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# Expression and purification of bioactive high-purity human midkine in *Escherichia coli*[*]

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**Abstract:** Midkine is a heparin-binding growth factor, which plays important roles in the regulation of cell growth and differentiation. The non-tagged recombinant human midkine (rhMK) is therefore required to facilitate its functional studies of this important growth factor. In the present work, rhMK was expressed in *Escherichia coli* (*E. coli*) BL21 (DE3). The expression of midkine was efficiently induced by isopropyl-β-D-thiogalactopyranoside (IPTG). After sonication, midkine was recovered in an insoluble form, and was dissolved in guanidine hydrochloride buffer. Renaturation of the denatured protein was carried out in the defined protein refolding buffer, and the refolded protein was purified using S-Sepharose ion-exchange chromatography. The final preparation of the rhMK was greater than 98% pure as measured by sodium dodecylsulfate-polyacrylamid gel electrophoresis (SDS-PAGE) and reverse phase high performance liquid chromatography (RP-HPLC). The purified rhMK enhanced the proliferation of NIH3T3 cells.

**Key words:** Expression, Purification, Human midkine, *Escherichia coli*
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## INTRODUCTION

Midkine is one of the developmentally regulated heparin-binding growth factor. It plays important roles in various biological process, including neuronal survival and neurite outgrowth (Kaneda *et al*., 1996; Muramatsu *et al*., 1993), carcinogenesis (Choudhuri *et al*., 1997; Kadomatsu *et al*., 1997), and tissue remodeling (Ohta *et al*., 1999; Yoshida *et al*., 1995). Midkine is strongly expressed during the mid-gestation period of mouse embryogenesis, especially when the epithelial-mesenchymal interaction takes place (Mitsiadis *et al*., 1995). As embryogenesis proceeds, midkine expression progressively decreases in most tissues. Although midkine is extensively expressed in a wide variety of human cancers and other neoplasms, its expression in normal adult tissues is rather restricted (Garver *et al*., 1993; 1994). In adult mice and humans, midkine is only expressed in the kidney, testis, stomach, and small intestine (Muramatsu *et al*., 1993; Nakagawara *et al*., 1995; Nakamoto *et al*., 1992). Human midkine comprises 121 amino acids and has a calculated molecular mass of 13240 Da (Kurtz *et al*., 1995; Muramatsu, 1994). It contains two domains with five intra-domain disulfide bridges (Fabri *et al*., 1993). The domain structures of N- and C-terminal halves of human midkine molecule have been chemically synthesized and analyzed in aqueous solutions using nuclear magnetic resonance (NMR) (Iwasaki *et al*., 1997). The amino acid sequence of human midkine has about 50% homology with that of human pleiotrophin, and these two proteins form a new protein family (Kurtz *et al*.,

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1995; Muramatsu, 1994).

As broad functions of midkine have been described in the research areas of cancer, inflammation, and degenerative diseases (Kadomatsu and Muramatsu, 2004), available simple method is urgently needed to produce large quantity of the recombinant protein for in vivo animal studies to reduce the high cost of the commercial products. The *Escherichia coli* (*E. coli*)-derived recombinant midkine is commercially available, demonstrating the success of *E. coli* expression system in making the protein.

In the present work, the recombinant human midkine (rhMK) expressed as inclusion bodies in *E. coli* was solubilized and renatured by using the dilution method. Final purification of the protein was done by one-step of ion-exchange chromatography with a purity of >98%. The biological activity of the purified protein was determined by the proliferation of NIH3T3 cells.

