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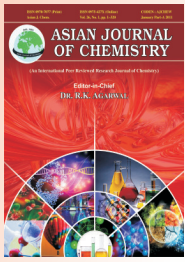

High Expression in *E. coli* and Biological Activity of T-19 Peptide

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To obtain antitumor peptide of Tumstatin (T-19 peptide) and study its biological activity are reported in this paper. According to the nucleotide sequence of T-19 peptide and *E. coli* favorite to base, the gene of T-19 was synthesized and inserted into the vector pTYB2.After the recombined vector T-19-pTYB2 was transformed into BL-21 (DE3) *E. coli* competent cells. Transformed *E. coli* BL-21DE3 were induced by isopropyl-β-thiogalactopyranoside (IPTG) and high expressed under the condition 28 ºC, 0.1 mmol/L IPTG, 6 h. By 1,4-dithiothreitol (DTT) induced, T-19 peptide was obtained from chitin affinity chromatograph. The rate of its protein purification was as much as 90.83 % by high performance liquid chromatography. Its biological activity was identified by HE stain, TUNEL assay, transmission electron microscopic (TEM). T-19 peptiade can inhibit human hela cell proliferation,promote human hela cell apoptosis and have no affect on endothelial cell. It would be an efficient method for tumor therapy in future, which will lay foundation on its mechanism of action research and clinical tumor therapy.

Keywords: T-19, Protein expression, Protein purification, Tumor therapy, BL-21 (DE3) *E. coli*.

INTRODUCTION

Tumstatin, a NC1 domain fragment of the type IV collagen α3 chain, was discovered as good-pasture antigen in 1980's[1]. By 2000, tumstatin was found that it can repress the proliferation of melanoma cell directly[2]. It can bind the integrin αvβ3 to mediate the intercellular interaction independent of vitronectin, fibronectrin and RGD sequences[3]. But it was a large molecular weight protein (28 KD) and it was an antigen of good-pasture itself, which made it unsecured in clinical application. Further studies indicated T-19 peptide had antitumor activity independent of Tumstatin. When being a part of the whole Tumstatin, T-19 peptide could not display antitumor activity, the reasons of which may be that the active region was covered and not to bind to the receptors[4]. So we designed and artificially synthesized the base sequence of T-19 peptide and construct its gene engineering bacteria of *E. coli* BL-21(DE3). The T-19 peptide was then expressed in *E. coli* BL-21(DE3) and purified with chitin affinity chromatograph. Finally, the soluble T-19 peptide was obtained and the initial research was carried out on its antitumor activity, which will help to identify its biological activity.

EXPERIMENTAL

Synthesis of T-19 gene: According to the nucleotide sequence encoding 185-203 amino acids of Tumstatin and the codon *E. coli* favored, the gene of T-19 peptide was designed to enhance its expressing level in genetic engineering bacteria. 3'termini of the designed T-19 gene was blunt. 5'termini of T-19 gene was NdeI restriction site. Then the T-19 gene was synthesized by DNA synthesizer. The sequence as follows:

5'-T A TG GCT AGC CCT TTC CTA GAA TGT CAT GGA AGA GGA ACG TGC AAC TAC TACTCA AAC TCC -3' (61bp). 3' AC CGA TCG GGA AAG GAT CTT ACA GTA CCT TCT CCT TGC ACG TTG ATG ATGAGT TTG AGG -5' (59bp)

Construction of recombined T-19-pTYB2 plasmid: The synthesized T-19 gene was phosphorylated, annealed and became double strand DNA as target gene. The vector pTYB2 (NEB Corporation) was cleaved by NdeI and SmaI restriction endonuclease (Promage Corporation). After T-19 gene and above vector pTYB2 purified by gel DNA extraction kit, both of them was ligased by T4DNA ligase under 16 ºC, over night. The recombined T-19-pTYB2 plasmid was transformed in JM109 *E. coli* competent cells and identified by means of restriction endonuclease and gene sequencing.

Construction BL-21 (DE3) *E. coli* engineering expression bacteria of T-19-pTBY2: After the recombined T-19-pTYB2 plasmid was identified , the reconstructed T-19-pTYB2 plasmid was extracted from JM109 *E. coli* clone bacteria by gel DNA extraction kit and transformed in BL-21 (DE3) *E. coli* competent cells. Then the BL-21 (DE3) *E. coli* Engineering expression bacteria of T-19-pTYB2 was constructed.

