/v) gel]. The protein bands were revealed by staining with Coomassie Brilliant Blue R-250. Results of SDS-PAGE showed, the flow-through fraction from the fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type that had been eluted with $0.1 \text{ mol} \cdot \text{L}^{-1}$ NaCl-Buffer A through CM-52 cellulose column, an obvious major strap and a minor strap. Other flow-through fractions only had one strap. Furthermore, all the straps had no change with or without 2-ME in the electrophoresis sample loading buffer (Fig. 1).

Determination of molecular weight

The molecular weights of new components of trichosanthin were determined by MALDI-TOF MS analysis using LDI-1700 MALDI-TOF MS. The result showed that the molecular weights of new components of trichosanthin from the fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type were 33388.9 and 28648.9 (Fig. 2). The molecular weights of new components of trichosanthin from artificial planting type were 32116.0 and 26964.9 (Fig. 3).

N-terminal amino acid sequence analysis

The N-terminal amino acid sequences of new components of trichosanthin were determined by using the automated Edman degradation method with ABI PROCISETM 492 cLC protein sequencer apparatus. The result showed that the N-terminal amino acid sequence of new component of trichosanthin from the fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type was D-C-P-S-F-D-L-S-T-A-T-Q-D-

S-Y-A-S-F-I-T-P-L-A, whose molecular weight was 33388.9. The N-terminal amino acid sequence of new component of trichosanthin from the fresh root tubers of *Trichosanthes kirilowii* Maxim of artificial planting type was D-C-P-S-F-D-L-S-T-A-T-Q-D-S-Y-A-S-F-I-T, whose molecular weight was 32116.0.

Comparison of the yield

750g fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type was used in the present study and we got 0.898g new component of trichosanthin, resulting in a yield of $1.2 \text{ mg} \cdot \text{g}^{-1}$. We got 0.227g new component of trichosanthin from 750g fresh root tubers of *Trichosanthes kirilowii* Maxim of artificial planting type, resulting in a yield of $0.3 \text{ mg} \cdot \text{g}^{-1}$.

Comparison of the molecular weight and N-terminal amino acid sequence

The molecular weights and N-terminal amino acid sequences of these two new components of trichosanthin were compared with those of TCS injection, TAP29, and Trochoanguin (Table 1).

Study on the allergic reaction in guinea pigs

The grade I allergic reaction was found by using the new component of trichosanthin to sensitize and attack guinea pigs, the grade II allergic reaction was observed when injectable trichosanthin was used, and the grade IV allergic reaction was found by using egg albumin (Table 2).

DISCUSSION

Trichosanthin (TCS) is an active component that was isolated from the root tuber of *Trichosanthes kirilowii* Maxim. At present, the methods to purify TCS include acetone precipitation, ion exchange chromatography, HPLC, Blue-Sepharose CL-6B chromatography, lot crystallization, etc [26-28]. But these methods were time-consuming and the purification procedure produced poor yields. In this research, new components of trichosanthin were purified from fresh root tubers of *Trichosanthes kirilowii* Maxim by ammonium sulfate precipitation, CM-52 ion exchange chromatography and G-75 gel filtration (molecular sieve chromatography). Thus, a convenient, quick, high-performance purification method has been established. Furthermore, the fresh root tubers of *Trichosanthes kirilowii* Maxim being used in this research were gathered from Henan Province of China. According to Chinese pharmacopeia [29], the output is large and the quality is good in this place.

The molecular weights and N-terminal amino acid sequences were also determined. Moreover, the molecular weights and N-terminal amino acid sequences of these two new components of trichosanthin from fresh root tubers

of *Trichosanthes kirilowii* Maxim of wild type and artificial planting type were compared with those of injectable TCS and other high-homology proteins.

