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Original article

Molecular cloning, expression, purification and osteoblasts proliferation activity of sika deer thymosin beta10

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Abstract

Thymosin beta 10 (Tβ10) is a member of the β-thymosin family. As an actin-binding peptide, thymosin β10 is involved in many important biological activities. Transcriptome sequencing results suggest that Tβ10 may play important roles in the growth of deer antler. In this study, Tβ10 cDNA was isolated from sika deer, and complete open reading frame consisting of 129 nucleotides was obtained by PCR amplification. The predicted peptide was 42 amino acids in length. The *sdTβ10* cDNA was cloned and expressed in *Escherichia coli* resulting in a 6 kDa recombinant-His tagged protein. The recombinant, non-glycosylated peptide was overexpressed in a soluble form and purified by immobilized metal ion affinity chromatography. Functional studies revealed that recombinant Tβ10 stimulated osteoblasts proliferation. This study provides the first evidence that recombinant sika deer Tβ10 promotes proliferation in an osteoblasts cell model.

Key words: affinity chromatography, cell proliferation, prokaryotic expression, sika deer, thymosin 10

Introduction

Thymosins are a group of naturally occurring, small bioactive proteins. They were first isolated from the calf thymus in 1966 and divided into three distinct classes based on their isoelectric point (Goldstein et al. 1966, Goldstein 2007). Thymosin beta 10 (Tβ10) is a member of the β-thymosin family (the isoelectric point between pH5.0 and pH7.0) and a Tβ4/ Tβ15 homologue (Hannappel 2007). Tβ10 was first described in 1983 from mammalian tissues (Erickson-Viitanen et al. 1983). This protein consists of about 43 amino acid residues and is mainly localized in the cytoplasm of the cell (Tsitsiloni et al. 1992). Tβ10 contains two helices and forms α-helical structures. Tβ10 is considered to be a multi-functional protein that is involved in many important biological activities. A conserved motif, LKKTET, is critical for binding to G-actin (Hoch et al. 2016). An actin-binding site containing peptide with an additional amino acid, LKKTETQ, has several biological functions beyond actin-binding (Sosne et al. 2010). Tβ10 is believed to

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Description	RPKM Value											
	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	C1	C2
40S ribosmonal protein S29-like	31955	20299	22285	12002	14584	13131	21905	14234	17990	27071	11481	19040
60Sribosmonal protein L37-like	15892	10318	10035	8020	10357	10297	9678	8534	10600	13723	8042	11399
Collagen alpha-1(I) chain precursor	13454	21412	17910	24475	16562	17506	26015	19613	21563	12240	20106	18777
Thymosin beta 10, isoform CRA_a	11123	8759	6908	5584	8580	8073	6259	5420	6786	6889	7727	7068
40S ribosmonal protein S15	9199	7653	7569	4342	6401	5901	8779	5230	7370	8131	6413	6573
60S acidic ribosomal protein P1-like	8451	6316	5470	4500	7514	6331	5303	4793	7038	7636	7718	4748
Collagen alpha-2(I) chain precursor	8214	10688	10063	12161	9078	9843	11983	11448	10961	7747	10502	9474
40S ribosmonal protein S20-like	7305	5474	3162	5529	6031	6528	4669	5170	5098	7209	4439	4491
Ribosomal protein L35-like	6852	5624	4832	4145	5672	5613	5088	4182	6348	6954	6858	4341
40S ribosmonal protein S27-like	6729	5007	5025	3551	4737	4055	4592	3625	4800	7242	4062	4951
Collagen alpha-1(III) chain precursor	6605	4239	4119	2579	3370	3412	2725	3987	3617	4167	2809	2814
NaDH dehydrogenase subunit 5	6088	4014	3534	4439	4872	4200	3144	5698	4458	7555	3683	5738
40S ribosmonal protein S11	6030	4681	5010	3891	4347	4052	4918	4035	5585	6641	4942	4964
40S ribosmonal protein S18-like isoform 1	5817	4761	4520	3474	5065	4422	4029	3329	4490	5752	4786	3819
60S ribosmonal protein l13a	5727	4138	4316	3203	4454	4170	4364	3419	4197	4927	4440	3268
60S ribosmonal protein L23	5598	3288	3420	2714	3984	3620	2482	3349	3903	6375	4570	4469
Macaca mulatta hypothetical protein LOC698789	5498	3597	3294	2075	3688	3761	3093	2147	3198	3976	2914	2806
60S ribosmonal protein L37a-like	5493	3450	3423	2812	4646	3508	2890	3175	3563	5460	1992	3717
60S ribosmonal protein L31-like	5075	3553	3110	2754	4142	3726	3102	2601	3790	4109	4192	3047

