

Research Article

Expression and Hydroxylamine Cleavage of Thymosin Alpha 1 Concatemer

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Human thymosin alpha 1 ($T\alpha 1$) is an important peptide in the development and senescence of immunological competence in human, and many studies have reported the expression of this peptide. In this study, we designed and synthesized the $T\alpha 1$ gene according to the *E. coli* codon usage preference and constructed a $6\times T\alpha 1$ concatemer. The latter was inserted into an *E. coli* expression vector pET-22b (+), and transformed into *E. coli* BL21 (DE3). After induction with IPTG, the concatemer protein was successfully expressed in *E. coli* then cleaved by hydroxylamine to release the $T\alpha 1$ monomer. Gly-SDS-PAGE and mass spectrometry confirmed that the recombinant protein was cleaved as intended. The bioactivity of the $T\alpha 1$ monomer was analyzed by lymphocyte proliferation and by mitochondrial activity in two different tumor cell lines. This study provides a description of the preparation of a bioactive $T\alpha 1$, which may prove useful in future biomedical research.

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1. INTRODUCTION

Thymosin alpha 1 ($T\alpha 1$), a 28-amino-acid peptide with molecular weight (MW) of 3108 Da, was first isolated from calf thymus in 1977 by Goldstein et al. [1, 2]. $T\alpha 1$ is mainly produced in vivo by thymic epithelial cells and is stable at 80–90°C [3]. $T\alpha 1$ has no aromatic amino acids (see Figure 1), and N-acetylation is important for protecting towards proteolysis. $T\alpha 1$ plays a role against viral infection, against tumors, and in immunomodulation. Upon stimulation of $T\alpha 1$, T cells become mature in vitro and express IL-2, IL-2R, and CD2 [4, 5]. $T\alpha 1$ inhibits viral replication at least in hepatitis virus B-transfected HepG2 tumor cells [6, 7]. In addition, $T\alpha 1$ antagonizes T cell apoptosis induced by dexamethasone, CD3 monoclonal antibody treatment [8], or addition of sera from tumor-bearing mice [9]. It also downregulates tumor weight in mice [10], enhances major histocompatibility complex (MHC) class I antigen expression in tumor cells and antigen-presenting cells [11–13], and partly restores cellular immunity [14]. $T\alpha 1$ is used in combination with IFN- α for patients with chronic hepatitis virus B or C infection [15, 16]. $T\alpha 1$ is used to reduce adverse effects from chemotherapy or radiotherapy [17]. Recently, $T\alpha 1$ has even been used as an adjuvant for vaccines against influenza and hepatitis B [18].

In the past two decades, although the successful expression of the recombinant $T\alpha 1$ (r $T\alpha 1$) and Prothymosin (thymosin comprises the N-terminus of Prothymosin) in *Escherichia coli* has been reported [19, 20]. However, $T\alpha 1$ was expressed with a fusion protein or in a concatemer form [21, 22]. The expression of the $T\alpha 1$ monomer peptide has not been reported, because it is difficult to extract the micromolecular peptide from the fermentation broth. The fusion protein must be removed because fusion protein could influence the bioactivity of the peptide. The concatemer protein has not been processed further. All of these issues made it difficult to prepare active recombinant $T\alpha 1$. In this study, we have expressed $T\alpha 1$ concatemer protein and cleaved the concatemer protein with hydroxylamine to obtain active $T\alpha 1$ monomer peptide.

2. MATERIALS AND METHODS

2.1. Materials

The pET-22b (+) vector, *E. coli* BL21 (DE3) (*hsdS gal* [λ clts857 ind1 Sam7 nin5 lacUV5-T7]), the HepG2 liver carcinoma cell line, and the SPC-A-1 lung carcinoma cell line were kindly provided by Institute of Biochemistry

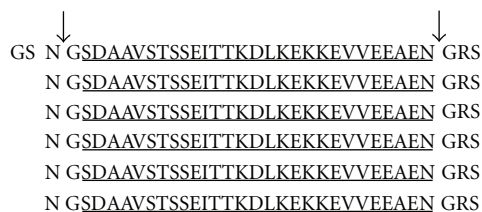


FIGURE 3: The sequence and hydroxylamine cleavage sites of the concatemer protein. The concatemer protein contained a total of 200 amino acids, and the molecular weight of the concatemer protein was approximately 23kD. The $6 \times T\alpha 1$ sequence is underlined, and the cleavage sites Asn-Gly are indicated with a vertical arrow.

