Protein Expression and Purification 84 (2012) 1–8

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Intein-mediated expression, purification, and characterization of thymosin α1–thymopentin fusion peptide in *Escherichia coli*

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ARTICLE INFO

Article history:
Received 14 February 2012
and in revised form 16 April 2012
Available online 25 April 2012

Keywords:
Thymosin α1–thymopentin
Intein
Self-cleavage
Protein refolding
ESI-MS
Immunoregulatory activity

ABSTRACT

Thymosin α1–thymopentin (Tα1–TP5) fusion peptide has been proved to be an immune regulator based on its higher immunoregulatory activity than Tα1 and TP5. To obtain Tα1–TP5 more effectively and economically, Tα1–TP5 was genetically fused to a self-cleaving intein-chitin binding domain tag for purification via chitin beads in *Escherichia coli*. After affinity purification, the target peptide was released from the chitin beads via self-cleaving intein ((INTervening protEIN) induced by dithiothreitol. Further, Tα1–TP5 was purified by Superdex 30 and identified by Tricine-SDS-PAGE and electrospray ionization-mass spectrometry. Finally, about 7.6 mg Tα1–TP5 purified from the soluble fraction and inclusion bodies was obtained from 1 L culture media. The purity was 95% after a series of chromatographic purification steps. *In vitro*, the purified Tα1–TP5 could stimulate the proliferation of mouse splenic lymphocytes. Overall, this work demonstrated that Tα1–TP5 was purified with low cost and high efficiency, greatly expanding its potential use as an immune regulator.

Introduction

Thymopentin (TP5) is a synthetic peptide consisting of five amino acid residues (H_2N–Arg-Lys-Asp-Val-Tyr–OH). As an immunomodulator, TP5 is used clinically to treat diseases relating to abnormal immune response, such as serious post-operative infections, type II diabetes mellitus, and various autoimmune diseases [1,2]. Thymosin alpha 1 (Tα1), a biologically active peptide consisting of 28 amino acid residues, exhibits similar immunoregulatory activity as TP5. As a biological response modifier, Tα1 has the activities of immunoregulation, antitumor, protection against oxidative damage and so on, and is widely used in clinic treating many kinds of disease such as cancers and infectious diseases [3].

Although TP5 has been successfully applied clinically, it has very short half-life ($t_{1/2}$) in human plasma. Accordingly, frequent intramuscular injections or intravenous infusion is necessary, both of which are inconvenient and stressful for patients. Presently, many ways have been tried to enhance the $t_{1/2}$ and bioavailability of TP5, such as the creation of TP5 analogs, cyclopeptides, novel TP5 oral delivery systems and release systems [4–8]. In order to prolong the $t_{1/2}$ of TP5 and get a new peptide with better immunoregulatory activity, a Tα1–TP5 fusion peptide was designed and expressed in *Pichia pastoris* by our research team [9]. Previous study showed that Tα1–TP5 had longer $t_{1/2}$ and higher activities than TP5 and Tα1. However, the secretory expression level of Tα1–TP5 in *Pichia pastoris* was low, and the high price and non-specific cleavage of thrombin [9] as well as the complex downstream purification process limited the scale up of Tα1–TP5. Thus, it is necessary to establish an expression system with higher yield and lower cost.

Fortunately, several of these problems can be solved by using intein-mediated protein purification. Inteins (INTervening protEINS) are natural protein sequences which are capable of post-translational self-excision from the precursor protein through a process known as "protein splicing" [10,11]. The intein-mediated self-cleavage system has been recently developed as a powerful tool for protein expression, purification, ligation and amidation [12–16]. In practice, the self-cleaving reaction can be induced at the intein's N-terminus by dithiothreitol (DTT) addition, which will reduce disulfide bonds in proteins containing them, effectively inactivating those targets. Recently, the system of intein-mediated expression of proteins and polypeptides has been successfully employed [17–23].