Fig. 1. The RPKM value of top 20 most highly expressed transcripts in sika deer antler transcriptome. A1-A6: uniseriate antlers at ~15 day, 30 day, 45 day, 60 day, 75 day, 90 day of growth. B1-B4: two-branched antlers at ~45 day, 60 day, 75 day, 90 day of growth. C1-C2: three-branched antlers at ~75 day, 90 day of growth.

participate in cytoskeleton modulation and organization, cell proliferation, anti-apoptosis, angiogenesis, cell morphology and motility (Santelli et al. 2009).

Altered Tβ10 expression has been associated with various cancers. Compared with adjacent normal tissues, Tβ10 expression is up-regulated in human pancreatic cancer (Li et al. 2009b), non-small cell lung cancer (Gu et al. 2009), and hepatocellular carcinoma (Wang et al. 2014) among others. However, in ovarian cancer and metastatic cervical carcinoma, the expression of Tβ10 is down regulated (Lee et al. 2001, Hang et al. 2011). Thus, Tβ10 could be used as a prognostic cancer marker. Tβ10 also plays a critical role in the developing tissues. Tβ10 mRNA levels are significantly up-regulated during the early stage of mouse embryonic postimplantation (Carpintero et al. 1996) and are primarily localized in neuronal cells (Anadon et al. 2001). High levels of Tβ10 are also found in the human fetal brain (Hall et al. 1990), human newborn salivary glands (Fanni et al. 2011), developing kidneys (Gerosa et al. 2010) and marrow mesenchymal stem cells (MSCs) (Kalwitz et al. 2009). During inflammation or an immune response, Tβ10 expression is up-regulated (Nakanishi et al. 2005).

Sika deer (*Cervus nippon hortulorum*) velvet antler, produced in Jilin Province of China, is one of the most popular traditional Chinese medicines in Asia. The main biological characteristic of velvet antler is its rapid growth, and various growth factors are abundantly expressed or show increased biological activity. In various countries of Asia, deer antler has been used for centuries and clinical observations showed multiple functions, including strengthen bones (Wu et al. 2013). Meanwhile, Antlers are the only mammalian organs that are capable of regeneration (Li et al. 2009a, Kierdorf et al. 2011). Because of the aforementioned factors, we aimed to investigate the important activity factor in deer antler. During transcriptome database analysis, we identified that *Tβ10* was highly expressed in different growth phases of sika deer antler (Fig.1). We speculated that Tβ10 may play an important role in the bone growth. Then we determined and cloned the sika deer Tβ10 gene. This gene was expressed in *Escherichia coli* and the soluble protein was efficiently purified. Cell proliferation analysis confirmed its proliferation activity on MC3T3-E1 mouse osteoblast cells. These results provide the theoretical basis for antler growth and pharmacological research.

Materials and Methods

Strains, plasmids, enzymes and reagents

Plasmid PET-28a, competent *E. coli* cells DH5 and BL21 (DE3), restriction endonucleases *Nco* I and *Xho* I, Tag DNA polymerase, T4 DNA ligase, pMD18-T, DNA ladder and protein ladder were purchased from TAKARA Biotechnology Co., Ltd. (Dalian, Liaoning, China). Anti-His antibody was purchased from BioLegend (Emeryville, CA, USA). All chemicals used in this study were of analytical grade. Transcriptome sequencing was performed by BGI (Shenzhen, China).