Ampicillin (BBI, Toronto, Canada). The overnight culture was inoculated into 250 mL LB medium in a 1 L flask with 50 $\mu\text{g/ml}$ Ampicillin. After 2 hours of incubation, isopropyl-1-thio-beta-D-galactoside (IPTG; BBI, Canada) was added to the culture at a final concentration of 1.0 mM, when the cell density had reached $\text{OD}_{600\text{nm}} = 0.6$. After incubation at 37°C for 8 hours, the bacterial cells were harvested by centrifugation (5000 $\times g$, 15 minutes) at 4°C. The inducibility of the concatemer gene was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

The cell pellet was resuspended in 100 mL buffer A (50 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], and 0.1 M NaCl) and then sonicated (800 W, 20 minutes). After sonication, the sedimented material (containing the concatemer protein) was washed once first with 100 mL buffer B (50 mM Tris-HCl [pH 8.0], 1 mM EDTA (pH 8.0), and 0.5% Triton-X 100) and then with 100 mL buffer C (50 mM Tris-HCl [pH 8.0], 1 mM EDTA (pH 8.0), and 2 M urea) by centrifugation. The sedimented material was then dissolved in 100 mL buffer D (50 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], and 8 M urea) with stirring for 1 hour at 25°C. The soluble protein was then dialyzed (Dialysis Membrane MWCO 10 000, SPECTRUM, Tex, USA) with double distilled H₂O and cryodesiccated.

2.4. Hydroxylamine cleavage reaction and analysis of cleavage products

The concatemer protein was then cleaved with hydroxylamine. The cleavage sites are shown in Figure 3. The cleavage buffer [21] contained 0.1 M trizma base and 2.0 M hydroxylamine-HCl. The pH of the cleavage buffer was adjusted with 5 M NaOH solution to a pH of 9.0, and the reaction was performed at 45°C for 4 hours. The reaction was terminated by adjusting the pH to 4.0 with concentrated formic acid and cooling to 4°C [21]. Using Sephadex-G25 (GE) column chromatography, the products of cleavage were desalted. Column chromatography revealed 2 peaks; one peak corresponded to the T α 1 monomer and the other contained the incompletely cleaved concatemer protein. The molecular mass of the T α 1 monomer was analyzed by Gly-SDS-PAGE and mass spectrometry.

2.5. Assay for proliferation of mice splenic lymphocytes

The proliferation response of splenocytes was determined by MTT assay [23]. Spleens were removed from mice and dispersed through nylon mesh to generate a single-cell suspension. Cells were pelleted (1500 $\times g$, 6 minutes), and the cell pellet was resuspended in erythrocyte lysis solution (0.15 M NH₄Cl, 0.02 M Tris, sterilized with a 0.22 μm filter) for 5 minutes to remove the red cells. After lysis, the cell suspension was incubated in a culture flask for 2 hours, and then the suspension was transferred to a new flask. Splenic macrophages adhered to the flask; this procedure enriched for lymphocytes.

Splenic lymphocytes were incubated in RPMI 1640 medium with 5 $\mu\text{g/ml}$ concanavalin A (ConA; BBI, Canada) for 12 hours, and then harvested by centrifugation (1500 $\times g$, 6 minutes). The cell pellet was resuspended in RPMI 1640 medium at a concentration of 1×10^6 cells/mL. A 96-well culture plate (Costar, Inc., Calif, USA) was used for incubation of 180 μL lymphocytes in RPMI 1640 medium. 50 $\mu\text{g/ml}$ of the proteins (cleaved T α 1 and synthesized T α 1) were added to the wells. After 24-hour or 48-hour incubation at 37°C, 20 μL of MTT (0.5 mg/mL), solution was added to each well, and the microplates were incubated for 4 hours at 37°C. After incubation, the plates were centrifuged (1400 $\times g$, 18°C, 10 minutes). The supernatants were removed, and 200 μL of dimethyl sulfoxide (DMSO) was added to each well and incubated for 15 minutes at room temperature. After incubation, the solubilized reduced MTT was measured colorimetrically at 570 nm using a Benchmark microculture plate reader (Bio-Rad, Calif, USA).

