American Journal of Medical Sciences and Medicine, 2014, Vol. 2, No. 1, 1-6
Available online at http://pubs.sciepub.com/ajmsm/2/1/1

DOI:10.12691/ajmsm-2-1-1

Orthogonal Test Design for Optimization of the Expression of Thymosin α1 and Thymosin α1- iRGD Gene in Engineered *E.coli* BL21 Strain

Xingzhen Lao[1], Meng Liu[1], Fang Zhang[2], Heng Zheng[1,*]

[1]School of Life Sciences and Biotechnology, China Pharmaceutical University, Nanjing, Jiangsu, PR China
[2]School of Pharmacy, Nanjing University of Traditional Chinese Medicine, Nanjing, Jiangsu, PR China
*Corresponding author: zhengh18@hotmail.com

Received November 28, 2013; Revised December 25, 2013; Accepted December 30, 2013

Abstract In order to increase the expression of fusion protein of thymosin α1(Tα1) and thymosin α1-iRGD (Tα1-iRGD) by the engineered E. coli BL21 strain, containing pET32a-Trx-Tα1 and pET32a-Trx-Tα1-iRGD plasmid, respectively. The key parameters that influenced the expression of Tα1 and Tα1-iRGD were optimized by employing an orthogonal experiment [$L_{25}(5)^3$], including OD_{600nm} before induction, lactose concentration and induction time, each with five levels. The intensity of target protein band was scanned as a quantitative measure method of the protein expression, after electrophoresis separation of total soluble protein on SDS-PAGE. For Tα1 fusion protein, the optimal conditions were OD_{600nm}=0.6 before induction, 2.5 mmol/L lactose concentration, 4 hours induction time. Target protein expression levels could be achieved 32.8% of the total soluble proteins. For Tα1-iRGD fusion protein, the optimal conditions were OD_{600nm}=0.8 before induction, 7.5mmol/L lactose concentration, 4 hours for induction. Under the condition, the amount of expressed protein could reach 33.8% of the total soluble proteins. Whereas before optimization, the expression level of Tα1 and Tα1-iRGD fusion proteins were 20.6% and 9.0% of the total soluble proteins, respectively. The orthogonal test was proved to be an effective method to optimize the expression of target proteins.

Keywords: *thymosin α1, thymosin-iRGD, gene expression, orthogonal experiment, anticancer*

Cite This Article: Xingzhen Lao, Meng Liu, Fang Zhang, and Heng Zheng, "Orthogonal Test Design for Optimization of the Expression of Thymosin α1 and Thymosin α1- iRGD Gene in Engineered *E.coli* BL21 Strain." *American Journal of Medical Sciences and Medicine* 2, no. 1 (2014): 1-6. doi: 10.12691/ajmsm-2-1-1.

1. Introduction

Thymosin alpha 1 (Tα1), a 28-amino acid peptide first described and characterized by Goldstein et al., is known to improve the immune response [1,2]. Tα1 has been used clinically under the trade name Zadaxin for the treatment of chronic hepatitis B viral (HBV) and hepatitis C virus (HCV) infections [3,4]. Additionally, Tα1 shows remarkable effects in the treatment of other diseases such as non-small cell lung cancer (NSCLC), hepatocellular carcinoma, AIDS, malignant melanoma, drug-resistant tuberculosis, and DiGeorge's syndrome [5,6].It was also found that Ta1 and its derivatives have been shown to reduce tumor cell growth and used in the treatment of a variety of cancer in vitro and in vivo, such as liver [7], lung [8], breast [9], colorectal cancer [10], glioblastoma [11]. The Tα1 content from isolated thymosins (thymosin fraction 5) is very low, in the range of 0.56-1.0% [12]. Therefore, chemical synthesis by solid-phase technique has been used to produce large amounts of Tα1 for clinical trials [13]. It possesses the advantages in high efficiency, no endotoxin and/or DNA contamination. However, this method showed disadvantages in long chemical synthesis process with many protection and deprotection steps, resulting in a relatively low productivity and high cost of the final product. Recently, genetic engineering expression of Tα1 in different hosts including *Escherichia coli*(*E.coli*), *Pichia pastries* and plants [14,15] has attracted attention due to its potential for producing bioactive Tα1 with relatively high efficiency.