Antler tissue sampling

The different stage antler harvested at the incipient stage (5 cm long on the top of a full grown pedicle from a 4-5 years old hypnotic sika deer) was collected in a sika deer farm (Changchun, Jilin Province, China). The antler tip (distal 2 cm) was cut into 5-mm thick slices and then was further cut into strips 1 cm across. The central pieces were quickly frozen in liquid nitrogen and then RNA was extracted for transcriptome sequence.

Expression plasmid constructs

After isolating the total RNA from antler (A1 stage) growth centre by using TRIzol (Invitrogen, Carlsbad, CA, USA), the cDNA was synthesized by RT-PCR. The *Tβ10* gene was amplified by PCR with the following nucleotide primers: forward 5'-CCATGGCAGACAAGCCCGACAT-3' and reverse 5'-CTCGAGTCACTTTGCTTGCTTCTCCT-3'. The products were inserted into the cloning vector pMD18-T and then digested with *Nco* I and *Xho* I. The products with sticky ends were inserted into the expression vector pET-28a (+).

Expression and purification of recombinant sdTβ10

Plasmid pET-28a-sdTβ10-His_6 was transformed into *E. coli* BL21 (DE3). Then the bacteria were induced to express recombinant protein by adding various concentrations isopropyl-β-D-thiogalactopyranoside (IPTG) to a culture with an OD_{600} of 0.6-0.8 and incubating for different times at 37°C. The cells were harvested by centrifugation at 5000×*g* for 2 min. A portion of the pellet was prepared for SDS-PAGE analysis and the optimum pellet was washed with 100 mM Tris-Cl (pH 6.5) twice. Then, the cells were resuspended in lysis buffer (50 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, pH 6.5, 0.5 mg/m*l* lysozyme and 0.13 mM PMSF). After mixing for 10 min on ice, sodium deoxycholate (1.33 mg/m*l*) was added and the suspension was mixed for 20 min at 37°C. Then DNase I was added to a concentration of 2000 U/m*l* at 37°C with shaking at 150 rpm for 2 hr. The sample was then centrifuged at 12,857×*g* for 20 min at 4°C.

The suspension was passed through a 0.22 μm filter and then applied to a 1 m*l* HisTrap™ FF nickel ion affinity column (GE Healthcare) according to the manufacturer's instructions. After washing the column with binding buffer (100 mM Tris-Cl, 200 mM NaCl, 10% glycerin, 15 mM imidazole, pH 6.5), the fusion protein was eluted with elution buffer containing different concentrations imidazole at a flow rate of 1 m*l*/min. The elution sample was collected and injected into Sephadex G-25 medium for desalting at room temperature. The column was equilibrated with ultrapure water. The sdTβ10-His_6 was eluted at a flow rate of 1 m*l*/min and the eluate was monitored at 280 nm. The recombinant protein was freeze-dried and stored at -20°C prior to use. The protein concentration was determined by using a Bradford protein assay kit and BSA was used to construct the standard curve. The purity of sample was estimated by densitometric measurement of the proteins on SDS-PAGE using Dolphin-1D Gel Analysis Software (Wealtec, Sparks, NV, USA).

Mass spectrum analysis

A total of 1 mg frozen dry protein was dissolved in 1 m*l* buffer (water/methanol: 50/75) and mass spectrometric detection was carried out on a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an ESI source in positive ion mode. Nitrogen was supplied as the sheath gas and auxiliary gas at flow rates of 0.6 and 3 *l*/min, respectively. Nitrogen was also used as the collision gas, and the normalized collision energies were optimized to 45~55%. The capillary temperature was set at 320°C for all experiments. The optimized spray voltage and S-lens voltage was 3000 and 35 V, respectively. The Q Exactive mass spectrometer was operated in data-dependent acquisition mode using Xcalibur 2.2 software, and a single full-scan mass spectrum was collected using the Orbitrap (100-2000 m/z, 70,000 FWHM) passed through a 0.22 μm filter.