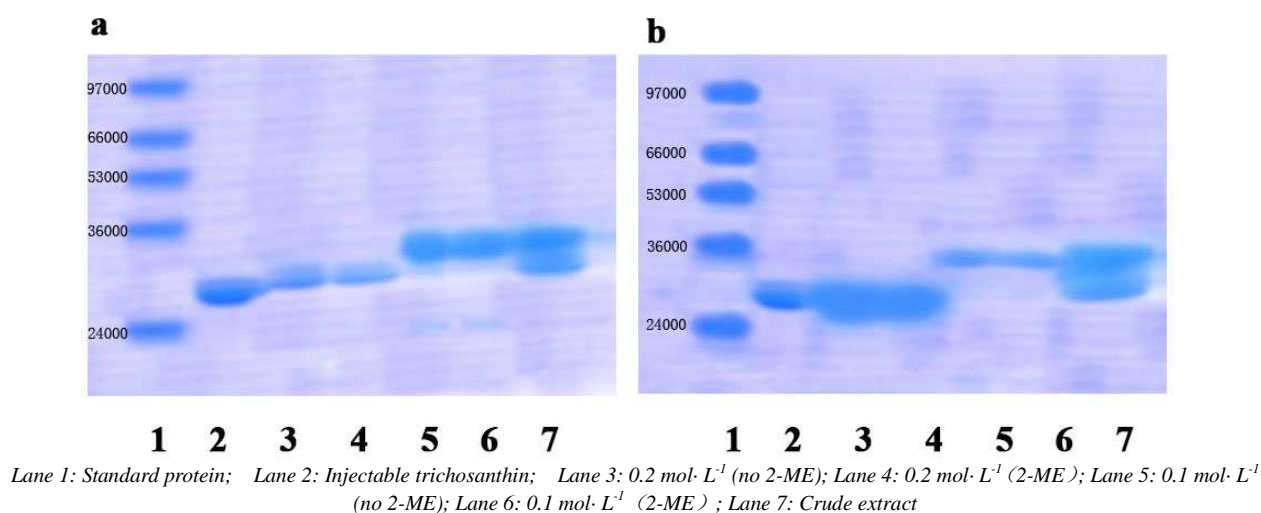
Collins et al. elucidated the primary structure of α -trichosanthin in 1990 [30]. Trichosanthin is composed of 247 amino acid residues. Wangyou et al. confirmed that injectable trichosanthin was composed of 247(246) amino acid residues including 19 types of amino acids. It is a single subunit protein and the primary structure does not contain cysteine [1].

The result of this research showed that the molecular weights of these two new components of trichosanthin from fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type and artificial planting type were different (33388.9 and 32116.0 Da respectively), but their N-terminal amino acid sequences were same. The 2-residues of N-terminal ends of both were cysteine. According to SDS-PAGE results, the protein bands had no change with 2-ME or without 2-ME in sample loading buffer. The result revealed that these two new components of trichosanthin were both single-subunit protein. NCBI database search did not show same sequence with the new component. In contrast, we discovered that these two new components of trichosanthin were highly homologous with Trochoanguin and MAP30. Trochoanguin is a type I ribosome-inactivating protein and MAP30 is a type of anti-HIV protein. Trochoanguin is purified from the seeds of *Trichosanthes anguina*, and it contains two cysteine residues. Trochoanguin possesses various pharmacological properties including induction of abortion, anticancer and anti-HIV activities [31].

The result also showed that the molecular weights and N-terminal amino acid sequences of these two new components of trichosanthin from fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type and artificial planting type were different from trichosanthin (26000) that had been used to inject in clinic. It was also different from TAP29, another anti-HIV protein that was purified from *Trichosanthes kirilowii* Maxim that grew in America. It has been shown that the toxicity of TAP29 was lower than TCS [32]. Furthermore, we found that the yields were different between the wild and artificial planting type.

Recent research has shown that injectable TCS possessed not only various pharmacological properties but also immunogenicity [23]. It is prone to cause allergic reactions. In order to know the immunogenicity of the new component of trichosanthin, we performed the allergic test with white guinea pigs. In the allergic test of guinea pigs, we only found a first grade allergic reaction by using the new component of trichosanthin to sensitize and attack guinea pigs. In contrast we found a second grade allergic reaction when the injectable trichosanthin was used. The result showed that the immunogenicity in guinea pigs to the new component of trichosanthin was negative.

Fig. 1 Analysis of crude extract, injectable TCS and the flow-through fractions through CM-52 cellulose column by SDS-PAGE
a: Wild type; b: Artificial planting type;



In conclusion, this research has established a convenient, quick, high-performance method for purification of new components of trichosanthin. We confirmed that these new components of trichosanthin have a high homology with Trochoanguin, a type I ribosome-inactivating protein and MAP30, a type of anti-HIV protein. The allergic reaction in guinea pigs to the new component of trichosanthin was negative. It is possible that the new component of trichosanthin can be used as a new drug with wide spectrum anticancer effect and without allergic reaction. (The research on anticancer effect of this new component of trichosanthin are performing, and it is going to be reported in

other articles.)

