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Preparation of Thymosin-α1 Using a One-step Heat-treatment Method and CNBr Cleavage*

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Abstract Thymosin-α1 (Tα1), as a commercial peptide drug, has been chemically synthesized and widely used for enhancing immune responses and anti-tumor. The increasing therapeutic Tα1 need leads to concerns about its mass and low-cost production processes. To prepare peptide thymosin-α1 by a novel recombination expression and purification technique. The thymosin-α1 was designed into a tetraploid concatemer by genetic manipulation and expressed in *E. coli*, and the purified concatemer thymosin α1 was obtained by one-step heating. Subsequently, the concatemer thymosin-α1 was cleaved into monomers with cyanogen bromide dissolved in 50% − 70% trifluoroacetic acid. The thymosin-α1 monomer was further purified by high performance liquid chromatography with purity ⩾ 98%. Finally, the obtained thymosin-α1 could stimulate lymphocyte proliferation, which was almost as effective as the commercial thymosin-α1 (Zadaxin®).Finally, The recombinant thymosin-α1 was successfully obtained, which is similar to the commercial one, by genetic recombination, heat purification and appropriate cleavage.

Key words CNBr cleavage, concatemer peptide, *E. coli* expression, purification methods, recombinant thymosin-α1
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1 Introduction

Thymosin-α1 (Tα1), a 28-amino acid peptide, is an important constituent of thymosin fraction 5 that is known to improve the immune response[1-2]. Tα1 has been used in clinical trials to treat patients with infections, cancer, immunodeficiency and aging disorders[3-5]. Commercial Tα1 is chemically synthesized by solid-phase technique[6]. However, this method is comparatively expensive for use in clinical applications because of the cost of long peptide synthesis and the necessity for long-term usage[6-8].

Recombinant protein expression methods have been used for the production of short proteins, similar to Tα1, and have the potential to be less expensive than solid-phase synthesis. However, short proteins are apt to degradable in host cells resulting in very low yield compared to normal size proteins[9-10]. To solve this problem, peptide concatemers are generally adopted to avoid degradation and to increase the yield[11-13]. Some methods have been used to increase the production of recombinant proteins, including added insoluble tags, such as KSI(ketosteroid isomerase protein), which can lead to the formation of inclusion bodies[14].

For peptide digestion, Cyanogen Bromide(CNBr) is generally used due to its high efficiency and conservative cleavage site[15-17]. CNBr can specifically cleave peptides at the sites of methionine(M) residues by changing M residues to homoserine(HSe) or homoserine lactone(HSL) moieties[12,14]. The cleavage efficiency strongly depends on the solubility of the peptide, the amino-acid sequence, and the acidic solvents used, such as formic acid(FA), trifluoroacetic acid(TFA), or hydrochloric acid(HCl)[18-20].

In this paper, a soluble tetraploid concatemer of Tα1 was expressed in *Escherichia coli(E. coli)*. A one-step heat-treatment method was adopted in the purification of the recombinant protein. Purified concatemers were cleaved to monomers by CNBr. A convenient and economical method of production of Tα1 was developed.

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2 Materials and methods

2.1 Materials

PCR regents and DNA purification kits were purchased from Beibei Biotechnology Company (Zhenzhou, China). DNA and primers were synthesized by the Genewiz Company(Beijing, China). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs(Ipswich, MA, USA). His60 Ni Superflow Resin was from Clontech (Mountain View, CA, USA). Commercial Tα1 (Zadaxin®) was from SciClone (Beijing, China). CNBr, TFA, and all other chemicals were purchased from Sigma-Aldrich.

2.2 Construction of vectors

Tα1 gene (5'-TCGGATGCTGCGGTCGATACC-AGCAGTGAAATAACTACGAAAGACTTAAAAG-AAAAGAAAGAGGTTGTGGAAGAAGCCGAGC-AG-3') was synthesized according to the reported human Tα1 amino acid sequence (ACCESSION: 2MNQ_A) and the *E. coli* codon usage preference. The gene was amplified using primers P1(5'-ATACCTAATGTCGGATGCTGCGGTCGATACCAG C -3') and P2(5'- ATACAGCATCTGCTCGGCTTC-TTCCACAACCTCTT-3') and subsequently digested by isocaudarner *Bsl* Ⅰ and *Alw*N Ⅰ .The digestion was self-ligated by T4 DNA ligase and then cloned into the site of *Alw*N Ⅰ in the vector pET-31b skeleton in which the KSI fragment was removed previously to avoid expressing inclusion bodies. Colony PCR was done to identify the copy number of Tα1 gene. Gene orders were further confirmed by Sanger sequencing.