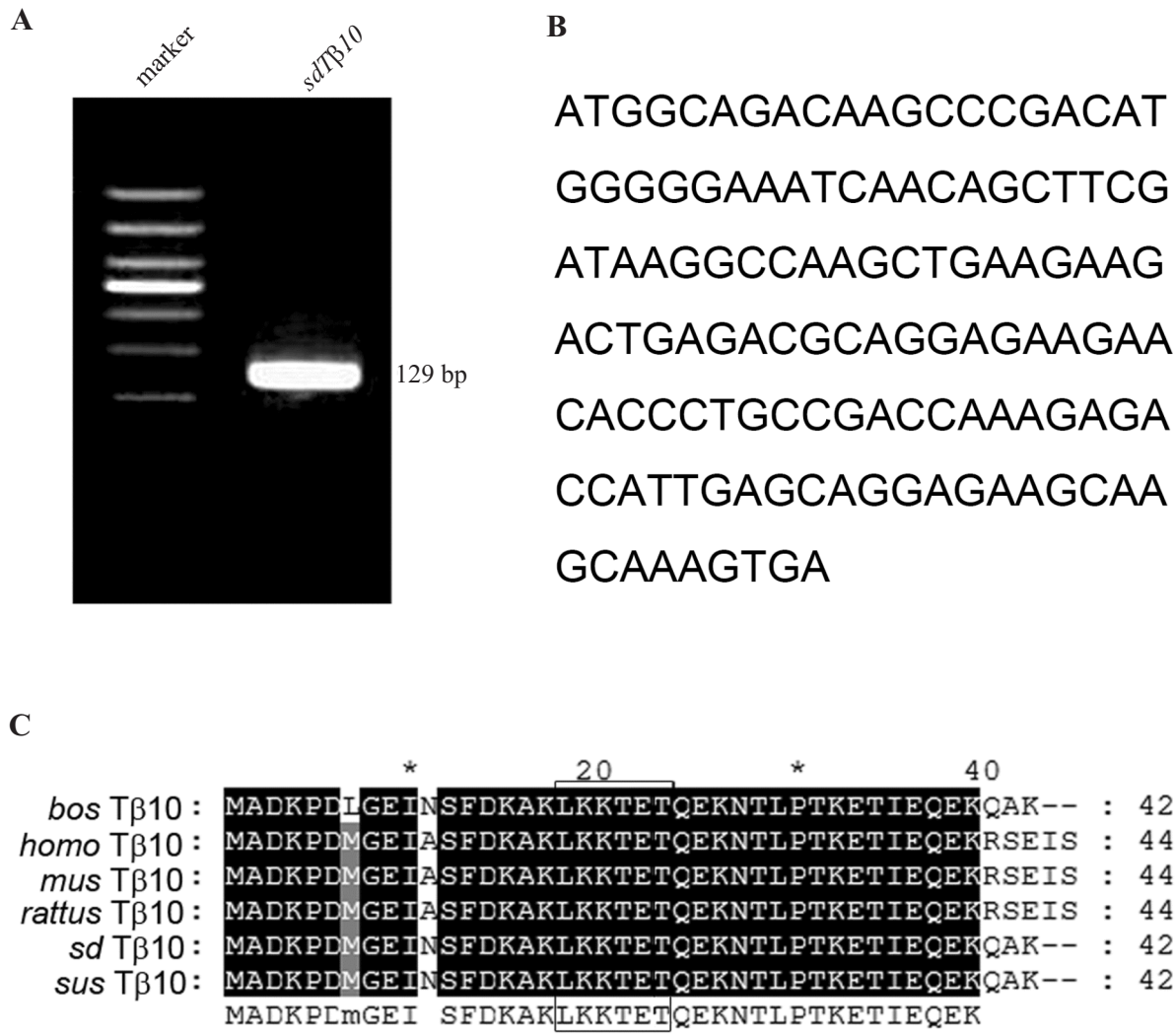

Fig. 2. Detection of the sika deer *T*β10 cDNA and sequence analysis. (A) PCR amplification of sika deer *Tβ10* by RT-PCR from total mRNA of antler (A1 stage). (B) Nucleotide sequence of sika deer *Tβ10* cDNA. (C) Alignment of the amino acid sequences of the sdTβ10 and Tβ10 from other species. Predicted actin-binding domain is shown in the black box.

