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# Expression, purification, and characterization of recombinant mouse nerve growth factor in Chinese hamster ovary cells

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ABSTRACT

Mouse NGF (mNGF) extracted from mouse submaxillary gland has been approved on the market in China for treating nerve damage caused by N-hexane poisoning for over a decade, and many researches showed the clinical effectiveness of mNGF for the treatment of other nerve system diseases. The extracted mNGF have risks of potential viral contamination due to the animal origin. Here, we report the successful expression, purification, and characterization of recombinant mNGF (rmNGF). An expression plasmid of mouse nerve growth factor (mNGF) was constructed and transfected into CHO-S cells. Stable transfectants were obtained using a two-phase selection scheme with the addition of different concentrations of methotrexate and puromycin. Recombinant mNGF (rmNGF) was purified from cell culture medium by a two-step procedure: cation exchange followed by size-exclusion chromatography. The purity of rmNGF was 98.6% determined by size exclusion high performance liquid chromatography (SEC-HPLC). The molecular weight, isoelectric point and N-terminal sequence of rmNGF were identical to the theoretical values entirely. In TF-1/MTS, the specific activity of the protein was approximately $1.7 \times 10^6$ U/mg against rhNGF (the reference standard). In DRGs, the specific activity was approximately $7.3 \times 10^5$ AU/mg against mNGF (the reference standard). Our results showed that a high quality of rmNGF with marked biological activity comparable with mNGF was produced, and laid the basis for further research and development of rmNGF.



## Introduction

Nerve growth factor (NGF)[1] is a member of the neurotrophins, which are a family of secreted proteins essential for proper development, patterning, and maintenance of the mammalian nervous system. The first isolation of NGF came from snake venom and mouse submaxillary glands [1]. In China, injection of mouse NGF (mNGF) has been approved for treating nerve damage caused by N-hexane poisoning and has been on the market for over a decade. Thus far, mNGF has been shown to be clinically effective for the treatment of peripheral nerve injury [2], craniocerebral trauma [3], acute spinal cord injury [4], optic nerve contusion [5], child facial paralysis [6], chronic peripheral polyneuropathy [7], carbon monoxide poisoning [8], persistent corneal epithelial defects [9], corneal ulcer [10], keratoconjunctivitis sicca [11], glaucoma [12,13], and radiation-induced temporal lobe necrosis [14]. However, commercial mNGF may be contaminated by viruses, such as ectromelia virus, mouse hepatitis virus and sendai virus, because it is isolated from mouse submaxillary glands. Recombinant mNGF is one of the way to reduce the risk of virus contaminations.

Thus far, recombinant human NGF (rhNGF) has been produced in yeast [15], *Escherichia coli* (*E. coli*) [16–19], and both insect and mammalian cells [20–23]. A phase II trial has revealed a positive effect of rhNGF on neuropathic pain and pain sensitivity in human immunodeficiency virus-associated sensory neuropathy [24]. A phase III clinical trial failed to demonstrate a beneficial effect of rhNGF on diabetic polyneuropathy [25].

However, despite the beneficial clinical effects of mNGF, only one study has produced recombinant mouse NGF (rmNGF) (in *E. coli*) [19]. *E. coli*-produced human NGF results in low biological activity [16]. This response may occur because *E. coli* produces recombinant protein in the form of inclusion bodies, which require further naturation possibly causing protein misfolding protein with low biological activity.

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[1] *Abbreviations used:* mNGF, mouse NGF; rmNGF, recombinant mNGF; NGF, nerve growth factor; rhNGF, recombinant human NGF; CHO, Chinese hamster ovary; FBS, fetal bovine serum; MTX, methotrexate; TBST, tris-buffered-saline with tween; CE, capillary electrophoresis; WCID, whole column imaging detection; CE-WCID, capillary electrophoresis of whole column imaging detection.

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The biological activity of mNGF is dependent on the formation of three disulfide bonds and a cysteine knot within two chains after cleavage of the signal and propeptide sequences from a precursor molecule [26]. N-glycosylation of the pro-segment of mNGF is necessary for subsequent processing and secretion and formation of the three mature forms of intra-chain disulfide bonds [27,28]. Mammalian cells have the capacity for correct folding and post-translational modification of recombinant proteins. The mammalian cell line, Chinese hamster ovary (CHO) cells, plays a key role in the production of recombinant proteins [18].

In the present study, we produced rmNGF in CHO-S cells. We provide a biotechnological approach for the production, purification, and characterization of biologically active rmNGF in CHO-S cells, thereby possibly allowing for future research and development of rmNGF.