## MATERIALS AND METHODS

### Construction of rhMK expression vector

The plasmid pET30a(+) and strain *E. coli* BL21 (DE3) (Novagen, USA) were used as expression vector and recombinant host strain, respectively. Reverse-transcription kit and high-quality *pfu* polymerase were purchased from TaKaRa Company (Dalian, China). The human midkine gene was cloned from the cDNA library, which was reversely transcribed total RNA of human bone marrow cells. The mature human midkine protein was encoded by the full-length sequence from 289 to 654 bp (GenBank accession No. NM_001012334) without the signal peptide coding sequences. According to the coding sequence, the polymerase chain reaction (PCR) primers were designed as follows: 5′-GGAATTC CATATGAAAAAGAAAGATAAG-3′ as the forward primer, and 5′-CCCAAGCTTAGTCCTTT CCCTTCC-3′ as the reverse primer (Fig.1). The *Nde*I and *Hin*dIII sites (underlined) were incorporated into primers to facilitate directional cloning of the PCR product into *Nde*I-*Hin*dIII sites of the expression vector pET30a(+). To avoid the His-tag of the vector, the reverse primer contains the stop codon of the gene. After PCR amplification, 5 μl of each reaction mixture was run on 1.2% (w/v) agarose gel to confirm the size and purity of PCR products. The DNA was purified with a DNA gel extraction kit following the protocol provided by the supplier (TaKaRa, Dalian, China). The purified PCR product and expression vector pET30a(+) were digested with *Nde*I and *Hin*dIII (TaKaRa, Dalian, China) overnight, then gel-purified and ligated together. The resulting plasmid was designed as pET30a-rhMK and transformed into *E. coli* BL21 (DE3). The transformed cells with recombinant plasmid were plated on Luria-Bertani (LB) containing 100 μg/ml kanamycin and 1.5% (w/v) agar and incubated at 37 °C for 12 h. The positive clones were selected, analyzed by restriction enzyme digestion, and finally confirmed by DNA sequencing.

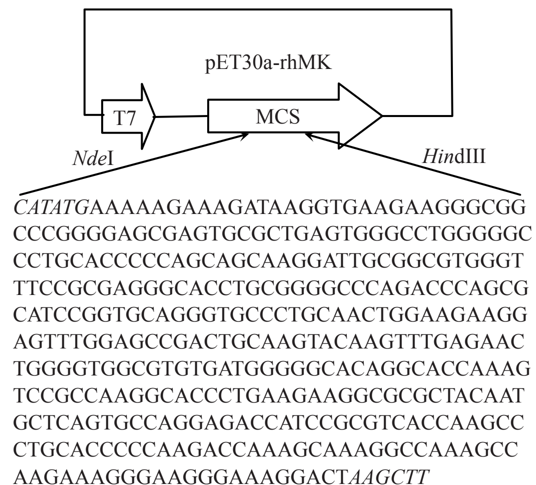


**Fig.1 Construction of the recombinant expression plasmid pET30a-rhMK. The human midkine gene was inserted into pET30a vector. The forward and reverse primers contain *Nde*I site and *Hin*dIII site (italic letters), respectively. T7: T7 promoter; MCS: multiple cloning sites**

### Expression of rhMK

The recombinant strain *E. coli* BL21 (DE3) containing the plasmid pET30a-rhMK was cultured in the LB liquid medium containing 100 μg/ml kanamycin with 250 r/min shaking at 37 °C overnight. 100 μl of culture was subcultured into 3 ml fresh LB liquid medium supplemented with 100 μg/ml kanamycin in a 15-ml tube. The culture was grown with 250 r/min shaking at 37 °C until the culture density, measured by the absorbance at 600 nm ($OD_{600}$), reached 1.0. The rhMK gene expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/L at 42 °C, and 3 h later

protein extract prepared from the culture was analyzed by 15% (w/v) sodium dodecylsulfate-polyacrylamid gel electrophoresis (SDS-PAGE), and then stained with Coomassie brilliant blue R250 (Sigma, USA).