Biological activity assay

The mouse osteoblast cell line MC3T3-E1 (ATCC, CRL-2593) was maintained in α-Modified Eagle medium supplemented with 100 μg/m*l* streptomycin, 100 U/m*l* ampicillin and 10% fetal bovine serum at 37°C and 5% CO_2. MC3T3-E1 cells at a density of 1×10^4 cells/m*l* were cultured in a 96-well plate for 24 hr. The culture medium was removed and cells were incubated for 24 hr in α-MEM with 0.5% fetal bovine serum. The cells were supplemented with recombinant sdTβ10-His_6 or sdTβ10-His_6 ΔL-T protein at different concentrations (from 0 to 64 μg/m*l*) and incubated for 48 hr. MTT (3-(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-diphenytetrazoliumromide) was added to cells for 4 hr at 37°C and 5% CO_2. After removing the medium, DMSO was added to the wells and they were kept at room temperature for 8 min. The absorbance was measured at a wavelength of 590 nm by using a microplate reader (Tecan Infinite 200PRO).

Sequence accession number

The nucleotide sequence of sika deer *Tβ10* identified in this study was submitted to GenBank with the accession number KY216190.

Results

Characterization and cloning of sdTβ10-His_6

In the sika deer antler transcriptome database, *Tβ10* was one of the 20 genes that RPKM (reads per