2.3 Expression and purification of recombinant concatemers

The fusion proteins were expressed in *E. coli* strain BL21. A single colony was inoculated into 10 ml LB medium and incubated overnight at 37° C. Then 5 ml culture solution was inoculated into 500 ml LB medium in a 1 L shake flask. When the A600 reached 0.6, IPTG was added up to a final concentration of 0.4 mmol/L. Cells were harvested by centrifugation after cultivation for 6 h. To purify Tα1 concatemers, 1 g of wet cell pellets were re-suspended with 20 ml PBS, and then heated in a boiling bath for 10−20 min. The supernatant was collected after centrifuging at 8 000 *g* at 4°C for 15 min.

2.4 Cleavage of concatemers

Purified proteins were dissolved in up to 10 ml of HCl, FA or TFA in 100 ml bottles. CNBr (0.2 g) was added to each bottle. Liquid nitrogen or nitrogen was added to remove oxygen. The bottles were wrapped with aluminum foil and shaken at room temperature for 18−24 h. The samples were dried by lyophilization or rotary evaporation, then re-suspended with alkaline solutions of 1 mol/L Tris-HCl (pH8.0), 1 mol / L $NaHCO_3$, or 1 mol / L NaOH, to neutral. The supernatants of the dissolved mixtures were collected after centrifugation at 8 000 *g* at 4° C for 15 min.

2.5 Reverse−phase HPLC analysis, purification, and molecular weight identification

Samples were then injected into an Aglient 1200 series system, analyzed and purified using a C18 column. The products were eluted using a linear gradient of 20% to 40% B over 30 min (A, H_2O/0.1% TFA; B, 80% CH_3CN / 0.1% TFA) with a column temperature of 25° C. The detection wavelength was set at 220 nm and the flow rate was 1 and 5 ml/min respectively. Cleavage yield was calculated using the formula: cleavage yield (%) = target peak area /total peak area × 100%. The eluted peptide peak was collected based on the HPLC analysis data, and the solutions were lyophilized. The lyophilized products were then identified by surface assisted laser desorption ionization time of flight mass spectrometry (SALDI-TOF-MS).

2.6 Biological activity assay

To evaluate Tα1's biological activity, the proliferation of splenocytes was tested according to previous reports[12,21-22]. Lymphocytes were isolated from human peripheral blood by lymphocyte separation medium. The blood samples and protocol were approved by a project of The National Natural Science Foundation of China (81472257), Medical Ethics Committee of Zhengzhou University, and volunteers signed an informed consent. The cell pellet was re-suspended in RPMI 1640 medium. 100 μl of the suspension was seeded in each well of 96-well plates at a concentration of 5×10^5 cells/ml. After 6 h, 100 μl of HPLC purified Tα1-HSL peptide solutions (50, 25, 12.5 and 6.25 mg/L) were added. Commercial hTα1(Zadaxin®) and RPMI 1640 culture media were the positive and negative controls, respectively. Each

group had three parallel wells. After incubation for 48 h at 37° C, 20 μl of MTT (5 g / L) solution was added to each well. After incubation for 4 h at 37° C, the plates were centrifuged (2 000 *g*, 25° C, 10 min). Supernatant (100 μl) was removed from each well, and then 100 μl of dimethyl sulfoxide was added dropwise. After incubation for 10 min at room temperature, the solubilized reduced MTT was measured at 570 nm by the microculture plate reader. The absorbance results were used to calculate the growth rate: growth rate = $A_{sample} / A_{control} \times 100\%$.