The arginine-glycine-aspartic acid (RGD) is a cell adhesion motif present in many proteins of the extracellular matrix (ECM). Peptides containing RGD fragment can specifically bind integrin αvβ3, which is specifically and highly expressed in tumor cells or tumor neovascular endothelial cells, but not in normal tissue blood vessels [16,17]. Therefore, RGD-based recognition has been successfully applied in tumor targeting during the past decades [18,19,20]. Recently, the more efficient tumor-penetrating peptide iRGD was reported by Sugahara et al [21,22], which initially binding to αv integrin and then penetrate into tumor cells by neuropillin-1-dependent manner.

In our previous work, the engineered bacteria *E. coli* BL21-pET32a -Tα1 and *E. coli* BL21-pET32a-Tα1-iRGD were constructed, and the activities of purified Tα1 and Tα1-iRGD were tested on different cancer cell lines. The results showed that the introduce of iRGD fragment

enhanced the basal ability of Tα1 to inhibit the proliferation of cancer cells in vitro, particularly of mouse melanoma cell line B16F10 and human lung cancer cell line H460 [23]. To accumulate enough amount of soluble form of Tα1-iRGD for further research and development, this report discusses the optimization of the induced expression condition of the engineered bacteria. Three factors as the lactose concentration, OD_{600nm} before the induction and induction time were chosen with five levels, and these conditions were optimized by orthogonal experimental design to get the suitable condition for gene expression.

2. Materials and Methods

2.1. Materials

All the restriction enzymes used in the construction of recombinant plasmid were purchased from Takara Biotechnology (Dalian) Co.,Ltd., and T4 DNA ligase was purchased from Promega (USA). Lactose was purchased from Nanjing Chemical Reagent Corporation. Ampicillin and Dithiothreitol (DTT) were purchased from Shanghai Sangon Biological Engineering Corporation. Nickel-chelating column was purchased from GE Healthcare Company.

2.2. Construction of Engineered Strains for the Expression of Tα1 and Tα1-iRGD

The synthetic genes coded for Tα1 and Tα1-iRGD were insert into pET32a plasmid behind Trx-His×6-$(Asp)_4$Lys tag, and *E.coli* BL21 harboring positive recombinant plasmid were grown at 37°C on Luria-Bertani medium containing 100μg/ml ampicillin, as our previous work reported [23]. The expression of fusion proteins were induced by the addition of lactose, and the conditions were optimized as below.

2.3. Protein Assay and Mass Spectroscopy

The cells were harvested by centrifugation (10,000g, 10min, and 4°C), suspended in ice-cold 10ml buffer A (50mM Tris–HCl buffer, pH7.9, including 0.5mM EDTA, 50mM NaCl, and 5% glycerin), and disrupted by sonication. After centrifugation at 12,000g for 20 min(4°C), the supernatant of the cell lysate was analyzed by 15% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie brilliant blue R-250 (Bio Basic Inc.), then scanned by BandScan 5.0(GLYKO). The target fusion proteins were purify by nickel-chelating affinity, and confirmed by mass spectroscopy (Brook ultrafleXII MALDI-TOF instrument).

2.4. Optimization of the Expression Conditions

Orthogonal Text Design, also known as Orthogonal Array Testing, is a systematic, statistical way of testing pair-wise interactions. It creates an efficient and concise test suite with fewer test cases without compromising test coverage by using Orthogonal Array, which is a statistical method of defining parameters that convert test areas into factors and levels. Orthogonal Arrays are represented by: $L_{Runs}(Levels)^{Factors}$, in which Runs means the number of rows or number of test cases in the array that will be generated by the Orthogonal Array technique. Each row represents a test case. Levels means the maximum number of values in an Orthogonal Array that can be taken on by any single factor. Factors means the number of columns or the number of parameters/variables in an array that need to be tested in the system. In this report, an orthogonal experiment [$L_{25}(5)^3$] test design was used for optimizing the expression of the exogenous gene. The key parameters concerned in this report including OD_{600nm} before induction(A), lactose concentration(B), and induction time (C). Five levels of each factor were designed as Table 1 shown. The experimental conditions for induced expression of the target protein were set according to the orthogonal table. All experiments were repeated three times, and the results were represented as average values.