In this work, the Tα1–TP5–*Sce* intein-chitin binding domain (CBD) fusion protein was expressed in *Escherichia coli*. Then the fusion protein was purified from the soluble fraction and inclusion bodies by one-step purification with a chitin affinity column. The active Tα1–TP5 was released and separated from the fusion partner by adding 30 mmol/L DTT. Tα1–TP5 was further purified by Superdex 30 and analyzed by electrospray ionization-mass spec-

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http://dx.doi.org/10.1016/j.pep.2012.04.013

trometry (ESI-MS). The approach described here is a low-cost, convenient and potential way for obtaining Tα1–TP5.

Materials and methods

Strains, vector, chemicals, media

E. coli DH5α (preserved in our laboratory) was used as the host strain for cloning and maintaining plasmid, and *E. coli* ER2566 (New England Biolabs, NEB) was used as host for protein expression. These strains were cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% sodium chloride) supplemented with ampicillin (100 μg/mL). For solid media, 1.5% agar was added. All enzymes were purchased from Fermentas (Canada) unless otherwise noted. The *E. coli* expression vector pTYB2 and chitin beads were also purchased from NEB. The Tα1–TP5 gene and primers were synthesized by Sangon Corporation (China). Tα1–TP5 peptide standard was synthesized by CL. XIAN BIO. SCIENTIFIC CO., LTD. (China). Isopropyl-β-D-thiogalactoside (IPTG), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), DTT and glutathione (oxidized) were purchased from Sigma. Other chemical reagents were of analytical grade.

Construction of Tα1–TP5 fusion peptide expressing prokaryotic vector pTYB2- Tα1–TP5

The sequence of the Tα1–TP5 gene was designed according to the *E. coli* codon usage preference as follows: 5′-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 AACCGT AAA GAT GTG TAT-3′. The fusion gene was amplified using PCR primer-F (5′-ACGTACATATGTCTGATGCAGCGGT-3′) and primer-R (5′-ATACACATCTTTACGGTTTTCCG-3′), with an *Nde* I site (underlined). PCR amplification was carried out for 30 cycles with the following parameters: initial PCR activation at 94 °C for 10 min, 30 cycles at 94 °C for 1 min, 50 °C for 30 s, 70 °C for 1 min and a final extension cycle at 72 °C for 10 min. *Pfu* DNA polymerase was used in the PCR reaction to generate a blunt end in the C-terminal of the target gene. The amplified DNA fragment was digested by *Nde* I and then ligated into the expression vector pTYB2, which was digested by *Nde* I and *Sma* I and dephosphorylated with calf intestinal alkaline phosphatase. The ligation mix was transformed into competent DH5α cells and the single bacterial colony was selected by overnight growth on LB agar plates containing 100 μg/mL ampicillin. The obtained recombinant plasmid pTYB2-Tα1–TP5 was confirmed by PCR and DNA sequencing.

Expression of Tα1–TP5–Sce intein-CBD fusion protein in E. coli

Recombinant protein was expressed in *E. coli* strain ER2566. A single bacterial colony was inoculated in rich liquid LB medium containing ampicillin (100 μg/mL) and grown overnight at 37 °C. Then, 10 mL overnight culture was inoculated into 1 L LB medium and when the OD 600 nm reached approximately 0.6–0.8, protein expression was induced with 1 mmol/L IPTG for 4 h. About a 4.5 g wet cells pellet was obtained from 1 L culture medium and resuspended in 100 mL lysis buffer (20 mmol/L Hepes, 500 mmol/L NaCl, 0.1 mmol/L EDTA, pH 8.0) and sonicated on ice using a sonicator for 20 min at output 30% with repeated 10 s on/5 s off. The lysate was then centrifuged at 20,000 g for 30 min at 4 °C. The supernatant and the insoluble protein fraction were examined by 10% Tris-glycine SDS-PAGE. Different temperatures and IPTG concentrations were tested to improve the yield of soluble fusion protein. BandScan 5.0 was used to estimate the expression level of the target protein.

