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Short Communication

Overexpression of Soluble Human Thymosin Alpha 1 in *Escherichia coli*

Pei-Fu CHEN, Hong-Ying ZHANG[1], Geng-Feng FU, Gen-Xing XU[1], and Ya-Yi HOU*

Medical School, Nanjing University, Nanjing 210093, China;
[1]School of Life Sciences, Nanjing University, Nanjing 210093, China

Abstract Synthesized gene of human thymosin alpha 1 ($T\alpha_1$) was inserted into pET-28a, pET-9c, pThioHis B, pGEX-2T or pBV222 and then inductively expressed in strains of *Escherichia coli*. Among the five expression systems, the BL21/pET-28a system provides the highest expression level of fusion protein in a soluble form, which is up to 70% of total expressed bacterial proteins as visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting fusion protein purified through nickel affinity chromatography accounts for 2.53% of the wet bacterial pellet weight and reaches 94.5% purity by SDS-PAGE. These results indicate the potential of this expression system for high-throughput production of recombinant Tα1.

Key words human thymosin alpha 1; *Escherichia coli*; fusion expression; Ni^{2+} affinity chromatography

Thymosin alpha 1 ($T\alpha_1$), a 28 amino acid peptide with a molecular weight (MW) of 3108 Da, was first isolated from calf thymus in 1977 [1,2]. N-terminal acetylated $T\alpha_1$ is mainly produced by thymic epithelial cells *in vivo* and keeps stable at 80–90 °C [3]. $T\alpha_1$ exerts effects of anti-viral infection, anti-tumor and immunomodulation. $T\alpha_1$ stimulates T cells to mature and express IL-2, IL-2R and CD2 [4,5] and inhibits, directly or indirectly, viral replication at least in hepatitis virus B-transfected HepG2 tumor cells [6,7]. It also antagonizes apoptosis of T cells induced by dexamethasone or CD3 monoclonal antibody [8] or sera of tumor-bearing mice [9], down-regulates tumor weight in mice [10,11], enhances major histocompatibility complex (MHC) class I antigen expression in tumor cells and antigen-presenting cells [12–16], and partly restores cellular immunity [17].

As a biological response modifier, $T\alpha_1$ is clinically used, especially in combination with IFN-α, for patients with chronic hepatitis virus B or C infection [18–21], which has been greatly threatening public health in China and other countries. $T\alpha_1$ is also of great value for malignant tumors such as non-small cell lung cancer (NSCLC) [11] or to reduce adverse effects from chemotherapy or radiotherapy [22–24]. It is even believed useful to prevent liver cirrhosis and hepatocarcinoma related to chronic viral hepatitis [21]. Recently, this polypeptide has even been employed as an adjuvant for vaccines against influenza and hepatitis B [19].

Enormous market demand for $T\alpha_1$ is predicted; however, the present yield of $T\alpha_1$ can not meet the increasing requirements for clinical applications. There are several reasons for this. $T\alpha_1$ for clinical use is mainly attained from animal thymus extraction or chemical synthesis. The amount of calf or swine thymus available seriously restricts the yield of $T\alpha_1$. Furthermore, quality variation of thymus-derived $T\alpha_1$ among batches or manufacturers is often found. In fact, $T\alpha_1$ from this source is often impure so that it may cause heterogeneous allergy. Although chemically synthesized $T\alpha_1$ can reach a high level of purity, it is necessary to get rid of by-products at each step, including incorrect joining and dextral compounds. The longer one peptide is, the more intricate the chemical synthesis process will be. $T\alpha_1$ comprises 28 amino acid residues, and its chemical synthesis incurs a high production cost. Prokaryotic expression systems have a characteristic high expression of foreign genes and are widely used for producing large amounts of proteins or peptides. In the past two decades, although the successful expression of the recombinant $T\alpha_1$ ($rT\alpha_1$) in *Escherichia coli* (*E. coli*) was reported [25–28], the

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*Corresponding author: Tel/Fax, 86-25-83686441; E-mail, yayihou@nju.edu.cn

express level was still very low.

In the present study, we compared prokaryotic expression levels of $T\alpha_1$ in five different vectors and reported one overexpressing clone.