Table 1. Orthogonal Experiment Design (three factors and five levels)

Levels	Factors		
	A,OD_{600nm} [a]	B, Lactose[b] (μmol/ml)	C, Induction time(h)
1	0.1	1	2
2	0.2	2.5	4
3	0.4	5	6
4	0.6	7.5	8
5	0.8	10	10

[a] The absorbance at 600nm before add the lactose;
[b] The final concentration after lactose solution was added.

2.5. Verification of the Optimized Conditions

To verify the orthogonal test design results, the induced expression of Trx-Tα1 and Trx-Tα1- iRGD were conducted at the optimal condition and repeated three times, respectively. The expression of proteins was measured by SDS-PAGE electrophoresis, followed with intensity scan, as mentioned above.

3. Results

3.1. Cloning, Expression, and Purification of Trx-Tα1 and Trx-Tα1- iRGD Fusion Proteins

Tα1 and Tα1-iRGD genes cloned in the frame between KpnI and HindIII restriction sites of pET32a plasmid (Figure 1), which produced fusion proteins of Trx-Tα1 and Trx-Tα1-iRGD, respectively. The correction of the genes was confirmed by gene sequencing (Figure S1).

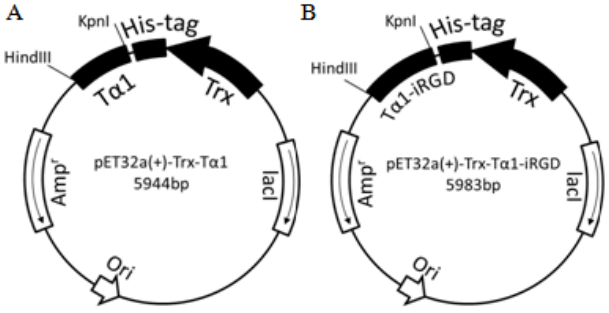

Figure 1. Construction of the recombinant plasmid, A. pET32a-Trx-Tα1; B. pET32a –Trx-Tα1-iRGD

Figure S1. Verification of gene sequences on recombinant plasmids. (A) and (B) were blast results of gene sequence on plasmid pET32a-Trx –Tα1 and pET32a-Trx-Tα1-iRGD, respectively. Query refers to gene sequence on plasmid (for clarity, only Tα1 and Tα1-iRGD sequence were shown). Sbjct was the synthetic DNA sequence coding for Tα1 (A) and designed Tα1-iRGD (B); (C) and (D) were sequencing chromatograms of recombinant plasmid pET32a-Trx - Tα1and pET32a-Trx -Tα1-iRGD, respectively

3.2. MALDI-TOF Mass Mpectroscopy

The molecular weight of Trx-Tα1 fusion protein was detected as 20,023.463 by using MALDI mass spectrometry, which is in agreement with the theoretical value (Figure 2A). Meanwhile, the molecular weight of Trx-Tα1-iRGD fusion protein was 21,183.711, which is consistent with the expected value as well (Figure 2B). These results confirm the successful expression of target proteins.

