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Production and characterization of highly purified recombinant thymosin beta 4 in *Escherichia coli*

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ABSTRACT

Thymosin β4 (Tβ4) is a small peptide composed of 43 amino acids. It has many important biological functions, such as promoting cardiac repair and wound healing, and therefore has great potential in clinical applications. In this report, we describe a novel and efficient way to produce highly purified and active Tβ4. It was expressed in a soluble form using a DsbA and hexahistindine tag in *Escherichia coli* (*E. coli*). Using high cell density cultivation, the final biomass concentration was about 50 g L^{-1} dry cell weight with the expression level of the fusion protein being 40%. To obtain highly purified protein, a purification process involving a five-step column procedure was implemented. The purity of Tβ4 was above 98% and all the host cell related impurities, such as endotoxin, host cell protein and residual DNA levels, were within the permissible range listed in the Chinese Pharmacopoeia. The E-rosette test demonstrated that the bioactivity of purified Tβ4 was consistent with other published work. This is the first report producing highly purified Tβ4 from genetically engineered sources.

Introduction

Thymosin β4 (Tβ4)[1], was first isolated from calf thymus by Low and his colleagues in 1981 [1]. It is one of the effective components of thymosin and has multiple biological functions. Tβ4 is composed of 43 amino acids with acetylserine at the NH_2 terminus. Tβ4 promotes cardiomyocyte migration, survival and repair giving it the required properties for therapies to treat acute myocardial damage [2]. Tβ4 is the first known molecule capable of organ-wide activation of the embryonic coronary developmental program in the adult mammalian heart [3]. Nicola Smart [4] showed that Tβ4 can promote vessel formation and collateral growth in mice, not only during development, but also critically from adult epicardium, suggesting that Tβ4 has considerable therapeutic potential in humans. Recently, significant attention has been paid to Tβ4 for its therapeutic applications [5–8].

Previously, the preparation of Tβ4 depended mainly on chemical synthesis, which is a costly process. However, genetic engineering methods have now been applied to the production of Tβ4. Tβ4 was expressed as a fusion protein with a hexahistidine tag in *Escherichia coli* and purified by Ni-affinity chromatography [9]. However, the tag could not be removed after the purification process and might affect the biological function of the protein. Another research inserted the Tβ4 gene into the pTXB1 vector, expressing it with the fusion tag, intein-CBD, at the C-terminal [10,11]. With the help of the fusion tag, Tβ4 was easily purified by chitin affinity chromatography and the tag removed through autocatalytic cleavage induced by DTT. However, only half of the fusion tag was successfully removed, indicating that the efficiency of autocatalytic cleavage by DTT was not high. This process also added five extra amino acids to the C-terminus of the Tβ4 protein after cleavage, which may change the function of Tβ4. Also, neither of these studies provided highly purified Tβ4 with little host cell related impurities.

The biological activity of Tβ4 has been determined using different approaches. Chick choriollantoic membrane assay has been used to investigate its ability to promote blood vessel formation [9,10]. This assay is the most widely used in vivo test system for studying angiogenesis, due in part to low cost and reduced ethical concerns compared to other in vivo assays. However, the number of branching blood vessels was difficult to determine. Another research evaluated the biological activity of Tβ4 by its promotion of T lymphocyte proliferation and differentiation [11]. However, the experiment was time consuming. Thymosin could enhance the percentage of rosette forming cell in the E-rosette assay, which is a useful indicator to evaluate the bioactivity of thymosin [13–15]. This method takes the advantages of simple operation, good stability and short period.

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[1] *Abbreviation used*: Tβ4, thymosin; HCDC, high cell density cultivation; IMAC, immobilized metal affinity chromatography; IEC, ion exchange chromatography; RPC, reversed phase chromatography; TFA, trifluoroacetic acid.

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In this article, highly purified Tβ4 was produced from the bench scale process. Tβ4 was expressed in *E. coli* with a fusion partner. In order to improve the process productivity, high cell density cultivation was developed in the fermentation process. A new purification strategy was used to effectively purify Tβ4. Its biological activity was evaluated using the E-rosette test and all the important pharmaceutical release criteria were systematically analyzed according to the methods of the Chinese Pharmacopoeia. All studies have been performed in accordance with the Chinese sFDA's Good Laboratory Practice Regulations. This article provides valuable information for expression and purification of other small peptides, especially for medical use.

Materials and methods

Plasmid construction

The coding region of Tβ4 (Genbank Accession No. NM_021109) was optimized using *E. coli* preferred codons. A ScaI site and a thrombin site were introduced at the N-terminal of Tβ4, and a XhoI site and a stop codon were introduced at the C-terminal. The sequence was synthesized by GENEWIZ, Inc. (Beijing, China). The 5′ and 3′ strands were annealed and ligated into the correspondingly digested pET39-b(+) plasmid (Novagen). The construct obtained encoded the Tβ4 protein, the DsbA protein and a hexahistindine tag at the N-terminus.

The recombinant plasmid pET39b(+)-Tβ4 was transformed into *E. coli* StarBL21 (DE3) plyss for expression. The transformant was selected on a LB agar plate containing kanamycin.