Materials and Methods

Construction of $T\alpha_1$ expressing plasmids

Depending on multiple cloning sites in the vectors, pBV222 (Virus Institute, Chinese Academy of Sciences, Shanghai, China), pThioHis B (Invitrogen, California, USA), pGEX-2T (Pharmacia, Uppsala, Sweden), pET-9c or pET-28a (Novagen, San Diego, USA), a pair of primers for the $T\alpha_1$ gene with appropriate restriction endonuclease recognition sites on their two flanks were synthesized (Genebase Company, Shanghai, China). *E. coli*-preferred codons were fully taken into account based on the cDNA of human $T\alpha_1$ gene (GenBank accession No. M14794). The $T\alpha_1$ cDNA sequence coding 28 amino acid residues is as follows: TCT GAT GCA GCG GTG GAC ACC AGC TCC GAA ATC ACC ACT AAA GAT CTG AAA GAA AAG AAA GAA GTT GTG GAA GAG GCG GAA AAC TAA. His_6 tag was added to vectors for subsequent convenient purification except pGEX-2T which had the histidine tag. An enterokinase cleavage sequence (Asp-Asp-Asp-Asp-Lys) was placed just prior to the first amino acid residue (Ser^1) of $T\alpha_1$. Plasmids encoding $T\alpha_1$ were enzymatically constructed as usual and transformed into competent cells of *E. coli* strains (**Table 1**). From single colonies growing on plates containing 100 μg/ml ampicillin or 50 μg/ml kanamycin, positive ones were screened by polymerase chain reaction (PCR) and finally confirmed by DNA sequencing (Sangon Company, Shanghai, China).

Induced expression

Clones that appeared to have high yields of the fusion protein in 3 ml-tubes after preliminary induction were chosen for larger scale expression. Bacteria were refreshed overnight and then inoculated by 0.5% (*V*/*V*) to 1000 ml Luria-Bertani medium containing proper antibiotics. As the bacterial culture grew at 37 ºC to about A_{600}=0.5, isopropyl β-*D*-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After shaking at 250 rpm for 6 h, bacteria were collected by centrifugation at 5000 rpm for 15 min. For DH5α/pBV222, bacteria were cultured at 35 ºC until A_{600}=0.5 and then induced at 42 ºC, 250 rpm for 6 h or 180 rpm overnight. Induction for each expression system was repeated twice.

Purification of fusion protein

The BL21/pET-28a expression system was selected for fusion protein purification. Bacterial pellets were suspended in 50 mM Na_3PO_4 (pH 8.0) containing 0.5 M NaCl, then frozen and quickly thawed twice and broken on ice by sonication until fully fragmentized as viewed under the microscope. Supernatant from the lysate (10,000 rpm for 15 min), either being further treated at 80 ºC for 30 min or not, was individually applied onto a nickel chelate resin column (Invitrogen, USA) and washed with binding buffer until the A_{280} of flow-through fell below 0.005 (Beckman Coulter, California, USA). The column was further washed with binding buffer containing 10 mM imidazole to an

Table 1 Expression systems (*E. coli*) for human thymosin α1

Vectors	pBV222	pET-9c	pGEX-2T	pThioHis B *	pET-28a
Insertion sites	*Eco*RI/*Sal*I	*Nde*I/*Bam*HI	*Eco*RI/*Sal*I	*Eco*RI/*Sal*I	*Eco*RI/*Sal*I
Host strain	DH5α	BL21	K802	Top10	BL21
Promoter	P_LP_R	T7	tac	trc	T7
Resistance	Amp^r	Kan^r	Amp^r	Amp^r	Kan^r
Induction type	42 °C	IPTG	IPTG	IPTG	IPTG
MW of FP (Da) [a]	4930	4650	31,100	16,600	6900
Composition	42 a.a.	40 a.a.	283 a.a.	151 a.a.	64 a.a.
Amount of FP	12.6%	20.6%	32.2%	38.2%	70%
$T\alpha_1$ yield [b]	7.9%	13.7%	3.2%	7.1%	31.4%

* the original sequence between *Bam*HI and *Eco*RI was cut away. [a] molecular weight (MW) of fusion proteins (FP) are calculated using number of amino acid (a.a.) residues multiplied by 110 for the fusion proteins from pGEX-2T and pThioHis B, or by summing MW of the individual amino acid residues for the other three fusion proteins. [b] the final $T\alpha_1$ yield is a theoretical value according to the MW ratio of $T\alpha_1$ and fusion protein.