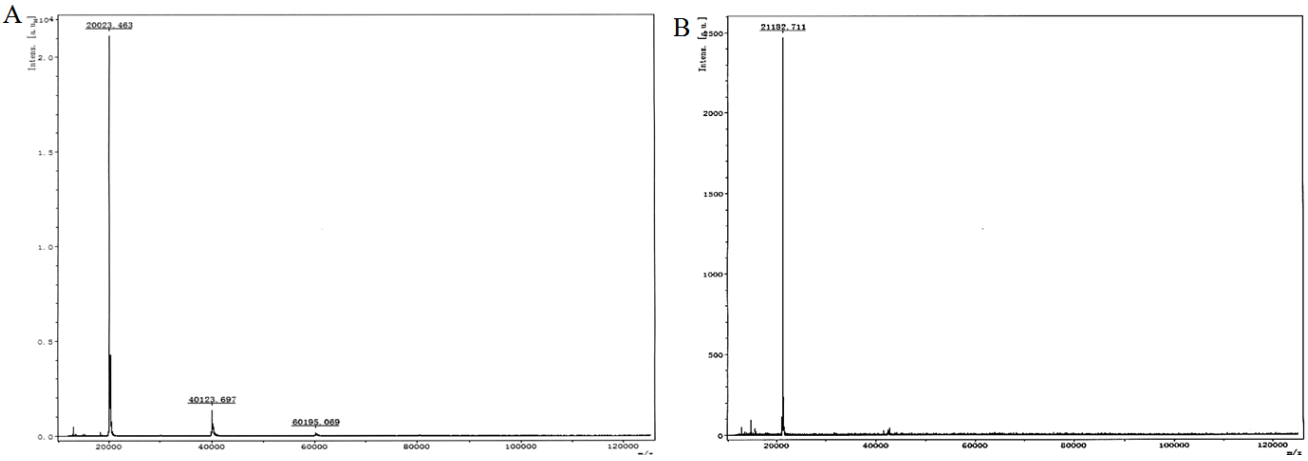

Figure 2. Mass spectroscopy result of recombinant Trx- Tα1 or Trx- Tα1-iRGD. A. Trx-Tα1; B. Trx-Tα1-iRGD

3.3. Optimization of the Expression of Tα1 FusionProtein

Three main factors directly influence the expression of target protein were chosen, including OD_{600nm} before induction(A), lactose concentration(B), and induction time(C). The results of orthogonal test of Trx-Tα1 expression were presented in Table 2.

Table 2. Orthogonal test result of the expression of Tα1 fusion protein

No.	Factors[a]			Expression of Tα1 (%)
	A	B	C	
1	1	1	1	7.3
2	1	2	2	17.6
3	1	3	3	18.4
4	1	4	4	19.1
5	1	5	5	19.6
6	2	1	2	17.2
7	2	2	3	16.8
8	2	3	4	17.3
9	2	4	5	14.3
10	2	5	1	3.2
11	3	1	3	15.2
12	3	2	4	18.9
13	3	3	5	14.9
14	3	4	1	10.7
15	3	5	2	20.2
16	4	1	4	17.6
17	4	2	5	17.6
18	4	3	1	16.2
19	4	4	2	18.2
20	4	5	3	18.0
21	5	1	5	17.4
22	5	2	1	18.2
23	5	3	2	18.01
24	5	4	3	17.07
25	5	5	4	12.23
k1	16.40	14.94	11.10	
k2	13.78	17.81	18.25	
k3	16.01	16.98	17.11	
k4	17.49	16.47	17.03	
k5	16.57	14.68	16.76	
R	3.71	3.13	6.01	

[a] The level of each factor, as show in Table 1.

According to the R values, the importance of three factors decreases in the order: C>A>B, indicating that the induction time is the most critical factor in Tα1 expression. The best conditions could be selected according k values. The optimal condition for the expression of Tα1 fusion protein was: OD_{600nm}=0.6 before adding lactose, using 2.5μmol/ml lactose as inducer, and harvesting cells after 4 hours induction.

3.4. Optimization of the Expression of Tα1-iRGD Fusion Protein

Table 3. Orthogonal test result of the expression of Tα1-iRGD fusion protein

No.	Factors[a]			Expression of Tα1-iRGD (%)
	A	B	C	
1	1	1	1	7.1
2	1	2	2	18.2
3	1	3	3	16.3
4	1	4	4	16.4
5	1	5	5	13.2
6	2	1	2	17.6
7	2	2	3	17.4
8	2	3	4	16.8
9	2	4	5	13.0
10	2	5	1	10.2
11	3	1	3	13.7
12	3	2	4	11.6
13	3	3	5	10.3
14	3	4	1	14.6
15	3	5	2	16.7
16	4	1	4	12.1
17	4	2	5	10.1
18	4	3	1	15.9
19	4	4	2	19.8
20	4	5	3	14.3
21	5	1	5	10.3
22	5	2	1	17.8
23	5	3	2	20.1
24	5	4	3	18.3
25	5	5	4	11.7
k1	14.24	12.16	13.12	
k2	15.00	15.02	18.48	
K3	13.38	15.88	16.00	
k4	14.44	16.42	13.72	
K5	15.64	13.22	11.38	
R	2.26	4.26	7.10	

[a] The level of each factor, as show in Table 1.