2.6. Assay for mitochondrial activity of HepG2 and SPC-A-1 cells

The influence of T α 1 on mitochondrial activity was measured using MTT reduction, a common experimental method to quantify mitochondrial activity. The cells were seeded in a 96-well culture plate at 1×10^6 cells/mL. After 24-hour, 48-hour, and 72-hour exposures to T α 1 at a concentration of 0.1 mg/mL, the cells were incubated with MTT (0.5 mg/mL) for 4 hours at 37°C. The formazan precipitate was then dissolved in 200 μL DMSO, and absorbance at 570 nm was measured using the Benchmark microculture plate reader (Bio-Rad, Calif, USA).

2.7. Statistic analysis

The data are presented as mean \pm SEM, for at least 3 replicate experiments and evaluated using the Student's *t*-test. *P*-values below 0.05 were regarded as statistically significance (two-tailed test).

3. RESULTS

Hydroxylamine-induced cleavage at the Asp-Gly dipeptide site inserted between the 2 moieties of recombinant fusion proteins had been used for both analysis and preparation of

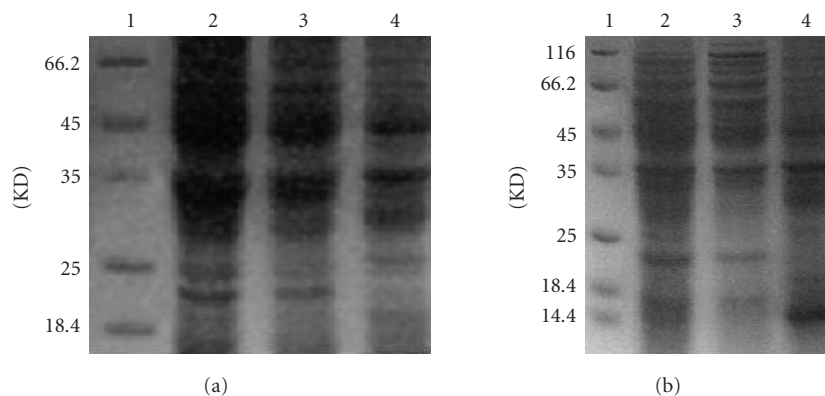


FIGURE 4: Overexpression of the recombinant concatemer protein. (a) Lane 1: prestained molecular mass markers. Lanes 2 and 3: full bacterial protein from 100 μ L transformed cells after 1.0 mM and 0.5 mM IPTG induction for 8 hours at 37°C. Lane 4: full bacterial protein from 100 μ L transformed cells without induction of IPTG for 8 hours at 37°C. (b) Lane 1: prestained molecular mass markers. Lane 2: full bacterial protein from 100 μ L transformed cells after 1.0 mM IPTG induction for 8 hours. Lane 3: sedimented material from Sonication of 100 μ L transformed cells after induction with IPTG. Lane 4: suspended material from Sonication of 100 μ L transformed cells after induction with IPTG.

mature protein. Cleavage occurs at the Asn-Gly bonds and results from the tendency of the Asp side chain to cyclize the formation of a substituted succinimide that is susceptible to nucleophilic attack by hydroxylamine. The infrequency of Asn-Gly bonds in most proteins results in the production of very large fragments that may overlap CNBr-produced fragments [24]. In the complete amino acid sequence of T α 1 [1] (see Figure 1), only 1 Asp was found at the C-terminus. However, the hydroxylamine-induced cleavage process was performed conveniently. Hydroxylamine-induced cleavage is high specificity and safe, however, the efficiency of hydroxylamine cleavage is low. So we expressed a concatemer protein of 6 repeats of T α 1 to increase the yield of T α 1 after cleavage and to decrease the cleavage sites.

3.1. Characterization of recombinant concatemer protein

The optimal conditions for inducing production of recombinant concatemer protein were at an IPTG concentration of 1.0 mM 37°C for 8 hours. Under these conditions, the recombinant protein (~23 kD) comprised 31% of the total bacterial protein (see Figure 4(a)). The concatemer protein was identified primarily in the inclusion bodies (see Figure 4(b)).

