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Recombinant expression and purification of heparin binding proteins: Midkine and pleiotrophin from *Escherichia Coli*

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ABSTRACT

Midkine (MDK) and Pleiotrophin (PTN) belong to a class of heparin-binding growth factors and are highly expressed in a number of cancers. Bioactive and recombinant MDK and PTN are critical reagent for cancer drug discovery studies. MDK and PTN belong to a newly evolving family of secreted neurotrophic and developmentally regulated heparin-binding molecules. PTN is related to MDK with 45% sequence identity and both proteins have been shown to be involved in promoting neurite outgrowth. MDK is a cysteine-rich 13 kDa protein containing five disulfide bonds and PTN is 19 kDa protein containing ten disulphide bonds. In this study, we expressed recombinant human MDK (rhMDK), mouse MDK (rmMDK) and human pleiotrophin (rhPTN) in *Escherichia coli BL21(DE3)pLysS* strain. Soluble rhMDK, rmMDK and rhPTN were expressed at a high-level in this strain and the protein was purified (~90%) by a one-step purification using heparin affinity chromatography. A total of 4 mg purified MDK and 7 mg of purified PTN were obtained with the overall yield from 1 L of bacterial culture. Activity of purified rhMDK and rhPTN was confirmed by a cell proliferation assay using NIH3T3 cells.

Introduction

Midkine (MDK)[1] and pleiotrophin (PTN) promote the growth, survival, and migration of various cells, and play roles in neurogenesis and epithelial mesenchymal interactions during organogenesis [1]. MDK belong to a newly evolving family of secreted neurotrophic and developmentally regulated heparin-binding molecules [2], which also includes PTN. MDK and PTN are low molecular weight proteins with closely related structures. PTN is related to MDK with 45% sequence identity and both proteins have been shown to be involved in promoting neurite outgrowth [3,4]. Moreover, both PTN and MDK have 10 conserved cysteine residues and their corresponding disulfide bonds are important for their functions [5]. They are mainly composed of two domains held by disulfide bridges, and there are three antiparallel β-sheets in each domain [6,7]. Various studies have shown that MDK and PTN are involved in mitogenesis, transformation, survival, migration and angiogenesis [7,8]. Furthermore, MDK and PTN are over-expressed in a number of human cancers [7,8]. MDK and PTN share receptors, and show similar biological activities that include fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic, and chemotactic ones. MDK is detected in human carcinoma specimens from pre-cancerous stages to advanced stages. Strong expression of PTN is also detected in several carcinomas, although, in general, MDK is expressed more intensely and in a wide range of carcinomas than PTN. Thus, it is a good market for evaluating the progress of carcinomas. Furthermore, antisense oligonucleotides for MDK and ribozymes for PTN show anti-tumor activity.

Therefore, MDK has become an attractive target for developing cancer therapeutics. Active recombinant human MDK (rhMDK), mouse MDK (rmMDK) and human pleiotrophin (rhPTN) is a critical reagent for cell assay development as well as generation of antibodies for therapeutic drug discovery. Most of the biological activities of MDK and PTN, heparin-binding function are located at the C-terminal domain. Expressing and purifying such a protein may be challenging, mainly because the disulfide bonds have to be properly formed to preserve its activity. Traditionally, proteins with disulfide bonds are purified under denaturing environment and then refolded [9,10]. There is only a single report that rhMDK was expressed in *Escherichia coli* as soluble expression [2] and many reports of MDK recovery from inclusion body with denaturing and reducing reagents are available [11–14]. However there are no reports on recombinant expression of PTN as soluble protein in *E. coli*.

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[1] *Abbreviations used:* MDK, midkine; rhMDK, recombinant human MDK; rmMDK, recombinant mouse MDK; PTN, pleiotrophin; rhPTN, recombinant human PTN; LB, Luria broth; IPTG, isopropyl thio-β-D-galactosidase; TG, Tris–glycine; PAGE, polyacrylamide gel electrophoresis; CV, column volumes; SEC, size exclusion column; PBS, phosphate buffered saline; CD, circular dichroism.

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The proteins were refolded during dialysis by gradually decreasing the concentration of denaturing and reducing reagents. Refolded rhMDK was then further purified by heparin chromatography. Soluble rhMDK and rhPTN expressed and purified from yeast *Pichia pastoris* has also been reported [14,15]. The purpose of our study was to establish an expression system and a purification strategy for recombinant MDK's and PTN. In this way, recombinant MDK's and PTN protein generation will be more straightforward while preserving its native conformation and biological activities.

In this study, we show that high-level expression of soluble active PTN and MDK was readily achieved using *BL21(DE3)pLysS* strain. rhMDK, rmMDK and rhPTN proteins were obtained with high purity by heparin affinity chromatography without refolding. Secondary structure of recombinant untagged native rhMDK and rhPTN were also analyzed by circular dichrosim (CD). Activity of purified rhMDK and rhPTN were also confirmed by cell proliferation assay using NIH3T3 cells.