Fig. 2 MALDI-TOF MS analysis of the flow-through fractions from the fresh root tubers of *Trichosanthes kirilowii* Maxim of wild type through CM-52 cellulose column
a: $0.1 \text{ mol} \cdot \text{L}^{-1}$; b: $0.2 \text{ mol} \cdot \text{L}^{-1}$

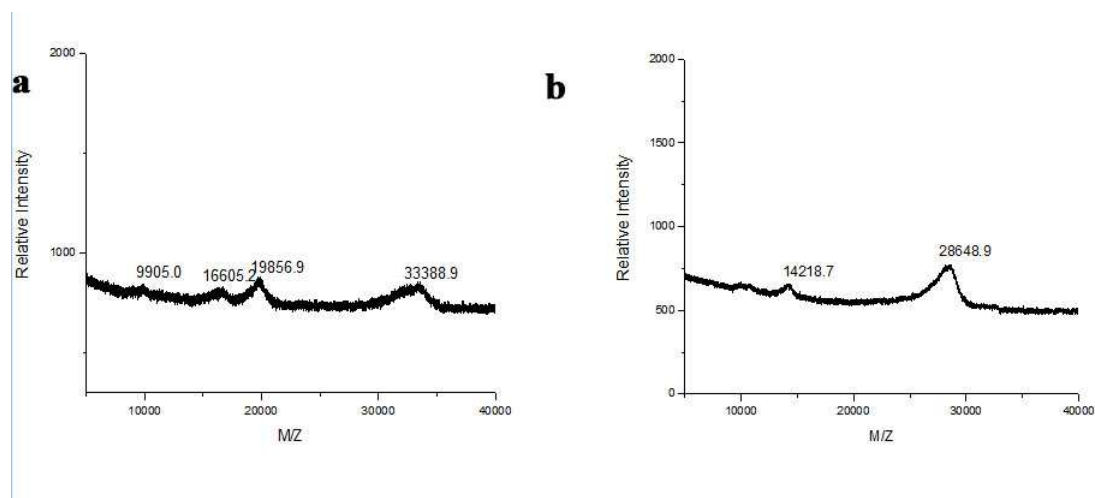


Fig. 3 MALDI-TOF MS analysis of the flow-through fractions from the fresh root tubers of *Trichosanthes kirilowii* Maxim of artificial planting type through CM-52 cellulose column
a: $0.1 \text{ mol} \cdot \text{L}^{-1}$; b: $0.2 \text{ mol} \cdot \text{L}^{-1}$

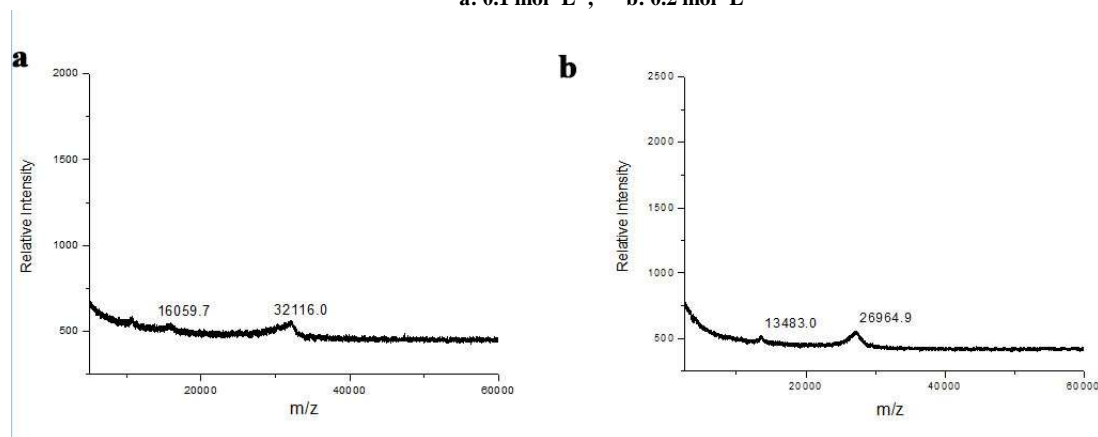


Table 1 Comparison of the molecular weight and N-terminal amino acid sequence

	Molecular weight	N-terminal amino acid sequence	Residue location
Injectable TCS	26000.0	D-V-S-F-R-L-S-G-A-T-S-S-S-Y-G-V-F-I-S-N-L-R-K	1-23
TAP 29	29000.0	D-V-S-F-R-L-S-G-A-T-S-K-K-K-V-Y-F-I-S-N-L-R-K	1-23
wild type	33388.9	D-C-P-S-F-D-L-S-T-A-T-Q-D-S-Y-A-S-F-I-T-P-L-A	1-23
artificial planting type	32116.0	D-C-P-S-F-D-L-S-T-A-T-Q-D-S-Y-A-S-F-I-T	1-20
Trochanterin	35000.0	S-F-D-L-S-T-A-T-K-K-S-Y-S-S-F-I-T-Q-L	3-21

Table 2 The allergic reaction of guinea pigs to different components

Groups (N = 3)	Grades of allergic reaction	Positive rate (%)
1 (egg albumin)	IV	100
2 (egg albumin)	IV	100
3 (injectable TCS)	II	100
4 (injectable TCS)	II	100
5 (new component of trichosanthin)	I	0
6 (new component of trichosanthin)	I	0

Acknowledgement

Supported by the National Natural Science Fund of China (No.39770913) and the fund of Jilin Provincial Science and Technology Department (No.20060925).