3.2. Analysis of the products of hydroxylamine cleavage

G-25 column chromatography was used to not only desalt but also remove the incompletely cleaved concatemer protein. Because the molecular weight of the T α 1 monomer was ~3.1 kD, it could enter the G-25 gel. However, the incompletely cleaved concatemer proteins, including 2 \times T α 1 (~7 kD), 3 \times T α 1 (~11 kD), 4 \times T α 1 (~15 kD), 5 \times T α 1 (~19 kD), and 6 \times T α 1 (~23 kD), could not enter the G-25 gel because the gel only allows the entry of molecules <5 kD. The Gly-SDS-PAGE could satisfactorily separate

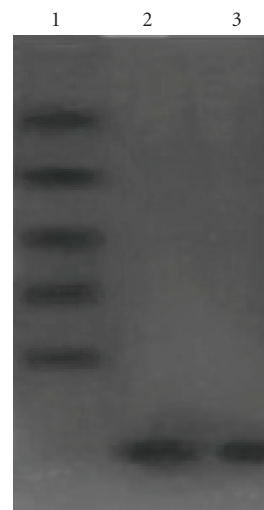


FIGURE 5: Analysis of hydroxylamine cleavage products. Lane 1: peak 1 of G-25 column chromatography revealed the protein with a molecular weight of more than 5 kD that was eluted early. Lane 2: peak 2 of G-25 column chromatography containing the protein with a molecular weight of less than 5 kD that was eluted later. Lane 3: the T α 1 synthesized as a standard protein.

polypeptides with molecular weights ranging from 3.5 to 68.0 kD with good linearity, and demonstrated the successful hydroxylamine-induced cleavage of the concatemer proteins (see Figure 5). However, not all the concatemer proteins were cleaved, and the incompletely cleaved proteins could be completely cleaved by another reaction (data not shown).

MS was performed to analyze the exact molecular weights of the products and the synthesized T α 1. The result showed that the products had only 1 peak of $m/z = 1042.19$, while the synthesized T α 1 had 2 peaks of $m/z = 1037.80$ and 1555.06 (see Figure 6). The molecular weight of the synthesized T α 1 was found to be approximately 3108 Da, it

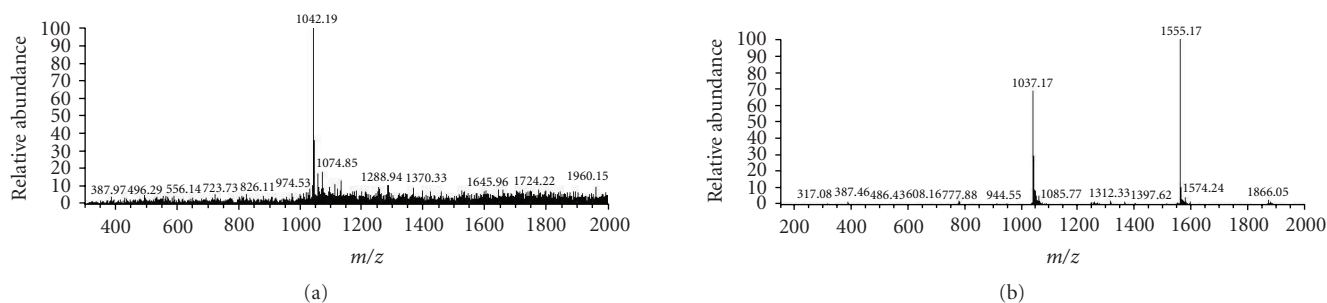


FIGURE 6: (a) Mass spectrum of the cleaved T α 1. The attachment of 3 protons per peptide molecule leads to only 1 of m/z peak; m/z = 1042.19. (b) Mass spectrum of the synthesized T α 1. The attachment of 2 and 3 protons per peptide molecule leads to 2 peaks of m/z = 1037.80 and 1555.06.

was assumed that the peptide might attach 2 or 3 protons $[(1037.80-1) \times 3 = 3110.4; (1555.06-1) \times 2 = 3108.12]$. Therefore, the cleaved T α 1 was believed to attach 3 protons, and the molecular weight of the products was approximately 3123.57 $[(1042.19-1) \times 3 = 3123.57]$. The difference in the molecular weights between the cleaved T α 1 and the synthesized T α 1 was approximately 15. According to a previous research [24], the structural difference between the products and the synthesized T α 1 was a -NH added to the N-terminus of the cleaved peptide (see Figure 9).