**Preparation of inclusion bodies**

400 μl of LB medium, supplemented with 100 μg/ml kanamycin, was inoculated with an overnight starter culture ($OD_{600}$<0.1). Cultures were grown at 37 °C with 250 r/min shaking to $OD_{600}$ of 0.8~1.0 and induced with 1 mmol/L IPTG (final concentration) at 42 °C for 3 h. Cells were harvested by centrifugation at 10000×*g* for 15 min. Cell pellet was resuspended in the lysis buffer (phosphate buffered saline (PBS) containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and 0.1 mmol/L phenylmethylsulphonyl fluoride (PMSF), pH 7.4) at a final concentration of 50 mg/ml and sonicated (Sanyo Soniprep 150, 15 bar; 30 s working and 30 s resting on ice for a cycle, 35 cycles). After centrifugation at 12000×*g* for 15 min at 4 °C, the undissolved inclusion bodies were collected, washed with the pellet wash buffer (PBS containing 0.5% (v/v) Triton X-100, 10 mmol/L EDTA and 50 mmol/L NaCl, pH 7.4), and centrifuged at 12000×*g* for 5 min at 4 °C. The inclusion bodies were then solubilized in Buffer G (20 mmol/L $Na_2HPO_4$, 1 mmol/L EDTA, 50 mmol/L NaCl, and 0.1 mmol/L PMSF, pH 8.0) containing 6 mol/L guanidine hydrochloride (GdnHCl) per gram pellet, shaking gently at room temperature for 1 h. The insoluble pellets were removed by centrifugation at 15000×*g* for 20 min at 4 °C, and the dissolved inclusion bodies were prepared for the following purification.

**Refolding and purification of rhMK**

The protein refolding was proceeded by drop-wise dilution into defined protein folding buffer. The protein solution was dropped by pumping into 10-fold volume refolding buffer (20 mmol/L $Na_2HPO_4$ and 1 mmol/L EDTA, pH 7.4) under vigorous (magnetic stirrer) agitation. pH value of the solution was maintained at 7.4. Then an equal volume dilution buffer (20 mmol/L $Na_2HPO_4$ and 1 mmol/L EDTA, pH 8.0) was added into the folding buffer, and final pH of the solution was maintained at 8.0. This protein solution was stored at 4 °C for 24 h.

The refolded protein solution was centrifuged at 18000×*g* and 4 °C for 30 min. The collected supernatant was loaded on an S-Sepharose column with a volume of approximately 20 ml. The column was pre-equilibrated with Buffer A (20 mmol/L $Na_2HPO_4$ and 1 mmol/L EDTA, pH 8.0). Sample was loaded at a flow rate of 0.5 ml/min and the column was then washed with 2 column volumes of Buffer A. The column-bound proteins were eluted using a programmed gradient of Buffer B (20 mmol/L $Na_2HPO_4$, 1 mmol/L EDTA, and 1 mol/L NaCl, pH 8.0) at a flow rate of 1 ml/min. Fractions containing eluted proteins were quantified by the Bradford method (Bradford, 1976).

**SDS-PAGE and Western blotting**

SDS-PAGE was performed using 15% (w/v) resolution gel on the PowerPac Basic (Bio-Rad, USA). Briefly, the protein samples were loaded on the gel, electrophoresed at 120 V for 1 h, and then stained with Commassie brilliant blue R250. For the Western blotting experiments, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes on a tank-blotting devices (Bio-Rad) at 400 mA for 60 min. Horseradish peroxidase-conjugated polyclonal anti-hMK antibody (R&D, USA) was diluted at 1:1000 in PBS containing 0.05% (v/v) Tween 20 (PBST). After blotting, the membrane was blocked at room temperature for 1 h with 5% (w/v) non-fat milk powder diluted in PBST. The blot was then incubated at 4 °C overnight in the presence of the antibody. The membrane was washed three times for 5 min each with PBST. For the electrogenerated chemiluminescence (ECL) reaction, immunoblots were developed for 1 min in Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, USA). The rhMK protein was visualized using enhanced chemiluminescence on X-ray film (Kodar X-Omat BT Film, Rayco Medical Products Co., Ltd., USA).