Purification of Tα1–TP5 fusion peptide from the supernatant

Tα1–TP5 fusion peptide was purified from the soluble fraction by affinity chromatography using chitin beads (New England Biolabs, NEB) resin following manufacturers instruction. After sonication, about 100 mL supernatant (5.98 mg/mL) was applied to 10 mL chitin beads with a flow rate of 0.6 mL/min. After removing unwanted proteins with the washing buffer (20 mmol/L Hepes, 500 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1% Triton X-100, pH 8.0) for 30 columns, chitin beads were washed three columns by cleavage buffer (20 mmol/L Hepes, 500 mmol/L NaCl, 0.1 mmol/L EDTA, pH 8.0) with 30 mmol/L DTT. The beads were incubated at 4 °C and the effect of time on the cleavage efficiency was investigated.

Purification of Tα1–TP5 fusion peptide from inclusion bodies

The sediment (2 g) was washed three times with 25 mL washing buffer A, then washed twice with buffer B to remove Triton X-100 (Table 1). Finally, the sediment was suspended and solubilized in 20 mL denaturing buffer C (Table 1) by stirring at 37 °C for 2 h. Insoluble residual was removed by centrifugation at 20,000 g for 30 min. 0.4 mg/mL denatured recombinant protein solution was diluted 1:10 in refolding buffer D to buffer L (detailed in Table 1) at 4 °C and mixed vigorously for 30 s. The mixture was then placed at 4 °C for 24 h for refolding and cut overnight by 30 mmol/L DTT. The cleavage percentage was analyzed by BandScan 5.0. For large scale purification, refolded recombinant protein was applied to the chitin beads and Tα1–TP5 was released from the fusion protein after intein-mediated cleavage. For further removal of unwanted proteins and peptides as well as salt, 30 mL effluent (0.2 mg/mL) from the column was lyophilized and loaded onto a Superdex 30 prep grade (GE Healthcare) (80 cm × 1.6 cm), eluted by 100 mmol/L NH_4HCO_3 with a flow rate of 0.5 mL/min. The relevant fraction was collected and lyophilized before SDS-PAGE analysis.

Characterization of Tα1–TP5

ESI-MS (Applied Biosystems, USA) was used to determine the molecular mass of Tα1–TP5 obtained from the soluble fraction and inclusion bodies. Simply, 10 μL Tα1–TP5 (0.1 mg/mL) was injected (10 μL/min) into an ESI quadrupole mass spectrometer. The spray voltage was 4.5 kV and source temperature was maintained at 120 °C. The mass range 100–1500 m/z was scanned every 3 s using a cone voltage of 20 V.

Biological activity of Tα1–TP5

The bioactivity of Tα1–TP5 was determined by the MTT assay *in vitro* [24]. Balb/c mice were purchased from the Experimental Animal Center of Shandong University, and treated in accordance with Chinese state legislation on the care and use of laboratory animals. Spleen cells were isolated from 6- to 8-week-old Balb/c mice, and collected by centrifugation at 2000 r/min for 3 min. 100 μL of the suspension was seeded in each well of 96 well plates at a concentration of 6–8 × 10^6 cells/mL. Then, 100 μL of Tα1–TP5 that was purified from the supernatant and inclusion bodies and diluted to different concentrations was added to all but the control wells. The synthetic Tα1–TP5 and RPMI 1640 mediums were used as positive control and negative control, respectively. The 96-well plate was incubated at 37 °C for 48 h in a cell-culture incubator with 5% (v/v) CO_2 and then 20 μL MTT was added into each well and incubated for 4 h. Then 150 μL DMSO was added to each well. After gentle shaking for 10 min, the plate was placed onto a microtitre plate reader (Bio-Rad 680) and measured at 570 nm. The results were used for calculate growth rate.