3.3. T α 1 cleaved by hydroxylamine stimulated the proliferation of mice splenic lymphocytes

To determine the bioactivity of the T α 1 monomer on lymphocyte proliferation, the mitochondrial activity was compared in the presence with the absence of the T α 1. The bioactivity of T α 1 preparations was analyzed by MTT assay. Preliminary results showed that the peptides could stimulate the mice spleen lymphocyte proliferation [22]. After a 24-hour treatment with T α 1 cleaved by hydroxylamine, mitochondrial activity was upgraded to 119.1% and after 48 hours, it was 110.4%. The numbers of the synthesized T α 1 were 117.5% and 116.0% at 24 hours and 48 hours, respectively (see Figure 7). Both of the peptide preparations could stimulate the proliferation of mice spleen lymphocytes to a similar degree.

3.4. T α 1 cleaved by hydroxylamine exhibits decreased mitochondrial activity in SPC-A-1 and HepG2 cells

In this study, we used human liver tumor cells HepG2 and human lung tumor cells SPC-A-1 as model, because the liver tumor and lung tumor are the most general tumor. To determine the role of mitochondria during incubation, the mitochondrial activity was examined. After the treatment with the cleaved T α 1 for 24 hours, 48 hours, and 72 hours, the mitochondrial activity of the SPC-A-1 cells decreased to 82.4%, 27.1%, 11.6%, respectively, and the corresponding activities with synthesized T α 1 were 62.0%, 15.1%, and 9.8%, respectively. On the other hand, after the treatment for 24 hours, 48 hours, and 72 hours, the mitochondrial activity of HepG2 cells decreased to 86.6%, 18.4%, and

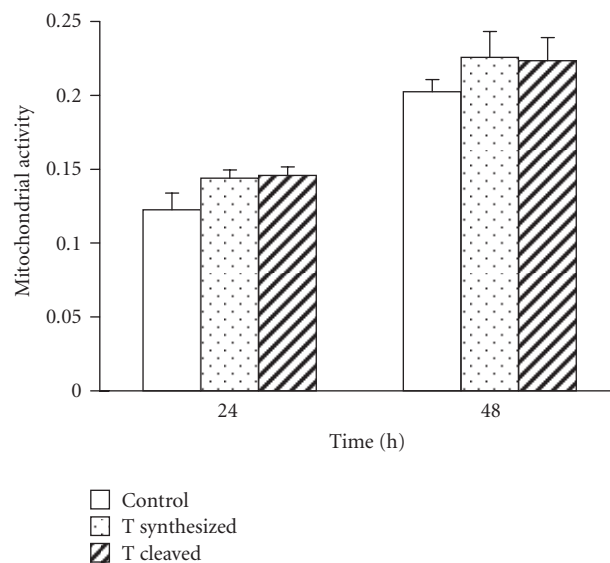


FIGURE 7: T α 1 (cleaved and synthesized) stimulated the proliferation of mice spleen leukomonocytes. The mice spleen leukomonocytes were treated with the indicated concentrations of T α 1 for 24 hours, and 48 hours. MTT assay was used to measure the mitochondrial activity. Significant stimulation of the mitochondrial activity, as compared to the control, occurred after 48 hours of T α 1 treatment. All the results are expressed as the mean \pm SEM of data obtained in 3 independent experiments ($P < .05$, relative to control).

10.4%, respectively, and corresponding activities with the synthesized T α 1 were 59.2%, 13.1%, and 10.0%, respectively (see Figure 8). Both of the peptide preparations decreased the mitochondrial activity remarkably, as compared to the control. The difference in the activity between the 2 peptides was not significant; however, the process of synthesizing T α 1 was rather intricate.

4. DISCUSSION

The requirements of T α 1 for clinical applications are increasing. For clinical use, T α 1 is mainly obtained by chemical synthesis and animal thymus extraction. However, in fact, T α 1 from an animal is often impure and may therefore cause