REFERENCES

- [1] Wang Y (2000) Trichosanthin, 2nd edn. Beijing: Sci Pub, pp 27-36
- [2] Krishnan R, McDonald KA, Dandekar AM et al (2002) *J Biotechnol* 97(1):69-88
- [3] Shaw PC, Lee KM, Wong KB (2005) *Toxicol* 45(6):683-689
- [4] Zhou H, Jiao Z, Pan J et al (2007) *Clin Immunol* 122(3): 312-322
- [5] Zhou X, Yang N, Lu L et al (2007) *Immunol Lett* 110(1): 74-81
- [6] Wang YY, Ouyang DY, Huang H et al (2005) *Biochem Biophys Res Commun* 331(4): 1075-1080
- [7] Wang Y, Mi SL, Lou MY et al (2005) *Front Biosci* 10: 2279-2284
- [8] Zhang C, Gong Y, Ma H et al (2001) *Biochem J* 355(3): 653-661
- [9] Zhang CY, Gong YX, Ma H et al (2000) *Analyst* 125(9): 1539-1542
- [10] Wang P, Yan H, Li JC (2007) *Biochem Biophys Res Commun* 363(1): 101-105
- [11] Wang P, Li JC (2007) *Life Sci* 81(14): 1130-1140
- [12] Bi LQ, Li HJ, Zhang YH (1998) Effect of trichosanthin of cell cycle and apoptosis of murine melanoma cells. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 18(1): 35-37
- [13] Tu SP, Jiang SH, Qiao MM et al (2000) *Cancer* 19(12): 1105-1108
- [14] Sun J, Tu SP, Wu YX et al (2001) *Shanghai Med J* 24(5): 292-294
- [15] Zhang S, Hu MJ, Wu YX et al (2000) *Chin J Dig* 20(6): 380-382
- [16] Zhou XY, Zhang TY, Shi HY et al (2003) *Acta Academiae Medicinae Nantong* 23(4): 371-374
- [17] Li J, Xia X, Ke Y et al (2007) *Biochim Biophys Acta* 1770(8): 1169-1180
- [18] Luo WD, Ren CM, Zhu M et al (2005) *Journal of Experimental Hematology* 13(2): 278-281
- [19] Li J, Xia X, Nie H et al (2007) *Biochim Biophys Acta* 1770(1): 63-70
- [20] Chan WY, Huang H, Tam SC (2003) *Toxicology* 186: 191-203
- [21] An Q, Lei Y, Jia N et al (2007) *Biomol Eng* 24(6): 643-649
- [22] Zhang XY, Wu Y, Yan JY et al (2006) *Biochem Biophys Res Commun* 343(4): 1251-1256
- [23] An Q, Wei S, Mu S et al (2006) *J Biomed Sci* 13(5): 637-643
- [24] Laemmli UK (1970) *Nature*. pp 680-685
- [25] Wang QM, Li L, Wang J et al (1999) *Journal of Anhui Tcm College* 18(3): 47-49
- [26] Yeung HW, Poon SP, Ng TB et al (1987) *Immunopharmac Immunotoxicol* 9: 25-46
- [27] Maraganore JM, Joseph M, Bailey MC (1987) *J Biol Chem* 262 (24):11628-11633
- [28] Song JF, Liu T, Shen X et al (1998) *Electrophoresis* 19(7) :1097-1103
- [29] Yuan Z, Tu Y, Peng ZB et al (1999) *Collection of Chinese Medicine*. Beijing: Chinese Medicine Sci Pub, pp 432
- [30] Collins E J, Robertus J D, Lopresti M et al (1990) *J Biol Chem* 265: 8665-8669
- [31] Chow LP, Chou MH, Ho CY et al (1999) *Biochem J* 338 (1): 211-219
- [32] Lee-Huang S, Huang PL, Kung HF et al (1991) *Proc Natl Acad Sci U S A* 88 (15): 6570-6574