T-19 peptide expression: T-19-pTYB2 transformed in BL-21 (DE3) *E. coli* was plated on Luria Bertani (LB) agar plates containing 100 μg $mL^{-1}$of ampicillin and incubated at 37 ºC for 14-18 h. Isolated colonies on the agar plates were selected and used to inoculate 10 mL of LB media containing 100 μg mL^{-1} of ampicillin, which was maintained in rotary culture at 37 ºC overnight. Then 5 mL of transformed culture was used to inoculate 250 mL of LB media containing 100 μg mL^{-1} of ampicillin (culture: LB = 1:50). This large-scale culture was maintained at 37 ºC until it reached an optical density (OD 600 = 0.5). Protein expression was induced under different concentration of IPTG , culture temperature and culture time to obtain the favorite T-19 peptide expression condition. The rate of T-19 peptide expression was detected by SDS-PAGE.

T-19 peptide purification: T-19 peptide expression was induced by IPTG at a final concentration of 0.1 mmol L^{-1}, 28 ºC, 6 h. Induced cells were harvested by centrifugation at 5000 g. Then the cell pellet was thawed on ice and resuspended in phosphate buffer (50 mM NaH_2PO_4, 10 mM imidazole and 2.5 M NaCl, pH 8) containing lysozyme at a concentration of 100 μg mL^{-1} and the solution was incubated on ice for 0.5 h. Following enzymatic lysis, the cells were sonicated for six cycles of 10 s each and then centrifuged at 19000 g for 0.5 h at 4 ºC. The lysate supernatant was harvested and introduced into chitin-affinity column (NEB Corporation). Though affinity column balanced, sample added, column washed, 30-50 mM DTT induced, T-19 peptide was obtained from chitin affinity chromatograph. The rate of protein purification was detected by SDS-PAGE and high performance liquid chromatography.

Determination of biological activities of T-19 peptide

HE stain: Human hela cells and human endothelial cells in good condition were digested by 0.5 % pancreatic protease and cell supernatant was made following conventional methods. About 1.5×10^4 human hela cells and human endothelial cells were cultured in 6-well plates. All cells were maintained in a RPMI1640 cell culture medium supplemented with 10 % FCS at 37 ºC and 5 % CO_2. At the same time, microscopic glass was put in every well. After the cells were allowed to equilibrate for 24 h, experimental wells were replaced with the media containing purified T-19 peptide at concentration of 44 μg mL^{-1}, the control wells were added the same volume PBS. By 48 h, microscopic glass had been taken out to HE staining. Cell morphology of human hela cells and human endothelial cells had been observed under light microscope as soon as microscopic glass was dried at 37 ºC.

Tunnel assay: Human hela cells and human endothelial cells in good condition were digested by 0.5 % pancreatic protease and cell supernatant was made following conventional methods. About 1.5×10^4 human hela cells and human endothelial cells were cultured in 6-well plates. At the same time, microscopic glass was put in every well. After the cells were allowed to equilibrate for 24 h, experimental wells were replaced with T-19 peptide at concentration of 44 μg mL^{-1}.The control wells were added the same volume growth media. By 24 h, microscopic glass had been taken out and fixed by 4 % paraformaldehyde for 20-30 min after air drying. The cell apoptosis was determined followed the methods of tunnel assay kit. Typical morphological manifestations of apoptosis and necrosis were observed in light microscopy and fluorescence microscopy (Fig. 1).