**Reverse phase high performance liquid chromatography (RP-HPLC) of rhMK**

The purity of the fractions was analyzed by an Agilent RP-HPLC system. The HPLC system was equipped with a high sensitivity ultraviolet (UV) detector and also a solvent mixture gradient module for solvent delivery, with an injector. Purified midkine sample (5 μg) in 0.1% (v/v) trifluoroacetic acid was applied onto the ZORBAX StableBond-C18 column (150 mm×4.6 mm; Agilent, USA), and eluted

with a linear gradient of acetonitrile [10% (v/v) to 30% (v/v)] in 0.1% (v/v) trifluoroacetic acid. The column was kept at room temperature. The flow rate was set at 1.00 ml/min. Absorbance was read at 280 nm and profile was compared with the standard.

**Biological activities**

One of the biological activities of midkine is to promote cell growth. To assay this activity, NIH3T3 cells were plated into 96-well multiplates ($5\times10^3$ 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 (50, 500, and 5000 ng/ml) of rhMK. The effect of rhMK on cell proliferation was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich Chemical Co., Shanghai, China) cell viability and proliferation assay (Mosmann, 1983). 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 measured at an absorbance of 570 nm with a microplate reader (Bio-Tek, USA).

## RESULTS

**Expression of rhMK**

*E. coli* containing the recombinant plasmid pET30a-rhMK was cultured in LB medium at 37 °C and IPTG was added when the culture reached the $OD_{600}$ of 0.8~1.0 to induce expression of rhMK. The expression of a 17-kDa protein was induced (Fig.2a). The identity of the expressed protein was investigated by Western blot using commercial available anti-hMK antibody. The un-induced and induced *E. coli* protein extracts were separated by SDS-PAGE and transferred onto PVDF membrane. Western blot with anti-hMK antibody was performed, one band on the membrane corresponding to the size of rhMK was detected in the bacteria extract after IPTG induction (Fig.2b, lane 1), but not in the un-induced bacteria extract (Fig.2b, lane 2). The Western blot results confirm that the induced protein is rhMK.

The predicted molecular weight of the rhMK was 13 kDa. The rhMK showed anomalously decreased mobility at about 17 kDa when analyzed by SDS-PAGE (Fig.2a). The basis for this anomalous behavior is unknown. It might relate to the highly basic character of the protein. After IPTG induction, the bacteria containing rhMK were sonicated, and soluble cell lysate (Fig.2a, lane 3) and inclusion bodies (Fig.2a, lane 4) were analyzed by SDS-PAGE. The inclusion bodies were found to contain the most of the rhMK representing 60.9% of the total proteins in the inclusion bodies measured by densitometry scanning of the stain gel.

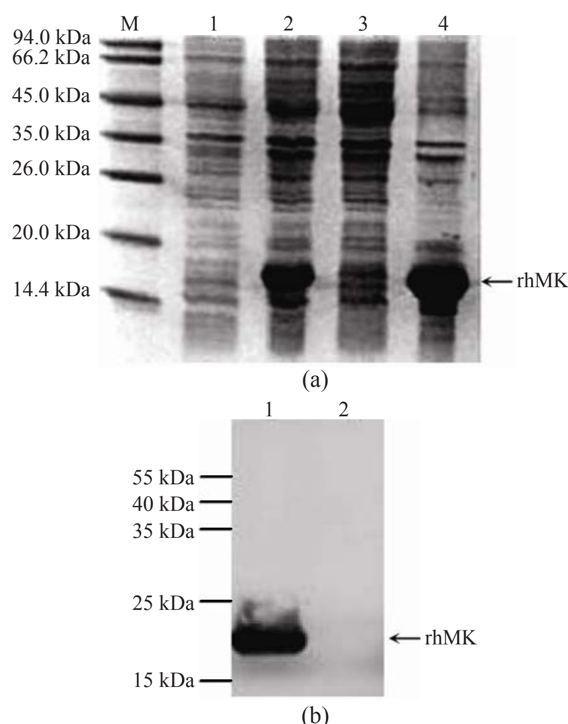


**Fig.2 (a) Expression of rhMK in *E. coli* BL21 (DE3). Lane M: low $M_w$ protein markers; Lane 1: un-induced *E. coli* cell lysate; Lane 2: *E. coli* lysate after IPTG induction; Lane 3: the supernatant of *E. coli* lysate after ultrasonication of the induced *E. coli*; Lane 4: the pellet fraction of the ultrasonication of the induced *E. coli*; (b) Western blot analysis of *E. coli* lysate with commercial anti-midkine antibody. Lane 1: *E. coli* cell lysate after IPTG induction; Lane 2: un-induced *E. coli* cell lysate**