Materials and methods

Expression of rhMDK, rmMDK, rhPTN

Nucleotide residue 67–432 (Amino acid 21–143) of human MDK (Entrez Gene ID 4192), 421–774 residues (Amino acid 23–140) of *Mus musculus* Mdk (NCBI Reference Sequence: NM_010784.4) and 460–867 residues (Amino acid 33–168) of *Homo sapiens* PTN (NCBI Reference Sequence: NM_002825.5) which corresponds to full-length gene minus the signal peptide sequence, was cloned via NdeI/XhoI sites into the pET30b vector (Novagen, Gibbstown, NJ). *E. coli* strains, *BL21(DE3)pLysS* (Stratagene, La Jolla, CA) were used for recombinant protein expression. *BL21(DE3)pLysS* is kanamycin sensitive, allowing selection and expression of recombinant plasmids which carry the kanamycin resistance gene. For expression in *BL21(DE3)pLysS*, the cells were cultivated at 37 °C in Luria Broth (LB) media containing (50 μg/ml) kanamycin, chloramphenicol (10 μg/ml) as a 5 ml culture overnight. Small scale expression was done at 18 °C overnight at different induction concentrations of IPTG, 0.5, 1, 2 and 4 mM when culture reached A_{600}nm 0.8. Large scale fermentations were performed with 50 ml starter culture grown overnight was inoculated in 1000 ml fresh LB media supplemented with (50 μg/ml) kanamycin, chloramphenicol (10 μg/ml), 0.5 mM IPTG when culture reached A_{600}nm 0.8. The cultures were induced at 18 °C overnight. Cells were then harvested by centrifugation at 4 °C and cell pellets were kept at −80 °C.

Analysis of rhMDK, rmMDK, rhPTN expression

To analyze the expression of recombinant proteins in the soluble fraction, 5 ml culture from each of these three *E. coli* constructs were centrifuged at 12,000 rpm for 15 min at 4 °C. Each cell pellet was suspended in buffer composed of 0.125 M Tris HCl, 10% 2 β-mercaptoethanol, 20% glycerol, 0.004% bromphenol blue, pH of approximately 6.8. Total cell lysates and soluble proteins were analyzed by SDS–PAGE and Western blot. SDS–PAGE was done according to the method of Laemmli [16]. Total cell lysates and purified proteins were treated with sample buffer in the presence of 2% β-mercaptoethanol. Samples were boiled for 5 min and electrophoresed on 15% polyacrylamide gels. Protein bands were visualized by Coomassie blue staining.

Purification of recombinant PTN and MDK

A total of 5 g wet weight of frozen cells from a 1 L *BL21(DE3)-pLysS* culture expressed at 18 °C were thawed on ice and resuspended in 200 ml of lysis buffer containing 20 mM Tris, pH 7.5, 200 mM NaCl, 5% glycerol, 200 μg/ml lysozyme, complete protease inhibitor cocktail tablet (Roche). After sonication on ice for 5 min the lysate was clarified by centrifugation at 18,000 rpm in Kubota rotor for 30 min. The supernatant was filtered through a 0.2 μm pore size filter apparatus (Nalgene, Rochester, NY). Single 5 ml Heparin HP columns (GE Healthcare, India), on Biologic Duoflow (BIORAD), were pre-equilibrated with 20 mM Tris, pH 7.5, 200 mM NaCl and 5% glycerol (Equilibration buffer) prior to loading the filtered supernatant. Flow rate was set at 5 ml/min throughout the run. Protein flow through was collected as the supernatant passed through the columns. After loading was completed, columns were washed with equilibration buffer for at least ten column volumes (CV) until UV 280 nm absorbance (A280 nm) became stable at baseline. Gradient elution from 0% to 100% of elution buffer (20 mM Tris, pH 7.5, 1 M NaCl and 5% glycerol) was applied for 10 CV. Protein fractions were collected at 5 ml each, and analyzed by SDS–PAGE using 15% gel. Tris–glycine (TG) gel was used for SDS–polyacrylamide gel electrophoresis (PAGE). MDK and PTN containing fractions were pooled and concentrated using Amicon Ultra centrifugal filter devices 5000 MWCO (Millipore, India). The concentrated pool was then injected on a size exclusion column (SEC) Superdex 75 16/60 (GE Healthcare), which was equilibrated in phosphate buffered saline (PBS). Flow rate was set at 1 ml/min throughout the run. SEC fractions were collected at 5 ml each and analyzed by SDS–PAGE on 15% gel. MDK fractions were pooled and concentrated to 1 mg/ml in PBS buffer. Aliquots of final MDK and PTN product were stored at −80 °C. Total protein concentration was quantified by Bradford assay using Bradford Assay Kit (Bio-Rad) according to the manufacturer's recommendation. Bovine serum albumin (SIGMA) was used as standard and purification buffer as blank. Protein samples, standard and blank were performed in duplicate.

Circular dichroism spectroscopy

CD spectra were measured over the range of 190–250 nm by using a JASCO 810 spectropolarimeter thermostatted with a water bath. The measurements were carried out in 1 mm path length cells and protein solutions of approximately 0.3 mg/ml in PBS buffer. All spectra were measured at 25 °C with a 16 s time constant and a scan rate of 10 nm/min. The spectral slit width was 1.0 nm. All measurements represent the computer average of three scans.

Biological activities

One of the biological activities of correctly folded PTN and MDK is to promote cell growth. To assay this activity, NIH3T3 cells were plated into 96-well plate (5×10^3 cells/well in 0.1 ml medium) with Dulbecco's Modified Eagles Medium (DMEM) containing 1% (v/v) fetal bovine serum (FBS) and cultured at 37 °C in a 5% (v/v) CO_2 for 24 h. The cells were washed once with serum-free DMEM, and the medium was subsequently replaced with DMEM containing different concentrations of rhMDK and rhPTN. The effect of rhMDK and rhPTN on cell proliferation were determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich Chemical) cell viability and proliferation assay Mosmann [17]. Briefly, after 3-d incubation, 20 μl of 0.5 mg/ml MTT solution was added to each well. After incubation at 37 °C in a 5% (v/v) CO_2 for 4 h, 150 μl of medium was removed from each well and replaced with an equal volume of dimethyl sulfoxide (DMSO) to dissolve the MTT crystals. The 96-well multiplates were