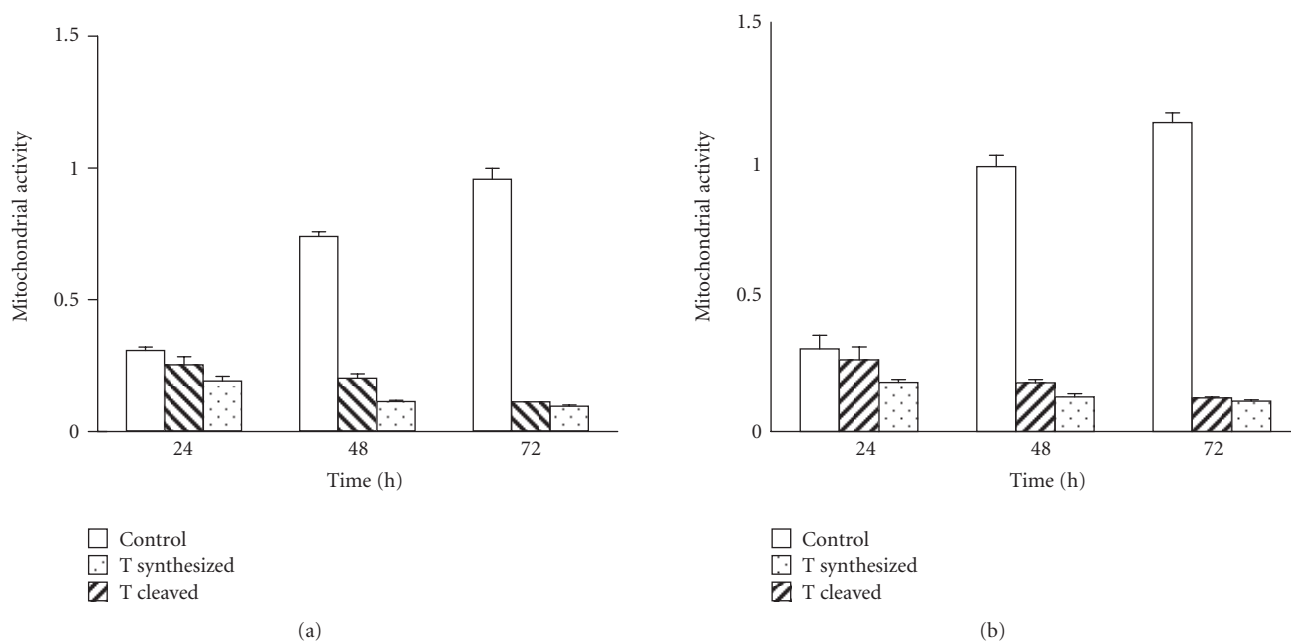


FIGURE 8: $T\alpha 1$ (cleaved and synthesized) induced a decrease in the mitochondrial activity in the SPC-A 1 and HepG2 cells. (a) The SPC-A1 cells were treated with the indicated concentrations of $T\alpha 1$ for 24 hours, 48 hours, and 72 hours. MTT assay was used to measure the mitochondrial activity. Significant decreases in the mitochondrial activity, as compared to the control, occurred after 48 hours of $T\alpha 1$ treatment. (b) The HepG2 cells were treated with the indicated concentrations of $T\alpha 1$ for 24 hours, 48 hours, and 72 hours. MTT assay was used to measure the mitochondrial activity. Significant decreases in the mitochondrial activity, as compared to the control, occurred after 48 hours of $T\alpha 1$ treatment. All the results are expressed as the mean \pm SEM of data obtained in 3 independent experiments ($P < .05$, relative to control).

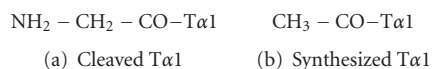


FIGURE 9: Differences in the structures of the synthesized $T\alpha 1$ and the cleaved $T\alpha 1$.

heterogeneous allergy. Although chemically synthesized $T\alpha 1$ can reach a high level of purity, it is necessary to eliminate the side products at each step, including incorrect joining and dextral compounds. The longer the peptide is, the more intricate the chemical synthesis process becomes. $T\alpha 1$ comprises 28 amino acid residues, and its chemical synthesis incurs a high production cost [19]. Prokaryotic expression systems allow for high expression of foreign genes and are widely used for producing large amounts of proteins or peptides. In this research, using a prokaryotic expression system BL21 (DE3)/pET-22b (+), we have established a new method to obtain relatively pure and large quantities of $T\alpha 1$.