The results of orthogonal test of Trx-Tα1- iRGD expression were presented in Table 3. According to the R values, the importance of three factors decreases in the

order: C>B>A. The best conditions could be selected according k values. The optimal condition for the expression of Tα1 fusion protein was: OD_{600nm}=0.8 before adding lactose, using 7.5mmol/L lactose as inducer, and harvesting cells after 4 hours induction.

3.5. Verification of the Optimized Conditions

Target protein expression levels could be achieved at 32.8% for Trx-Tα1 fusion protein (Figure 3A) and 33.8% for Trx-Tα1 -iRGD fusion protein (Figure 3C) at the optimized condition, versus 20.6% for Trx-Tα1(Figure 3B) and 9.0% for Trx-Tα1-iRGD (Figure 3D) obtained at the initial conditions, which were OD_{600nm}=0.4 before induction, using 5μmol/ml lactose and harvesting cells after 6 hours induction. As shown in Figure 3, under optimized condition, most of the target protein was found in the supernatant of the cell lysate, which can simplified the follow purification steeps.

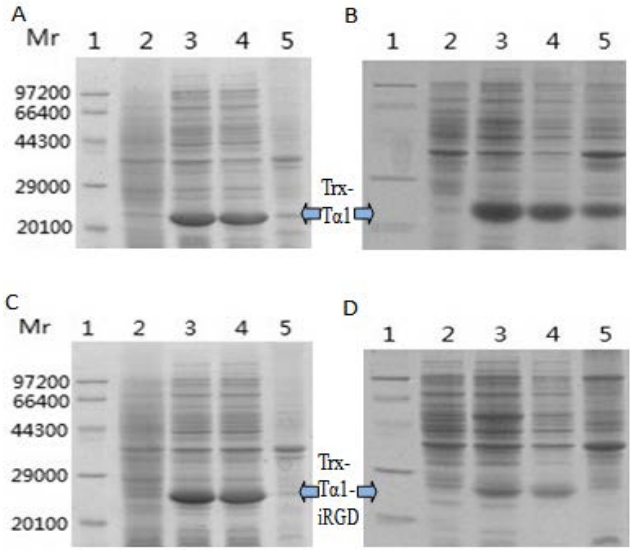

Figure 3. SDS-PAGE of cell lysate of Trx-Tα1 and Trx-Tα1-iRGD expression strains under optimized conditions (A,C) and initial conditions (B,D). Lane 1, molecular weight markers; lane 2, the total cell lysate of control strain harboring pET32a; lane 3, the total cell lysate of engineered strain; lane 4, supernatant of the cell lysate; lane 5, precipitation of the cell lysate

4. Discussion

In our previous work, a better anti- proliferation activity of Tα1-iRGD than Tα1 was observed by *in vitro* studies, which shows the potential application of Tα1-iRGD in treating solid tumors [23]. The further *in vivo* studies need large quantity of purified Tα1-iRGD, calls for effective expression of the target protein. In this study, we try to optimize the expression condition of Trx-Tα1 and Trx-Tα1-iRGD by orthogonal test design. The Trx and His-tag of the fusion proteins can be moved by enterokinase cleavage and nickel-chelating affinity column chromatograph as our previous reported [23].

There are many factors that influence the expression of exogenous gene, involved in the process of cell growth, gene translation and protein synthesis. For example, Romano D et al. optimized the expression of human D-amino acid oxidase in *E. coli.* by single factor experiment, considering the influence of medium ingredients, the time and the amount of inducer's addition, pH control and mechanical shear stress, respectively [24]. Volontè E et al. optimized the expression of glutaryl-7-amino-cephalosporanic acid acylase by single factor experiment, considering medium ingredients, the time and the amount of inducer's addition and temperature [25]. In this study, we used OD_{600nm} as a direct indicator to assess the growth status of the cells before add the inducer. We also considered the influence of the inducer (lactose) concentration and the time of induced expression. The result showed that the time of induced expression was the most important factor in both Trx-Tα1 and Trx-Tα1-iRGD expression. But the importance of lactose concentration and OD_{600nm} before induction differenced in different system.

Compare to single factor test, orthogonal test method can significantly reduce the number of trials, and has been widely used in the optimization of culture conditions [26], extraction processes [27], and drug formulations, etc. In this study, orthogonal test was proved to be an effective method to optimize the expression of Trx-Tα1 and Trx-Tα1-iRGD fusion proteins.