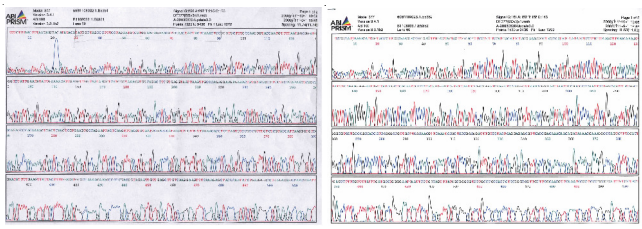

Fig. 1. Sequencing result of recombinant plasmid T-19-pTYB2

Cell morphology detection by TEM: Human hela cells and human endothelial cells in good condition were digested by 0.5 % pancreatic protease and cell supernatant was made following conventional methods. Both cells were divided into two groups, respectivly. Experimental group was replaced with T-19 peptide at concentration of 44 μg mL^{-1}. The control group was added the same volume growth media. Cells were cultured 37 ºC for an additional 24 h. Cells of two groups were washed two times by PBS to discard cell debris of apoptosis and necrosis. After that, they were digested by 0.5 % pancreatin and cell supernatant was made and centrifuged by 1000 g for 10 min. Cell precipitation of human hela cells and human endothelial cells was washed two times by PBS and then be fixed with glutaraldehyde at 4 ºC for 24 h. Cell precipitation was made ultra-thin section cutting of 50-80 nm. Cell morphology of two kinds of cells had been observed under TEM.

RESULTS AND DISCUSSION

Recombined plasmid identification: There was XhoI restriction site between NdeI and SmaI restriction site in native pTYB2 vector. Because the pTYB2 vector ligased to T-19 would lose XhoI restriction site, so XhoI restriction endonulease was used to identify weather T-19 recombined pTYB2 at first. On the other hand, 5'TAATACGACTCACTATAGGG3' as the forward primer, 5'CCATCACCTTATTACCAACCTC3' as the reverse primer, the sequencing results was completely consistent with the T-19 base sequence we designed.

T-19 peptide expression and purification: Recombinant plasmid T-19- pTYB2 transformed in BL-21 (DE3) was expressed by fusion protein. The molecular weight of recombinant plasmid was about 55KD. After T-19-pTYB2 plasmid was induced by IPTG in BL-21 (DE3), the rate of T-19 peptide expression was detected by SDS-PAGE. There was an obvious belt on the site of 55 KD. Under the optical condition 28 ºC, 0.1 mmol L^{-1} IPTG, 6 h, T-19 peptide expressed was as much as 40 %. By DTT induced, the intein underwent specific self-cleavage which released the T-19 peptide from the chitin-bound intein tag resulting in one single-affinity column purification. Due to T-19 peptide's small molecular weight, it had not been able

to be detected on a regular SDS-PAGE gel. Its purification ran up to 90.83 % by high performance liquid chromatography. The results of SDS-PAGE gel and curves of HPLC for T-19 peptide (Figs. 2 and 3)

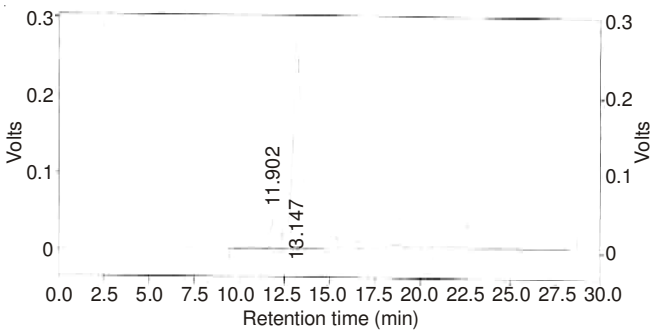

Fig. 2. T-19 peptide was analyzed by HPLC

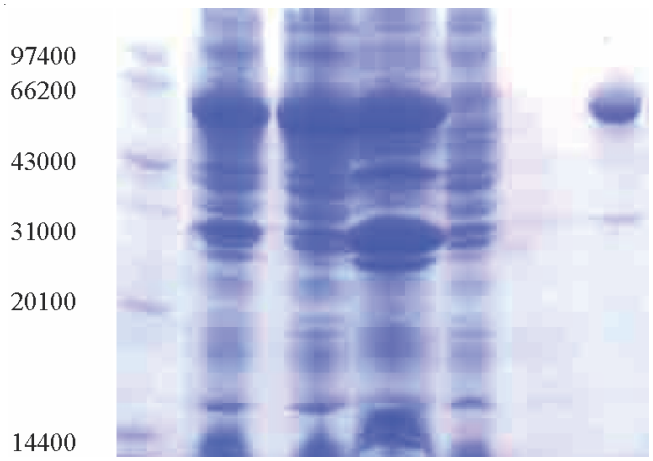

Fig. 3. SDS-PAGE analysis of T-19 peptide. 1: mark; 2: After induction; 3: Supernatant protein; 4: Precipitation; 5: mixed protein of *E. coli*; 6: T-19 peptide; 7: vector protein.