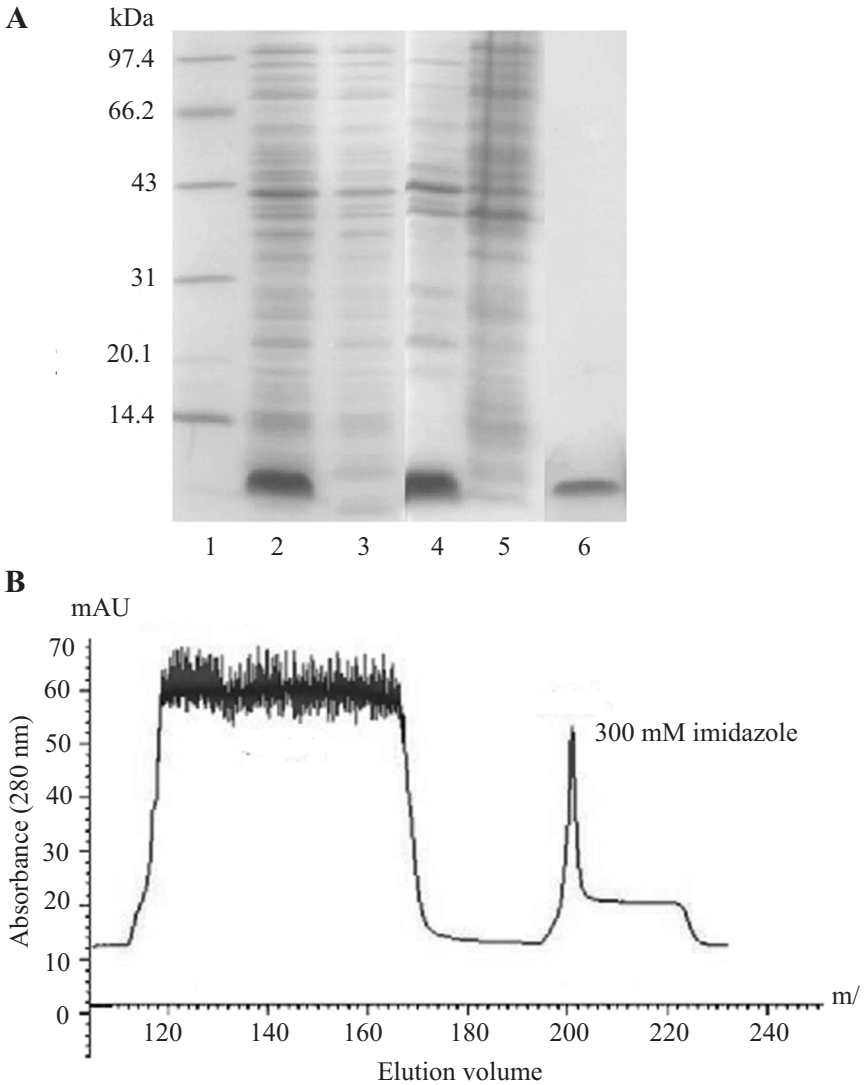

Fig. 3. Expression and purification of sdTβ10-His_6. (A) Samples collected from the purification steps of sdTβ10-His_6 were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1: protein marker; Lane 2: with IPTG induction; lane 3: without IPTG induction; lane 4: soluble protein fraction; lane 5: insoluble protein fraction; lane 6: purified fraction eluted with 300 mM imidazole. (B) Elution profile of sdTβ10-His_6 purified on Prodond nickel resin. The column was washed with wash buffer containing 15 mM imidazole. The protein was eluted with wash buffer with 300 mM imidazole.

kilobase per million mapped reads) (Li et al. 2015) value over 5000 in A1 stage (Fig. 1). Meanwhile, the RPKM value of *Tβ10* was continuous high in different growth phases. We predicted the sequence of the sika deer *Tβ10* gene from the transcriptome database. The *sdTβ10* cDNA was amplified and a 129 bp fragment was separated via 1.2% agarose gel electrophoresis (Fig. 2A). The purified PCR product was inserted into pMD18-T for DNA sequencing (Fig. 2B). The sdTβ10 is a 42 amino acid long protein with a molecular mass of 4.8 kDa and theoretical pI of 6.42. Its secondary structure was predicted to consist of 64.3% alpha helices, 9.5% beta sheet. 14.3% beta turn and and 11.9% random coils. The protein also has the conserved domain 18LKKTET23 that is critical for binding to G-actin. By sequence alignment analysis, the closest homologs of sdTβ10 were identified. sdTβ10 shares 97.6% and 100% identity with the Tβ10 from *Bos taurus* and *Sus scrofa*, respectively (Fig. 2C). The *sdTβ10* coding sequence was cloned into the prokaryotic expression vector, pET28a, and a C-terminal 6×histidine tag was added to the protein. Due to the actin-binding site has several biological functions beyond actin-binding, a mutant gene of deleted conserved domain (LKKTET) was synthesized and also cloned into pET28a.