High cell density cultivation for the expression of Tβ4

High cell density cultivation (HCDC) was performed in a 30 L bioreactor (Baoxing, Shanghai, China). A semi-defined medium, developed by our laboratory, was used in this study. Briefly, 15 L of medium was inoculated with 700 mL of seed culture grown to an OD_{600} of 3.5–4.0 in a shaking flask. The pH was controlled at 7.0 with ammonium hydroxide. The temperature was maintained at 37 °C before induction and at 30 °C after induction. The dissolved oxygen (DO) was maintained above 20% by increasing the stirrer speed, the air flow rate or the pure oxygen flow rate. The glucose solution of 500 g/L and yeast extract of 100 g/L were used for feeding. To avoid accumulation of acetate, a feedback glucose feeding strategy was used as previously described [16]. The strategy, based on superimposed pulses in the glucose feed, followed by evaluation of the responses in the DO signal, used the feedback control of the flow rate of glucose. At an OD_{600} of 80, the culture was induced with 1 mM IPTG. After a 4 h induction, the bacterial cells were harvested by centrifugation.

To determine the expression level, the sample was centrifuged after suspension in lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA). The soluble portion was subjected into 15% SDS–PAGE, and the protein bands were visualized by staining with Coomassie Brilliant Blue R250.

Purification of Tβ4

Multiple-step chromatography was used in the purification process. The whole procedure is illustrated in Fig. 1.

The bacterial cells were suspended in lysis buffer (50 mM Tris–HCl, pH 8.0) containing DNase. They were stirred slowly overnight. The extract was clarified by centrifugation at 9500 rpm for 30 min.

The fusion protein contains a histidine tag to facilitate purification by immobilized metal affinity chromatography (IMAC). Ni Sepharose 6 Fast Flow resin (100 mL; GE Healthcare) was packed into a column (35 mm/20 cm, Huamei, Shanghai, China). The resin was equilibrated with 0.5 M NaCl, 10 mM imidazole, 25 mM Tris–HCl buffer, pH 8.0 for 2 column volumes (CV). The supernatant was supplemented with 0.5 M NaCl and 10–50 mM imidazole and loaded onto the column. The concentration of the imidazole in the binding buffer was further optimized. The resin was washed with equilibration buffer until the UV absorbance of the effluent at 280 nm reached baseline. The fusion protein was eluted using 0.5 M NaCl, 200 mM imidazole, 25 mM Tris–HCl buffer, pH 8.0 and collected in a single peak based on A_{280} absorbance.

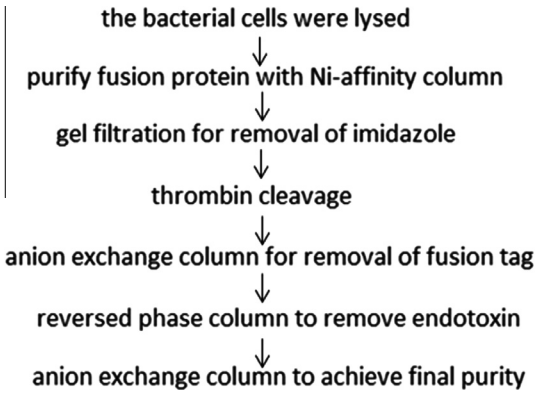

Fig. 1. The procedure of purification.

The eluate was desalted using gel filtration chromatography. Sephadex G-25 resin (400 mL; GE Healthcare) was packed in a column (50 mm/40 cm, Huamei, Shanghai, China). The resin was equilibrated with 25 mM Tris–HCl, 0.15 M NaCl buffer, pH 8.0 for 2 CV. After loading the eluate from the IMAC, the resin was washed with equilibration buffer and the target protein was collected according to the UV absorbance.

For cleavage of Tβ4 from the fusion tag, the fusion protein solution (8–10 mg/mL) was supplemented with thrombin (Hangkang Corp, Zhejiang, China) at a ratio of 3 U/mL and incubated at room temperature for 18 h.

Ion exchange chromatography (IEC) was used to remove impurities such as thrombin, fusion tag and host cell proteins. The column (50 mm/40 cm, Huamei, Shanghai, China) was packed with Q Sepharose XL resin (240 mL; GE Healthcare). The resin was equilibrated with 25 mM Tris–HCl buffer, pH 8.0 for 2 CV. The gel filtration eluate was diluted fourfold with water for injection and applied onto the column. After washing the column with 2 CV of equilibration buffer, bound Tβ4 was eluted with 1 mM NaCl, 25 mM Tris–HCl buffer, pH 8.0 and collected in a single peak based on A_{280} absorbance.

Reversed phase chromatography (RPC) was used to reduce endotoxin level. Source 30 RPC resin (50 mL; GE Healthcare) was packed into a XK26/20 column (GE Healthcare). The resin was equilibrated with 5% methanol and 0.1% trifluoroacetic acid (TFA) for 2 CV. The pH of IEC eluate was adjusted to 3.0 with 1 M HCl and applied onto the column. After washing the column with 1 CV of equilibration buffer, impurities were eluted with 5% methanol and 0.1% TFA for 4 CV. Tβ4 was eluted with 18% methanol, 0.035% TFA buffer and collected in a single peak.

Q Sepharose High Performance (25 mL; GE Healthcare) packed in a XK26/20 column (GE Healthcare), was used to remove organic solvents, such as methanol and TFA. The resin was equilibrated with 20 mM PB buffer, pH 8.0 for 5 CV. The RPC eluate was diluted threefold with water for injection and its pH was adjusted to 8.0 with 1 M NaOH before applied onto the column. After washing the column with 5 CV of equilibration buffer, Tβ4 was eluted with 1 mM NaCl, 20 mM PB buffer, pH 8.0.

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