**Renaturation and purification of rhMK**

In order to renature midkine, the denatured solution was diluted by the protein folding solution.

After renaturation, the protein solution was centrifuged for separating the unfolded insoluble proteins at 18000×*g* at 4 °C for 30 min, and fully folded soluble protein was purified using the S-Sepharose column. rhMK has a basic pI value of 9.72, and cation exchange chromatography was chosen for purification with buffer pH below 9.72. The protein binding conditions were optimized to be 20 mmol/L $Na_2HPO_4$ and 1 mmol/L EDTA, pH 8.0 (Buffer A) according to batch absorption/elution experiments. Buffer A was used to equilibrate the column. After loading the refolded protein solution at a flow rate of 0.5 ml/min, the column was washed with Buffer A until the absorbance at 280 nm returned to baseline indicating removal of unbound proteins. The recombinant protein was then eluted by slowly increasing ionic strength of the buffer from Buffer B. During the elution there were two protein peaks. The first one appeared when the conductivity reached 30 mS/cm (Fig.3a, Peak 1), and the second one at the conductivity of 70 mS/cm (Fig.3a, Peak 2). Analyzing the eluted proteins by SDS-PAGE and protein staining, rhMK was found to be eluted at Peak 1 (Fig.3b).

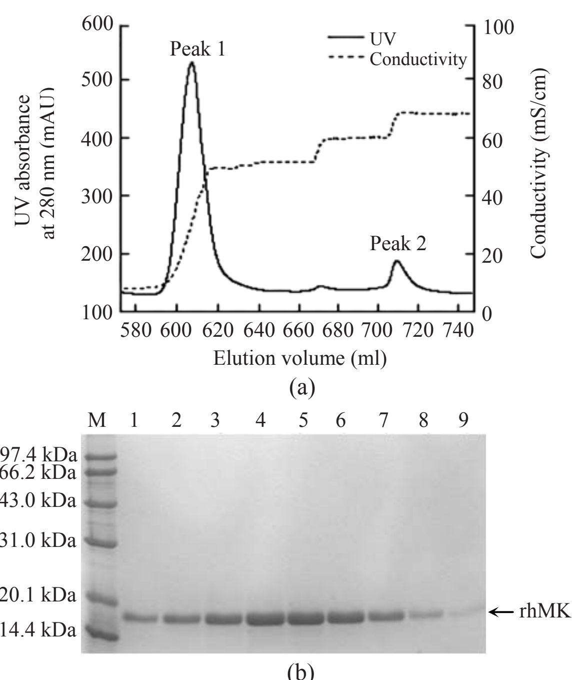


**Fig.3 (a) Purification profile of rhMK using S-Sepharose column. UV absorbance at 280 nm and conductivity were detected during the elution process. Two protein elution peaks were observed (Peaks 1 and 2); (b) SDS-PAGE analysis of Peak 1 proteins. Lane M: low $M_w$ protein markers; Lanes 1~9: the eluted fractions at Peak 1**

The protein samples from each step were analyzed by SDS-PAGE and protein staining with Coomassie brilliant. The protein bands stained on the gel were scanned using BandScan 5.0 software. The intensity of each band was recorded and transformed into digital data, and rhMK purity from each purification step was calculated using the numbers of the protein signal densities. With the purity improved, the rhMK yield decreased stepwise along the purification step. Each purification step contributed to the purification of rhMK. After denaturation and renaturation, the yield and purity of rhMK were greater than 27% and 90%, respectively. The final yield of rhMK was 6.2% compared to the starting material of the protein (Table 1). More than 1.8 mg of pure and biologically active rhMK was obtained from 50 ml culture medium.