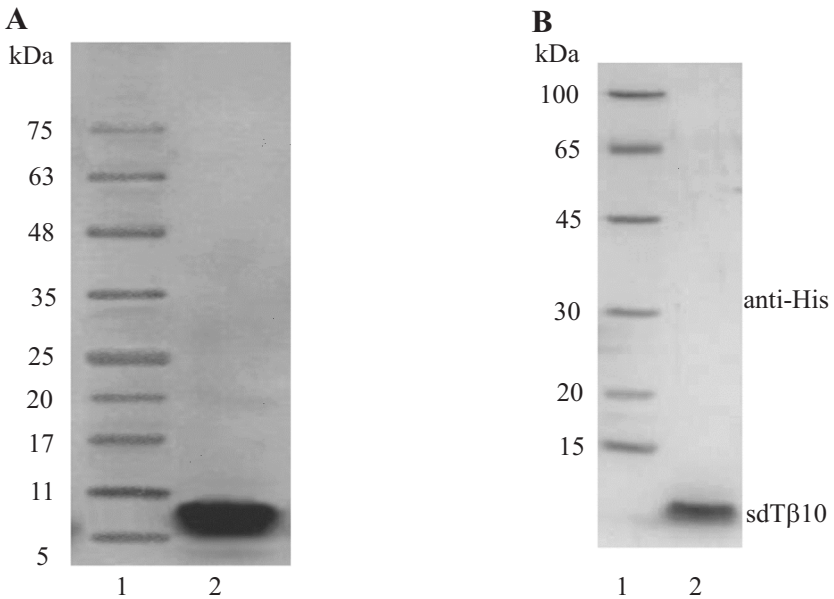

Fig. 4. SDS-PAGE and western blot analysis of sdTβ10-His_6 after desalting. (A) Samples collected from the desalting procedures were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining. (B) Samples collected from the desalting procedures were analyzed by western blot.

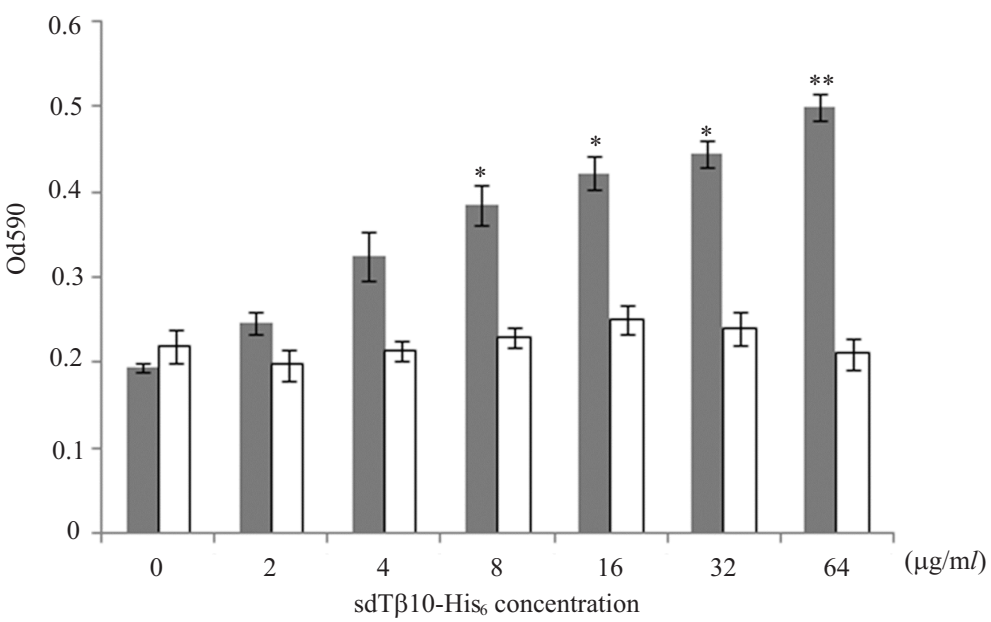

Fig. 5. Effects of different concentrations of sdTβ10-His_6 (gray) or sdTβ10-His_6 ΔL-T (white) on MC3T3-E1 cell proliferation. T n = 3, p<0.01(*), p<0.001(**), Student's t-test. Error bars in panels are defined as mean ± S.D.

Expression and purification of sdTβ10-His_6 protein

After optimizing, a 0.8 mM concentration of IPTG and induction at 37°C for 4 hr were considered to be the optimal conditions for sdTβ10-His_6 expression (Fig. 3A, lane 2). The sdTβ10-His_6 protein could be detected as a prominent band by Coomassie Blue staining by SDS-PAGE. Results indicated that the soluble protein was highly expressed (Fig. 3A, lane 4). The sdTβ10-His_6 recombinant protein was purified by affinity chromatography. The soluble protein bound to the beads effectively and was eluted with 300 mM imidazole (Fig. 3A, lane 6). The elution profile of the sdTβ10-His_6 measured at 280 nm is shown in Fig. 3B. The sample was loaded onto a G-25 column for desalination and was freeze-dried to concentrate it. The purified protein was analyzed by SDS-PAGE with

Coomassie Brilliant Blue staining (Fig. 4A). The final yield of the purified protein was about 28 mg/*l* and the total purity of the sdTβ10-His_6 prepared using this method was >90%. The sdTβ10-His_6 ΔL-T mutant was expressed and purified with the same procedure.