5. Conclusion

The orthogonal test design method was used to optimize the expression of Trx-Tα1 and Trx-Tα1-iRGD fusion proteins. It was shown that after optimization, the soluble form of expressed Trx-Tα1 and Trx-Tα1-iRGD fusion proteins were increased 59.2% and 275.6% than original condition. The *in vitro* experiments showed that purified Tα1-iRGD has higher anti- proliferative activity than Tα1 on several cancer cells [23], made it important to accumulate enough quantity of purified Tα1-iRGD, for further investigation of its potential application in solid tumors therapy. The genetic method of expression polypeptide through fusion protein could be an alternative method in producing bioactive peptide.

Acknowledgement

This work was supported by a project funded by the priority academic program development of Jiangsu Higher education institutions(PADP), the China National Nature Science Foundation (grant number 31300643 and 81102899).

Abbreviations

SDS-PAGE, Sodium dodecyl sulfate polyacryl- amide gel electropheresis;Tα1,Thymosin alpha 1; EDTA, Ethylenediamine tetraacetic acid; OD_{600nm}, the absorbance at 600nm.

References

[1] Goldstein,A.L, Low,T.L., McAdoo,M., Thurman,G.B., Rossio,J., Lai,C.Y., Chang,D., Wang,S.S., Harvey,C., Ramel, A.H., and Meienhofer,J., "Thymosin alpha1: isolation and sequence analysis of an immunologically active thymic polypeptide," *Proc Natl Acad Sci USA*, 74(2).725-729. Feb.1977.

[2] Goldstein,A.L., Slater,F.D. and White,A., “Preparation, assay, and partial purification of a thymic lymphocytopoietic factor (thymosin),” *Proc Natl Acad Sci USA*,56(3).1010-1017.Sep.1966.

[3] Goldstein,A.L., “Clinical applications of thymosin alpha-1,” *Cancer Invest*, 12(5).545-547. May.1994.

[4] Uicickas,Y.M., Quesenberry,J.C.P., Guo,D., Wells,K., Shan,J., Sanders,L., Skovron, M.L., Iloeje,U., Caldwell,C. and Manos,M.M., “Incidence of hepatocellular carcinoma among individuals with hepatitis B virus infection identified using an automated data algorithm,” *J Viral Hepatol*, 15(1). 28-36. Jan. 2008.

[5] Li,Y., Chen, H., Li,X., Zhou,W., He,M., Chiriva-Internati, M., Wachtel, M.S. and Frezza, E.E., “A new immunomodulatory therapy for severe sepsis: ulinastatin plus thymosin{alpha} 1,”*J Intensive Care Med*, 24(1).47-53. Jan-Feb.2009.

[6] Ancell, C.D., Phipps, J. and Young,L., “Thymosin alpha-1,” *Am J Health Syst Pharm*, 58(10). 879-885 May.2002.

[7] Qin, Y., Chen, F.D., Zhou, L., Gong, X.G. and Han, Q.F., “Proliferative and anti-proliferative effects of thymosin alpha 1 on cells are associated with manipulation of cellular ROS levels,” *Chem Biol Interact*, 180(3).383-388. Aug.2009.

[8] Moody,T.W., “Thymosin alpha 1 as a chemo- preventive agent in lung and breast cancer,” *Ann NY Acad Sci*,1112.297-304. Sep.2007.

[9] Moody,T.W., Tuthill,C., Badamchian.M. and Goldstein A.L., “Thymosin alpha1 inhibits mammary carcinogenesis in Fisher rats,” *Peptides*, 23(5).1011- 1014.May.2002.

[10] Garaci,E., Pica,F., Sinibaldi-Vallebona,P., Pierimarchi, P., Mastino,A., Matteucci,C. and Rasi,G., “Thymosin alpha(1) in combination with cytokines and chemotherapy for the treatment of cancer,” *Int Immunopharmacol*, 3(8). 1145-1150. Aug.2003.

[11] Sungarian,A., Cielo,D., Sampath,P., Bowling,N., Moskal,P., Wands,J.R. and de la Monte, S.M., “Potential role of thymosin-alpha1 adjuvant therapy for glioblastoma,” *J Oncol*, 2009.