**Table 1 Purification of recombinant human midkine using *E. coli* expression system**

| Purification step | Total protein (mg) | Total rhMK (mg) | rhMK purity (%) | Protein yield (%) |
|---|---|---|---|---|
| Cell lysate | 400.0 | 243.6 | 60.9 | 100.0 |
| Denaturation | 118.5 | 101.3 | 85.5 | 41.6 |
| Renaturation | 74.4 | 67.2 | 90.3 | 27.6 |
| Ion-exchange | 15.3 | 15.1 | >98.0 | 6.2 |

## Characterization of purified rhMK

To obtain the purity of the purified rhMK, column fractions were analyzed by SDS-PAGE and protein silver-staining. 1 μg purified protein was loaded onto the gel. The band corresponding to the predicted size of rhMK, but no other bands, was visualized (Fig.4a). As the protein silver-staining sensitivity is lower than 1 ng (Oakley *et al*., 1980; Ochs *et al*., 1981; Sammons *et al*., 1981), bands containing 1 ng or greater protein were detected. The result indicates that the purity of rhMK was >98%. The purified protein went through a sterile 0.22-μm filter and was stored at −80 °C. The endotoxin level of the final formulated rhMK protein solution was <0.3 EU/μg protein (data not shown). Purified midkine was also analyzed using a reverse phase column for HPLC. It showed a sharp single peak (Fig.4b). No other peaks were observed during the 45-min running period, demonstrating that no contaminant protein occurred in the final preparation of rhMK (data not shown). From this analysis, greater than 98% purity was calculated (Fig.4b).

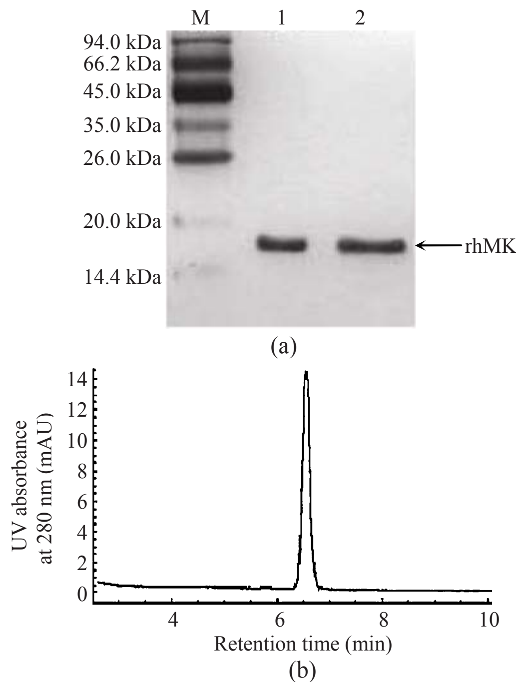


**Fig.4 The purified rhMK was analyzed by SDS-PAGE with protein silver-staining and RP-HPLC**
(a) 1 μg of purified rhMK column fractions were load on SDS-PAGE and protein silver-staining; no other bands could be observed except the main band of rhMK. Lane M: low $M_w$ protein markers; Lanes 1~2: purified rhMK column fractions; (b) Reverse phase HPLC analysis of purified rhMK. Purified rhMK (5 μg) was analyzed by RP-HPLC, using Agilent ZORBAX StableBond-C18 column (150 mm×4.6 mm), which showed a single peak with a retention time of 6.533 min

Midkine possesses a variety of biological activities. Its important role is to promote cell proliferation (Kurtz *et al*., 1995; Ratovitski *et al*., 1998). In this cell proliferation assay, the purified rhMK significantly stimulated the NIH3T3 cell growth and the activity of rhMK was concentration-dependent (Fig.5). The protein produced in this work showed the same bioactivity with the highly purified commercial rhMK (R&D, USA).