3 Results and discussion

3.1 Clone and purification of concatemers

The concatemer with four copies of Tα1 was adopted as SDS-PAGE analysis indicated that concatemers gave the similar protein yields after two copies (data not shown). The concatemer, in which Tα1 peptide was linked in tandem each other by a cleavage site methionine (M) for CNBr cleavage, is written as $(M\text{-}T\alpha1)_4$ (Figure 1).

Interestingly, it showed that $(M\text{-}T\alpha1)_4$ could be easily purified by boiling (Figure 2). The purity of recombinant concatemer in the supernatant improved with increasing the incubation temperature from 50 to 100° C (Figure 2a). In addition, there was no obvious difference in purity when the bacteria were incubated in the boiling water from 5 to 60 min (Figure 2b). The incubation in boiling water for 10 −20 min was sufficient to produce protein almost as pure as with the Ni-affinity column purification method (Figure 2b). The heat-treatment method could remove most of the other proteins leaving almost only the recombinant Tα1 concatemer. The results also indicated that the concatemer was thermostable.

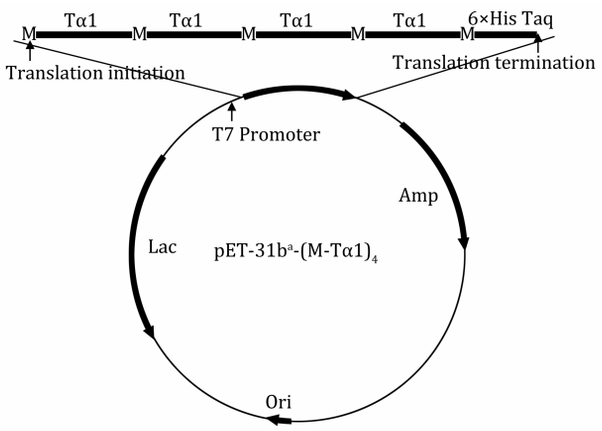

Fig. 1　Plasmid construction of （M−Tα1）$_4$

a, Plasmid pET-31b with KSI fragment removed.

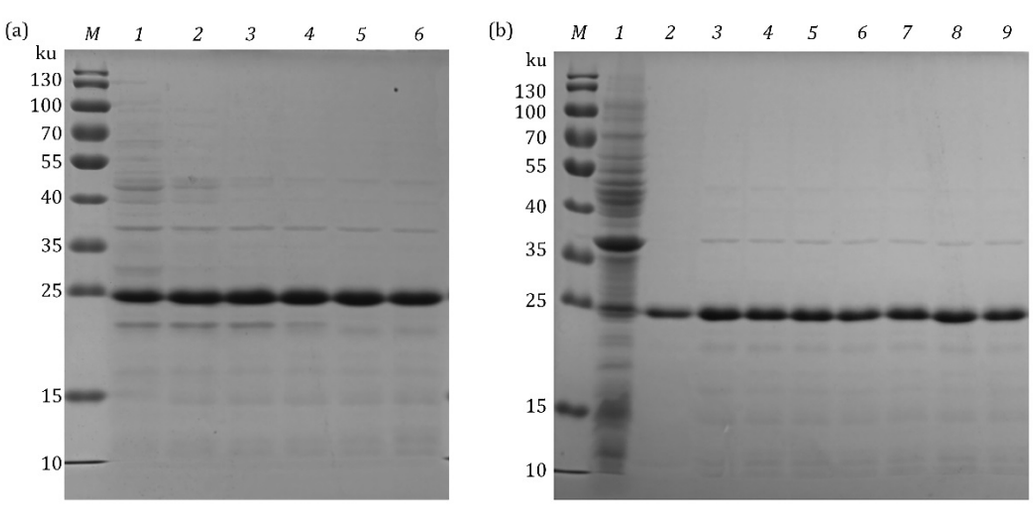

Fig. 2　SDS−PAGE analysis of （M−Tα1）$_4$ produced by different heat−purification processes

（a）*M*，Thermo Scientific Page Ruler Prestained Protein Ladder （SM0671）；Lanes *1-6*，soluble protein fraction of （M-Tα1）$_4$ separated by centrifugation after incubation at 50，60，70，80，90 and 100 ℃ for 10 min.（b）Lane *M*，Thermo Scientific Page Ruler Prestained Protein Ladder（SM0671）；Lane *1*，total protein of （M-Tα1）$_4$ after IPTG induction；Lane *2*，（M-Tα1）$_4$ purified by Ni-sepharose；Lanes *3-9*，the soluble protein fraction of （M-Tα1）$_4$ separated by centrifugation after incubation at 100 ℃ for 5，10，20，30，40，50 and 60 min.