When the plasmid was digested with isocaudamer BamH I (G*CTAG C) and Bgl II (A*GATC T), the 2 fragments had similar termini. When the 2 fragments were catalyzed with T4 DNA Ligase, the new sequence formed was GGATCT, and this new site could not be digested by BamH I or Bgl II (see Figure 2). Therefore, the new 2 \times concatemer gene could not be destroyed when the 4 \times concatemer gene was constructed. The new site GGATCT corresponded to the dipeptide Gly-Ser, located at the downstream of the $T\alpha 1$ gene. When concatemer protein was cleaved with hydroxylamine, the down-

stream amino acid of $T\alpha 1$ peptide was removed from this Gly site. When the cleavage reaction proceeded, the upstream amino acid of $T\alpha 1$ peptide was removed from another Gly site, which was added in front of the $T\alpha 1$ peptide.

It is common knowledge that extraction of the micro-molecular recombinant peptide from fermentation broth is difficult, when $T\alpha 1$ is expressed alone. Generally, a fusion protein is used under these circumstances, for example, a DsbA fusion protein [25]. However, the fusion protein must be removed because the peptide might be inactive, and this process incurs a high production cost. The concatemer protein of 6 repeats—6 \times $T\alpha 1$ —was prepared in this research to avoid the use of a fusion protein when expressing the recombinant protein, but the number of the repeats was not unique. Proteins with 4, 8, 12, and so forth repeats could also be constructed. The longer the concatemer protein is, the greater the yield of the $T\alpha 1$ monomer is. On the other hand, when a concatemer protein with more repeats is cleaved, the amount of the incompletely cleaved protein also increases (see Figure 5). However, these incompletely cleaved proteins can be cleaved by other 1 or 2 rounds of hydroxylamine cleavage.

Hydroxylamine was used to cleave the Asn-Gly peptide bond between the fusion protein and target protein [26]. In this study, hydroxylamine was used to cleave the concatemer protein of 6 \times $T\alpha 1$ to release the $T\alpha 1$ monomer. While chemical cleavage methods use relatively inexpensive and widely available reagents, cleavage conditions are often relatively harsh and amino acid side-chain modifications

may occur. This is especially true when hydroxylamine is used to cleave the Asn-Gly peptide linkages as other Asn or Gln residues can be modified to their hydroxamic acid forms [26]. Because the T α 1 peptide does not contain Gln, Met, or other Asn, chemical heterogeneity does not occur by conversion of the Gln or Asn to their hydroxamate forms. Moreover, there is no possibility of chemical heterogeneity by conversion of Met to its oxidized forms [27].

After hydroxylamine cleavage, a Gly remained at the N-terminus of T α 1. However, the natural T α 1 had an acetylated NH-terminal (see Figure 9). The molecular weight difference between the 2 groups was 15 Da, but there was no significant difference between the 2 bands revealed by the Gly-SDS-PAGE (see Figure 5). Hence, we used an MS to analyze the exact molecular weights of the 2 peptides. The result showed a difference was 15 Da (3123–3108 Da) (see Figure 6), indicating that the concatemer protein was cleaved satisfactorily.

To investigate the influence of the extra imino group on the T α 1, we compared the antitumor and immunomodulation activities between the synthesized T α 1 and the cleaved T α 1. The results showed no significant difference between the 2 peptides. Furthermore, we found that T α 1 was able to depress the proliferation of HepG2 cells by downregulating the oxidative stress (data not shown). Jörnvall [28] had analyzed 40 known α -amino-acetylated polypeptide chains and postulated that acetylation occurs during an early posttranslational stage and may have particular importance in this regard. However, the structural or functional significance of the N-terminal acetylation of T α 1 is not known, and a protective function of acetylation with regard to proteolysis is possible [1]. The change in the N-terminus of cleaved peptides might not influence the bioactivity of T α 1; however, it might influence the stability in vivo.

In summary, T α 1 with a novel structure was prepared via the expression and hydroxylamine cleavage of a T α 1 concatemer protein. This novel T α 1 could perform proliferation of mice splenic lymphocytes activities, identical to the natural T α 1. This study would provide the basis for the preparation of active T α 1 and other peptides.

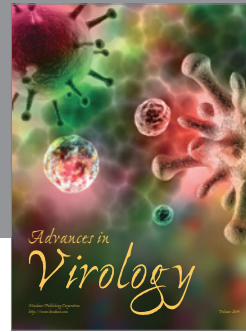
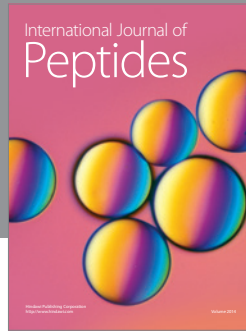
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