A_{280} of 0.01, followed by elution with 3 column bed volumes of 250 mM imidazole. The eluates were pooled and dialyzed twice against the same binding buffer or saline at 4 ºC.

Protein analysis

Bacterial lysates, supernatants, flow-through fractions and eluates were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the 18% separation gel. The gels were stained with 0.25% Coomassie brilliant blue R-250 and the amounts of fusion proteins were determined by ImageMaster VDS (Pharmacia, Uppsala, Sweden). The purified fusion protein was further measured by spectrometer (Beckman Coulter) and Lowry procedure.

Results

As revealed by the VDS densitometric analysis, the fusion proteins from DH5α/pBV222, BL21/pET-9c, K802/pGEX-2T, Top10/pThioHis B and BL21/pET-28a, with apparent MWs of 4.9 kD, 4.6 kD, 31.1 kD, 16.6 kD and 14.0 kD, accounted for approximately 12.6%, 20.6%, 32.2%, 38.2% and 70% in total cellular proteins, respectively (**Table 1** and **Fig. 1**). Before condensation, the fusion protein purified from the BL21/pET-28a system was determined up to 13.78 μg/μl. From 1.7 g wet bacterial pellets, 43 mg purified fusion protein was obtained using Ni^{2+} affinity chromatography, and the purity of the fusion protein was 94.5% as analyzed by SDS-PAGE. The fusion protein preparation from BL21/pET-28a was illustrated in **Fig. 2**.

Discussion

In this research we attempted to screen the best *E. coli* expression system among five different prokaryotic vectors. The fusion proteins from DH5α/pBV222, BL21/pET-9c, K802/pGEX-2T, Top10/pThioHis B and BL21/pET-28a, with apparent MWs of 4.9 kD, 4.6 kD, 31.1 kD, 16.6 kD and 14.0 kD, accounted for approximately 12.6%, 20.6%, 32.2%, 38.2% and 70.0% in total cellular proteins, respectively (**Fig. 1**). For DH5α/pBV222, it is important to shake bacterial culture overnight at 42 ºC with 250 rpm because the expression level of fusion protein, depending on the duration of induction, vary from nearly zero to 20% (data not shown). Although BL21/pET-9c could produce a slightly greater amount of the 4.6 kD fusion protein in total lysate than optimally induced DH5α/pBV222, the latter does not need the costly IPTG induction. Additionally, when 5 liters of BL21/pET-9c culture was induced with IPTG at the usual concentration of 0.4 mM, this system could only offer a fusion protein expression level of 12% (data not shown), which is equivalent to that of DH5α/pBV222. Both K802/pGEX-2T and Top10/pThioHis B expressed fusion proteins at middle levels but with the higher MWs, leading to too low theoretical yields of $rT\alpha_1$.

The BL21/pET-28a system, providing the highest

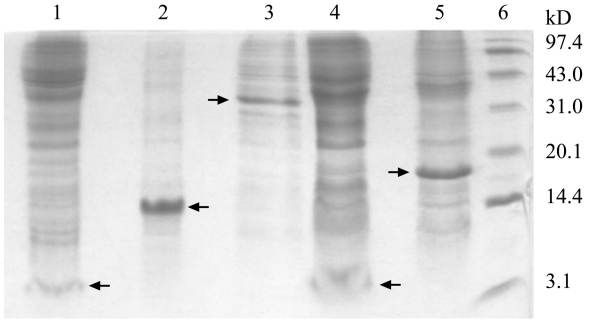

Fig. 1 Induced expression of fusion proteins in five different prokaryotic vectors

1, pBV222 (4.9 kD band); 2, pET-28a (apparent MW 14.0 kD); 3, pGEX-2T (31.1 kD band); 4, pET-9c (4.6 kD band); 5, pThioHis B (16.6 kD band); 6, protein marker (3.1 kD band represents chemically synthesized $T\alpha_1$). The highest expression level was achieved in BL21/pET-28a expression system, which was up to 70% of the total expressed protein.