3.2 Analysis of cleavage yield in different solvents

The total peak area was calculated from 5 to 20 min so as to eliminate the interference from solvent, and the target peak area was the sum of Tα1-HSL and Tα1-HSe.

FA was the most common cleavage reagent in the past. Whereas, the cleavage yield in 70% FA was only 21% (Figure 3e). We speculate the supernatant after heat treatment contained a mass of peptide, polysaccharide, lipid and nucleic acid may cripple the CNBr cleavage reaction. To test our hypothesis, we purified $(M\text{-}T\alpha1)_4$ through Nickel column(according to His60 Ni Superflow Resin & Gravity Columns User Manual provided by Clontech), and the cleavage yield increased significantly to 46% (P=0.03) (Figure 3f). In addition, two conjoint peak area appeared between 16 min and 17 min in Figure 3f was probably the results of side reactions as the methionine followed by serine in the amino acid sequence of $(M\text{-}T\alpha1)_4$[23-24]. The side reactions usually refers to peptides containing hydroxyl groups will react with FA and forming formyl esters[25].

Whereas, the interference from non-protein substance and side reactions seemed surmountable in TFA cleavage environment. $(M\text{-}T\alpha1)_4$ gave the highest cleavage yield (72%) in 70% TFA compared with other cleavage reagents(Figure 3). In 40% TFA group, almost all $(M\text{-}T\alpha1)_4$ was completely excised, but the main products were the mixture of Tα1-HSL and Tα1-HSe. When and after TFA concentration reaching to 50%, Tα1-HSL became the primary products. The cleavage yield rose up with the increasing of initial TFA concentrations, but plateaued at 50% TFA, and did not obviously increase at 60% or 70% TFA(Figure 4). Therefore, it appeared that 50% TFA was appropriate to cleave the tandem repeat Tα1 peptide. We also tried HCl to avoid side reactions[26], it is disillusionary that there was no obvious cleavage found in 0.5, 1 or 2 mol/L HCl (Figure 3b-d).

Due to the different amino acid residues between Tα1 and Commercial hTα1 leading to peak appearance time difference in HPLC analysis(Figure 3), we further identified the monomers by measuring the molecular weight of end-products using SALDI-TOF-MS(Figure 5). Three results, 3162.7, 3164.3, and 3164.7 u were matched to the true molecular mass of Tα1-HSL 3163.5 u (Relative Error<0.1%).