Identify of recombinant sdTβ10-His_6

Verification of the identity of the recombinant sdTβ10-His_6 protein after desalting was done by western blot (Fig. 4B). Including the polyhistidine tag, the calculated molecular mass of the recombinant sdTβ10-His_6 was 5929.44 Da. The sdTβ10-His_6 was analyzed by mass spectrometry to precisely determine its exact molecular weight. The molecular mass established for sdTβ10-His_6 was 5975.99 Da, which is within the range of the calculated value.

Biological activity of sdTβ10-His_6 protein

Antlers contain cartilage, bone, epidermis, blood vessels, nerves and other tissues. The biological activity of the sdTβ10-His_6 protein was determined to assess whether it could promote MC3T3-E1 cell proliferation. The results from the MTT assay showed that cell proliferation was enhanced after treatment with different concentrations (0 μg/m*l*~64 μg/m*l*) of sdTβ10-His_6 (Fig. 5). The rate of cell proliferation was 1.20 ~2.56 times higher than that of the negative control and sdTβ10-His_6 ΔL-T mutant. The p-values for the comparison between the control and the cells treated with 8, 16, and 32 μg/m*l* sdTβ10-His_6 were < 0.01 and those treated with 64 μg/m*l* sdTβ10-His_6 were < 0.001. These results indicated that recombinant sdTβ10 protein stimulated osteoblasts proliferation.

Discussion

Recently, transcriptome sequencing technology has emerged as a powerful tool for research traditional Chinese medicine (TCM). In sika deer antler transcriptome research, we found that Tβ10 was highly expressed in different growth phases. This was consistent with previous reports. Tβ10 was reported to be highly expressed in different tissue of red deer (*Cervus elaphus*) antler tip. The abundant expression of Tβ10 may be related to a role in the development of blood vessels and cartilage within antlers (Ded-Choudhury et al. 2015). However, Tβ10 in sika deer have not been reported. In this study, we have first cloned the *Tβ10* cDNA from sika deer antler by RT-PCR, and analysis of the sequence alignment demonstrated that Tβ10 was over 90% homologous with those from other mammals. *Tβ10* cDNA was introduced into the pET28a expression vector and optimal induction conditions were determined to obtain the highest possible yield. An apparent pure protein sample was obtained after one step affinity purification. MTT results showed that the recombinant Tβ10 protein promotes osteoblasts proliferation *in vitro*.

Deer antler is the only mammalian organ which can continuously regenerate, although the exact mechanism is not clear. Now, we considered that the high expression of Tβ10 in sika deer antler could promote bone cells proliferation and have positive activity on antler rapid growth. Meanwhile, Tβ10 was also highly expressed in MSCs, $CD34^+$ and $CD15^+$ cells, indicating that antler cells may have part capacity of MSCs. For thousands of years antlers have been used in Asian countries to promote rapid healing, treat developmental retardation, and strengthen weak bones. The active compounds of antler contain collagen, variety of polypeptides, and other constituents. Tβ10 is a new effect constituent of sika deer antler which can promote osteogenic growth.

Properties of the polypeptide include low immunogenicity, low molecular weight, strong activity and low cytotoxicity and thus it was widely used in the field of medicine. Our results complete the basic study of sdTβ10 expression and purification, which laid the foundation for orthopedics clinical application in the future. In addition, these results were useful for the research on the rapid growth of antler (such as cell proliferation, angiogenesis and cartilage development).

Acknowledgments

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