[12] Low, T.L., McClure, J.E., Naylor, P.H., Spangelo, B.L. and Goldstein, A.L., “Isolation of thymosin alpha 1 from thymosin fraction 5 of different species by high performance liquid chromatography,” *J Chromatogr*, 266.533-544.Aug.1983.

[13] Mutchnick, M.G., Lindsay, K.L., Schiff, E.R., Cummings, G.D., Appelman, H.D., Peleman,R.R., Silva, M., Roach, K.C., Simmons, F., Milstein, S., Gordon, S.C. and Ehrinpreis, M.N., “Thymosin alpha1 treatment of chronic hepatitis B: results of aphase III multicentre, randomized, double-blind and placebo-controlled study,” *J Viral Hepat*, 6(5).397-403. Sep.1999.

[14] Chen, Y., Wang, A., Zhao, L., Shen, G., Cui, L. and Tang, K., “Expression of thymosin alpha1 concatemer in transgenic tomato (Solanum lycopersicum) fruits,” *Biotechnol Appl Biochem*, 52(Pt 4), 303-312. Apr. 2009.

[15] Ni, Y., Shi, Z., Wang, D., Yao, M., Qiao, M. and Guo, P., “High expression of thymosin alpha1 by injecting recombinant PVX vector into the tomato fruit,” *Sheng Wu Gong Cheng Xue Bao*, 25(4).537-541.Apr. 2009.

[16] Pasqualini, R., Arap, W. and McDonald, D.M., “Probing the structural and molecular diversity of tumor vasculature,” *Trends Mol Med*, 8(12). 563-571. Dec. 2002.

[17] Zetter, B.R., “On target with tumor blood vessel markers,” *Nat Biotechnol*, 15(12).1243-1244. Nov. 1997.

[18] Arap,W., Pasqualini,R. and Ruoslahti,E., “Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model,” *Science*,279(5349). 377-380. Jan.1998.

[19] Zitzmann, S., Ehemann, V. and Schwab, M., “Arginine- glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells in vivo,” *Cancer Res*, 62(18).5139-5143. Sep.2002.

[20] Desgrosellier, J.S. and Cheresh, D.A., “Integrins in cancer: biological implications and therapeutic opportunities,” *Nat Rev Cancer*, 10(1). 9-22. Jan.2010.

[21] Sugahara, K.N., Teesalu, T., Karmali, P.P., Kotamraju, V.R., Agemy, L., Girard, O.M., Hanahan, D., Mattrey, R.F. and Ruoslahti, E., “Tissue-Penetrating Delivery of Compounds and Nanoparticles into Tumors,” *Cancer Cell*,16(6). 510-520.Dec. 2009.

[22] Sugahara, K.N., Teesalu,T., Karmali, P.P., Kotamraju, V.R., Agemy, L., et al. “Coadministration of a Tumor- Penetrating Peptide Enhances the Efficacy of Cancer Drugs,” *Science*,328(5981).1031-1035. May. 2010.

[23] Lao, X., Liu, M., Chen,J. and Zheng,H., “A tumor- penetrating Peptide modification enhances the antitumor activity of thymosin alpha 1,” *PLoS One*, 8(8):e72242.Aug.2013.

[24] Romano, D., Molla,G., Pollegioni,L. and Marinelli,F., “Optimization of human D-amino acid oxidase expression in Escherichia coli,” *Protein Expr Purif*, 68(1).72-78. Nov.2009

[25] Volontè, F., Marinelli, F., Gastaldo, L., Sacchi, S., Pilone, M.S., Pollegioni, L. and Molla,G., “Optimization of glutaryl-7-aminocephalosporanic acid acylase expression in E. coli,” *Protein Expr Purif*, 61(2).131-137. Oct.2008.

[26] Li,C.M., Guo,C.J., “Optimization of liquid culture medium for phellinus linteus mycelia by orthogonal test,” *Food SCi*, 29(5).311-314.Oct.2008.

[27] Cai, D.J., Shu, Q., Xu, B.Q., Peng, L.M. and He, Y., “Orthogonal test design for optimization of the extraction of flavonid from the fructus gardenia,” *Biomed Envion Sci*, 24(6).688-693. Dec.2011.