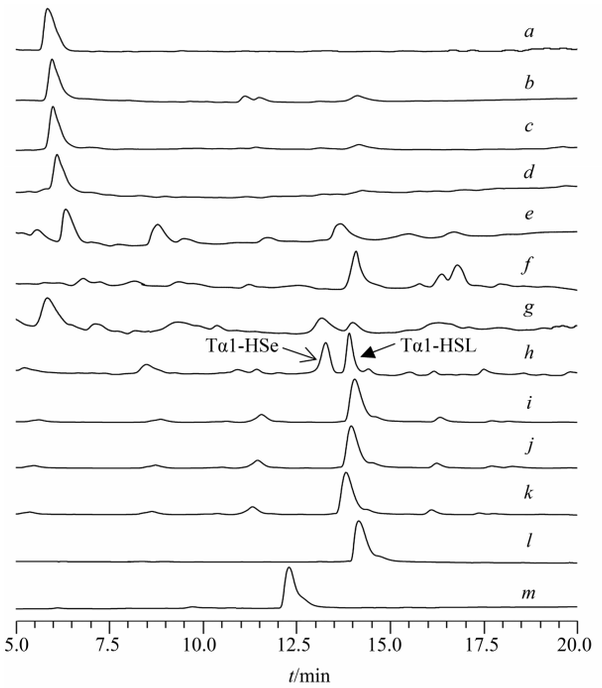

Fig. 3　Chromatographic traces obtained from HPLC analysis of samples

a: $(M\text{-}T\alpha1)_4$ purified by heating at 100 ℃ for 10 min; *b*–*e*, *g*–*k*: $(M\text{-}T\alpha1)_4$ (purified by heat treatment) cleavage with BrCN in different acid solutions. *b*: 0.5 mol/L HCl, *c*: 1 mol/L HCl, *d*: 2 mol/L HCl. *e*: 70% FA, *g*: 10% TFA, *h*: 40% TFA, *i*: 50% TFA, *j*: 60% TFA, *k*: 70% TFA; *f*: Nickel column purified $(M\text{-}T\alpha1)_4$ cleaved with BrCN in 70% FA; *l*: Tα1-HSL purified by HPLC from the product of *i*; *m*: Commercial hTα1.

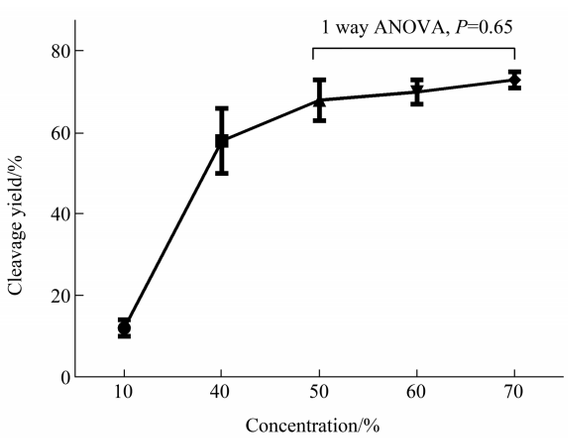

Fig. 4　Cleavage yield (%) of $(M\text{–}T\alpha1)_4$ purified by heat treatment in different concentration of TFA

After heat-treated $(M\text{-}T\alpha1)_4$ was cleaved with CNBr in 50% TFA, Tα1-HSL was purified by reverse-phase HPLC. We quantified the final yield of Tα1-HSL through comparing its peak area on HPLC with a commercial hTα1 standard. A total 5.6 mg of Tα1-HSL was obtained from 1 L *E. coli* (Table 1). The yield was superior in the production of peptides compared with the known recombinant expression methods[27-30].

Table 1 Production of Tα1−HSL （1 L of bacteria）.

Purification step	Quality/mg	Purity/%
Wet weight of bacteria	4.82×10^3	NA
Total recombinant fusion protein[1)]	48.2	NA
Fusion protein after heat-treatment[1)]	29.4	84
Yield of peptide monomer[2)]	5.6	≥98

[1)] Yields determined by SDS-PAGE protein bands to known standards. [2)] Heat purified $(M\text{-}T\alpha1)_4$ was cleaved with CNBr in 50% TFA and then pufied by HPLC. Results determined by peak area on reverse-phase HPLC compared with a commercial hTα1 standard.

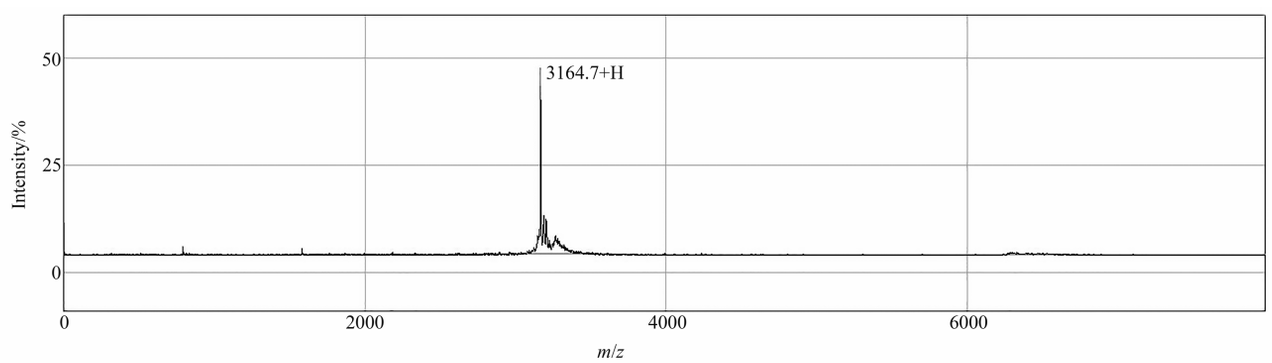

Fig. 5 Molecular mass of the peptide after HPLC purification determined by SALDI−TOF−MS