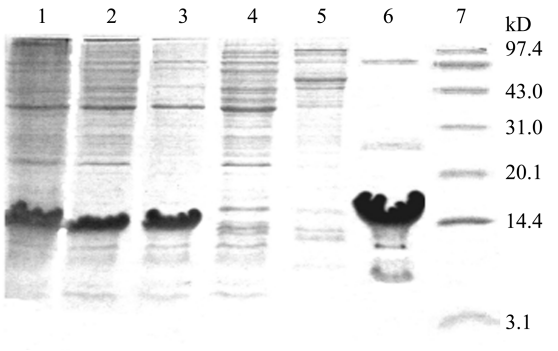

Fig. 2 Purification of fusion protein containing $T\alpha_1$ from BL21/pET-28a expression system

1, total bacterial lysate; 2, supernatant of lysate; 3, the second supernatant after heat treatment; 4, flow-through; 5, wash outflow by 10 mM imidazole; 6, eluate by 250 mM imidazole; 7, protein marker (3.1 kD band represents chemically synthesized $T\alpha_1$). Densitometric analysis showed the fusion protein (apparent MW 14.0 kD) had an amount of 94.5% after Ni^{2+} affinity chromatography. A small portion of the fusion protein degraded naturally (lane 6).

expression level (70%) as shown by the SDS-PAGE (**Fig. 1**, lane 2), could theoretically yield the greatest amount of rTα_1 from the total expressed proteins. Such a high expression level may be due to the strong transcription and full use of bacterial preferred codons, for instance, AAG→AAA, TTA→CTG and GAG→GAA. We note that pET-28a (5.3 kb) and pET-9c (4.3 kb) share the same *T7* promoter, pBR322 origin and host strain, but pET-9c gives a 17% lower theoretical Tα_1 yield than pET-28a (**Table 1**). The variations of transcription-related regulatory regions may thus be responsible for the difference. It is likely that a higher A+T content in the *lac* operator in pET-28a than that in pET-9c renders more efficient transcription once IPTG binds repressor protein molecules. Specifically, the A+T content increases from 47% to 58% when the *lac* operator is modified by GAGACCACAACGGTTTC→GGAATTGTGAGCGGATAACAATTC. Note that the *lac* operator is just the T7 transcription start (http://www.emdbiosciences.com). Particular host strains may be necessary for pET-28a, as, for example, no induced expression of the fusion protein was found in the DH5α strain (data not shown). In addition, this system results in a high amount of purified fusion protein (95% by SDS-PAGE). In our present study, the fusion protein available is 2.53% of wet bacterial pellet weight. This performance could be improved if conditions for fermentation and purification were optimized. Given a final rTα_1 recovery rate of 50% by high performance liquid chromatography (HPLC), the BL21/pET-28a expression system could give a final Tα_1 yield up to (2.53%×50%×3.1)/6.9=0.56% of wet bacterial pellet weight. This percentage would be 70 times as much as a previously reported yield from calf thymus tissues, which is 0.008% of homogenate [2]. A careful examination of pET-28a cloning/expression regions reveals that this fusion protein consists of 64 amino acid residues with a calculated MW 6.9 kD. It is not known why the pET-28a-derived fusion protein appears to be 14.0 kD instead of 6.9 kD, despite the fact that no cysteine exists in this molecule. The possibility of dimerization of the fusion protein molecules, the reasons of which keep unknown, could not be ruled out.

We found it acceptable to take 1 g bacterial pellet, 10 ml binding buffer and 0.5 ml resin for purification of the fusion protein. The supernatant from heat-treated lysate supernatant contained a much lower level of unwanted bacterial proteins than that of the unheated one (**Fig. 2**, lanes 2 and 3), showing that heat treatment is of great help to remove most miscellaneous proteins, but it is of no adverse affect to does not adversely affect the fusion protein. This may be mainly attributed to heat tolerance and high solubility of the fusion protein, in agreement with the heat stability of Tα_1 [3]. Unlike regular high expression of other proteins, no inclusion bodies form inside the present bacterial cells, making it easy to purify the fusion protein. However, there is a long way to go until pET-28a-derived Tα_1 can be optimally purified using large-scale fermentation.

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Ren-Bao GAN