Results of T-19 peptide biological activities *in vitro*

Results of HE stain: Under oil microscope, by HE staining, human hela cells with T-19 peptide were observed nucleolus condensation, nuclear fragmentation, cell surface with vacuole, it is difficult to find cell morphology changes in human umbilical vein endothelial cells (ECV304) with T-19 peptide, only a few cells appear volume smaller,which indicated that T-19 peptide probably can promote human hela cells apoptosis much stronger than human umbilical vein endothelial cells (Fig. 4).

Results of TUNEL assay: There was a large number of apoptotic cells which showed green fluorescence in experiment group of human hela cells under fluorescence microscopy. But human umbilical vein endothelial cells (ECV304) shows less in the field of fluorescent colour. By light microscopy, experiment group of human hela cells appears cell antennae geting shorter or disappear, changing colour to brown, reduced volume, cytoplasmic condensation, decreased cell density and so on. While the experiment group of human umbilical vein endothelial cells compared with control group,the cell morphology and colour change is not obvious and the cell density decreased slightly (Figs. 5 and 6).

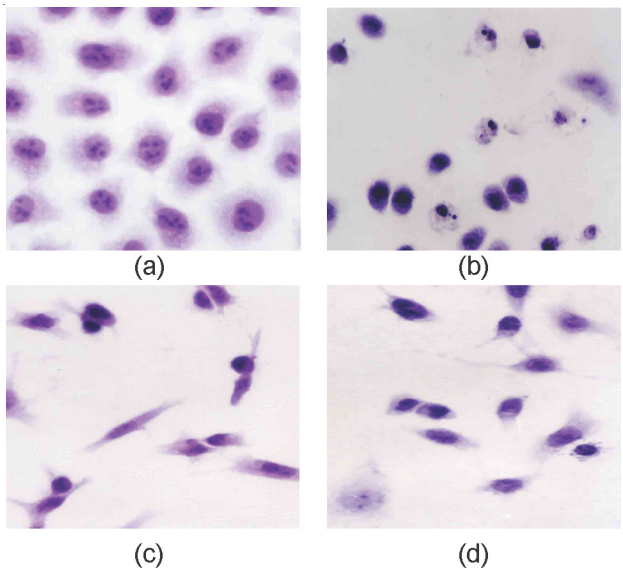

Fig. 4. HE staining of two kinds of cells (× 400). (A) hela cell control group (B) hela cell experiment group (C) ECV304 control group (D) ECV304 experiment group

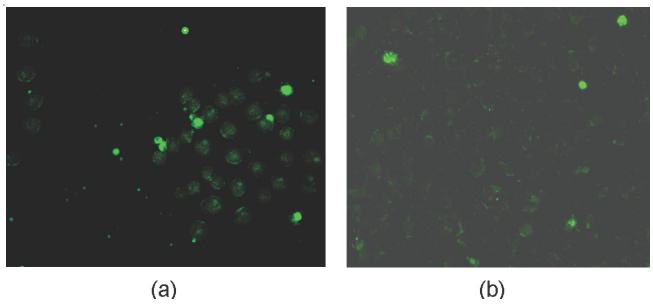

Fig. 5. TUNEL assay of two kinds of cells under fluorescence microscopy (A) hela cell experiment group (B) ECV304 experiment group

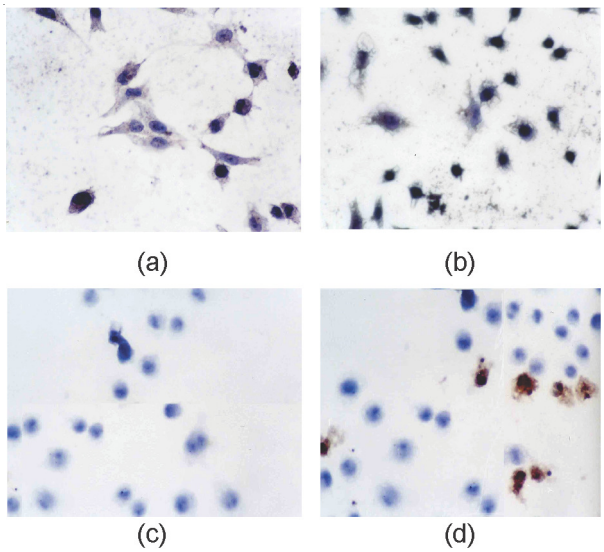

Fig. 6. TUNEL assay of two kinds of cells under light microscopy. (A) hela cell control group (B) hela cell experiment group (C) ECV304 control group (D) ECV304 experimentn group