3.3 Biological activity of monomer Tα1−HSL

Tα1 can improve the proliferation of splenocytes[4,12]. We compared the proliferation of human lymphocytes using purified Tα1-HSL and commercial hTα1(Figure 6). The results indicated that 6.25 mg/L Tα1-HSL or hTα1 could induce significant proliferation of human lymphocytes compared with the control group (P<0.05), exhibiting a dose-effect relationship. There was no obvious difference between Tα1-HSL and hTα1, at a series of concentrations, in the proliferation of human lymphocytes.

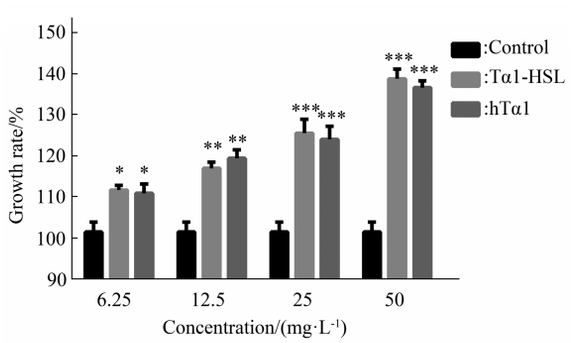

Fig. 6 Proliferation of human lymphocytes by MTT assay

Control group, lymphocytes cultured with RPMI 1640 medium only; Experiment group, lymphocytes cultured with RPMI 1640 containing various concentrations （50，25，12.5，and 6.25 mg/L） of Tα1-HSL or hTα1. The assays were repeated in triplicate and the data are reported as the mean ± S.E.M. All of groups consisting of three observations （*P<0.05，**P<0.005 and ***P<0.001）.

In conclusion , this study provides a better choice for mass production of Tα1. The recombinant concatemer $(M\text{-}T\alpha1)_4$ could be expressed as a soluble protein at high levels in *E. coli*. The purification was greatly simplified, just a boiling bath for 10−20 min, without the need for techniques such as ultrasonication or Ni chromatography. The concatemers could be efficiently cleaved in 50%−70% TFA with CNBr. In our study, peptide Tα1-HSL was prepared in high yield and high purity from prokaryote fermentation in a time-saving and low-cost way. The recombinant Tα1 was confirmed to have a similar effect on the proliferation of human lymphocytes as commercial hTα1(Zadaxin®).

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一步加热法和溴化氰切割生产胸腺肽α1*

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摘要　胸腺肽α1是一种已经商业化的化学合成多肽，它具有广泛免疫增强和抗肿瘤作用. 随着越来越多的临床治疗作用的发现，胸腺肽α1的大量和低价生产逐渐引起人们的重视. 我们通过一种新的重组和纯化技术来生产多肽胸腺肽α1，首先通过基因重组的方法在大肠杆菌中表达了四串体的胸腺肽α1，然后使用一步加热的方法将四串体的胸腺肽α1纯化. 随后，使用溴化氰在50%~70%三氟乙酸溶剂中将四串体的胸腺肽α1切割成单体. 使用高效液相色谱法将胸腺肽α1单体提纯后，其纯度可达到98%以上. 最后，利用胸腺肽α1能刺激淋巴细胞增殖的原理证实经我们的方法得到的重组胸腺肽α1，与商业化的胸腺肽α1（Zadaxin®）具有相似的生物活性. 我们通过基因重组、加热纯化、合适的切割成功获得了与商业化的胸腺肽α1相似生物活性的重组胸腺肽α1.

关键词　溴化氰切割，串联多肽，大肠杆菌表达，纯化方法，重组胸腺肽α1
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