## DISCUSSION

In the present study, we reported the expression and purification of human midkine using *E. coli* expression system. It costs very effectively, since culture by using the common LB medium resulted in higher yield of rhMK with ~43% of the rhMK expression when compared to the total protein, and

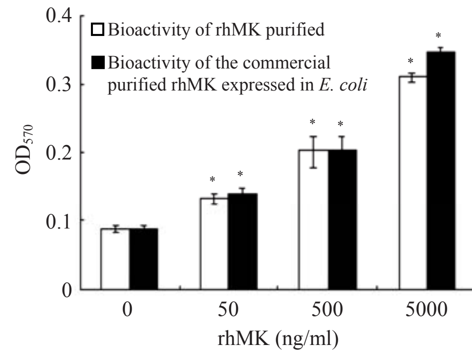


**Fig.5 Enhancement of NIH3T3 cell proliferation with rhMK**
Promotion of cell proliferation was shown against added amounts of rhMK. Data points are the mean±*SD* (error bars) of three independent experiments performed in triplicate; $^*P$<0.01, significant stimulation by rhMK

purification employed no special instruments or reagents (Fig.2a). After sonication, washing of the inclusion bodies, denaturation and renaturation of the protein, the rhMK had a high purity of 90.3%. After purification by one step of ion-change chromatography, the recombinant protein had a higher purity of >98%, and the endotoxin level was lower than 0.3 EU/μg protein. Amino acid methionine was added before the mature rhMK protein to fit the *Nde*I site in the vector. The biological activity of purified Met-midkine in this work was confirmed by NIH3T3 cell proliferation assay. As shown in Fig.5, the amino acid Met did not affect the bioactivity of midkine.

Several studies have been reported earlier for the purification of human midkine (Dansithong *et al*., 2001; Fabri *et al*., 1993; Maruta *et al*., 1993; Murasugi *et al*., 2000; Murasugi and Tohma-Aiba, 2003; Seddon *et al*., 1994). Murasugi and Tohma-Aiba (2003) used the yeast expression system for expressing human midkine by the method of high cell-density fermentation. Although more than 10 mg purified protein was obtained from 50 ml culture medium, the yeast cells containing the recombinant plasmid were cultured for at least one week. During the culture process, the cells were easily contaminated by other microbial with antibiotic resistance. They purified rhMK using special heparin affinity column and the final rhMK had the purity of >90% (Murasugi *et al*., 2000; Murasugi and Tohma-Aiba, 2003). Dansithong *et al*.(2001) purified rhMK using a heavy chain variable domain (VH) protein, which was anti-MK monoclonal antibody. The VH protein was produced

in *E. coli* and coupled with Sepharose 4B column to purify rhMK by the affinity chromatography. Some other researchers expressed human midkine in *E. coli* as a fusion protein and purified by heparin affinity chromatography (Fabri *et al*., 1993; Maruta *et al*., 1993; Seddon *et al*., 1994). Maruta *et al*.(1993) reported that midkine was overproduced in *E. coli* as a glutathione S-transferase (GST) fusion protein. The MK fusion protein extracted from the bacterial inclusion bodies with GdnHCl was renatured, refolded slowly, and cleaved by thrombin at the site where the GST links to the MK. Seddon *et al*.(1994) expressed human midkine in *E. coli* for the formation of insoluble aggregated protein that accounted for 25% of total cellular protein, then purified it by heparin affinity chromatography. Fabri *et al*.(1993) expressed human midkine as a fusion protein in *E. coli* and cleaved it by thrombin to generate 5 mg of midkine per liter of bacterial pellet.

In conclusion, compared to other studies reported in literature, our production process is simple, low-cost, and time-saving. It requires only one step of ion-exchange chromatography without using special heparin affinity column, and has a high purity (>98%) and a low level of endotoxin, with no need of further purification steps. The ability to produce milligram quantities of bioactive rhMK with a simple method will certainly facilitate its functions to be tested. Our purification strategy of rhMK should also have reference values for production of human production at a large scale.

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