Transmission electron microscopic observation on cell morphology: The characteristics of apoptosis could be observed in human hela cells of experimental group, such as reduction of cell surface microvilli, intracytoplasmic vacuoles,

irregular, fragmentation of apoptotic nuclear, formation of granular of nucleus chromatin, part of the plasma membrane containing organelles off. Human endothelial cell in experiment group were less apoptosis, the visible cells were vary in size, irregular shape and extracellular villus were reduced gradually (Fig. 7).

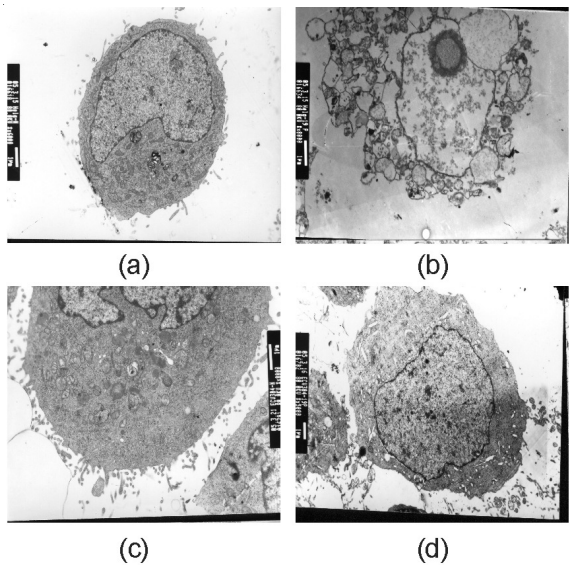

Fig. 7. Result of cell morphology of two kinds of cells under transmission electron microscopic. (A) hela cell control group (B) hela cell experiment group (C) ECV304 control group (D) ECV304 experiment group

Tumstatin was discovered to promote tumor cell apoptosis[5]. Further studies indicated T-19 peptide had antitumor activity independent of Tumstatin. The active domain of 19 peptide was located 189-191 amino acids, that is -SNS-, which constituted turn structure between two beta pleated sheets. If the spatial structure of -SNS- was not variable, T-19 peptide could maintain its antitumor activity[6]. Based on the achievements above, we tended to reconstruct Tumstatin's structure, genetic engineering methods would be applied to construct high protein expression *E. coli* gene enginerring bacteria. T-19 peptide was expressed by fusion protein and purified in just one single step of chitin affinity chromatograph. The advantage of this methods: minimum loss, high yield of fusion polypeptide, lower cost profit the competing market, which will lay foundation on its mechanism of action, biological activity and clinical tumor therapy.

Because T-19 peptide was of low molecular weight, it was not detected in a regular SDS-PAGE gel, which would bring about the difficulty to detect and identify its concentration, expression, purification. But the vector pTYB2 and chromatographic analysis matrix (chitin beads) that we choose to use decided that when the second peak of chromatographic analysis was coming, at the same time, what we collected was the purified T-19 peptide. A280/A260 determination and high performance liquid chromatography detection could derive its density and the rate of purification. The rate of its protein purification was as much as 90.83 %.

As reported, T-19 peptide had the strong inhibitory action to murine B16 melanoma cell[7]. Weather it could influence the proliferation of other tumor cells still needed the further research to confirm. Human hela cells as experiment cell and human endothelial cell as normal cell were chosed to test. By HE stain, TUNEL assay, transmission electron microscopic (TEM), T-19 peptiade can inhibit human hela cell proliferation, promote human hela cell apoptosis and have no affect on endothelial cell, which means T-19 peptide will have antitumor activity and have no side-effect on normal cells. It would be an efficient method for tumor therapy in future[8].

ACKNOWLEDGEMENTS

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