## Materials and methods

### *Cell culture*

CHO-S cells (Life technologies, America) were cultured in 125-mL Erlenmeyer shaker flasks in CD FortiCHO™ medium (Life technologies, America) supplemented with 8 mM L-glutamine (i.e., complete CD FortiCHO™ medium). Shaker flasks were incubated at 37 °C with 80% humidity and 8% $CO_2$ and shaken (130 rpm) on an orbital shaker platform. Cells were seeded at $2.0 \times 10^5$ viable cells/mL. Cells were subcultured 2 days later. Mono-clones of recombinant CHO-S cells were cultured in 125-mL Erlenmeyer shaker flasks in CD FortiCHO™ medium supplemented with 1 μM methotrexate, 50 μg/mL puromycin, and 8 mM L-glutamine. Cells were seeded at $2.0 \times 10^5$ viable cells/mL, and subcultures occurred 2 or 3 days later. TF-1 cells were grown in RPMI 1640 medium (Gibco, America) supplemented with 25 ng/ml mNGF and 10% fetal bovine serum (FBS) under 5% $CO_2$ at 37 °C. Cells were seeded at $1.0 \times 10^5$ viable cells/mL, and subcultures were made 3 days later.

### *Plasmid construction*

The cDNA of mNGF (sequence NM_001112698.1 published on pubmed) was cloned by polymerase chain reaction using a mouse cDNA clone (MC209031, Origene) with upstream primer TGCAG GATATC GCCACCATGTCCATGTTGTTCTACACTCTG and downstream primer CCTTAATTAATCAGCCTCTTCTTGTAGCC. The cDNA fragment was inserted into an expression vector pCHO 1.0 (Life technologies) at the EcoRV-PacI sites of the polylinker region. The construct was analyzed by restriction and sequence analysis to verify the correct sequence and orientation of the fragment. The constructed rmNGF expression vector was named pXL-mNGF.

### *The isolation of rmNGF expression CHO-S cells*

Both plasmids, pXL-mNGF and pCHO1.0 (Freedom pCHO1.0), were linearized by *Nru*I (R0192 M, NEB) and adjusted to 1 μg/μL for transfection. pCHO1.0 was set as the negative control. Transfection was completed in 125-mL flasks. CHO-S cells ($1 \times 10^6$ viable cells/mL) contained in 30 mL of pre-warmed complete CD FortiCHO™ medium were transfected with the rmNGF expression vector, pXL-mNGF, or the empty vetor, pCHO1.0. The transient transfection was performed by the FreeStyle™ max reagent (Invitrogen, America), according to the manufacturer's instructions. The expressed recombinant protein was evaluated 48 h later in the culture medium of transfected cells, via Western immunoblotting. Cells transfected with plasmid pXL-mNGF were collected for stable transfectants selection.

Stable transfectants were selected by methotrexate (MTX) (Sigma–Aldrich, America) and puromycin (Life technologies, America). The pCHO 1.0 expression vector contained the puromycin resistance gene and dihydrofolate reductase, which confers resistance to the puromycin and can be selected by the addition of puromycin and MTX. Stable transfectants were selected by a two-phase selection scheme. In the first selecting phase, 200 nM MTX and 20 μg/mL puromycin were added. In the second selecting phase, 1 μM MTX and 50 μg/mL puromycin were added.

Stable transfectants were isolated to mono-clones by limiting dilution. Mono-clones were seeded into 96-well plates, which were then observed under microscopy to ensure mono cells in each well. When mono cells proliferated to a certain degree, they were plated in 24-well plates, 6-well plates, T-25 flasks, T-75 flasks, and 125-mL flasks. The productivity of rmNGF protein was evaluated for each clone by enzyme-linked immunosorbent assay (ELISA) in 6-well plates.

### *Expression and purification of rmNGF*

Mono-clone C#10 was chosen to produce rmNGF in 1000-mL shaker flasks on a laboratory-scale. In the flask, cells of clone C#10 were seeded at $3 \times 10^6$ viable cells/ml and cultured in CD FortiCHO™ medium containing 1 μM MTX, 50 μg/mL puromycin and 8 mM L-glutamine. The culture was harvested on the fifth day. After centrifugation at 7000 rpm for 30 min, the cell culture supernatant was collected for the next purification.

rmNGF was purified by a two-step procedure including cation exchange chromatography and size-exclusion chromatography. As the first step in the purification scheme, a SP-sepharose fast flow column (GE Health care) was used. The column was equilibrated with mobile phase A (100 mM, 10 mM sodium citrate, pH 6.5) on a FPLC system (Amersham Pharmacia Biotech, USA) at 10 ml/min. The culture supernatant was first adjusted to pH 6.5 and a total volume of 1000 mL supernatant was loaded on to the column. The column was washed by 375 mL of mobile phase A to wash off non-adsorbed protein. Then the bound protein was eluted by using mobile phase B (100 mM, 10 mM sodium citrate, 1 M NaCl, pH 6.5) by increasing NaCl concentration to 1 M in a linear gradient. As a next step in the purification procedure, a Superdex 75 prep grade column (GE Health care, America) was employed on the eluted protein from step one using and mobile phase C (0.05 mol/L phosphate-buffered saline, pH 6.8) at 1 mL/min. Effluent fractions from both purification steps were collected and analyzed by 4–12% SDS–PAGE. The finally purified protein was analyzed by a serial of methods including purity analysis (SDS–PAGE and HPLC), protein quantification, Western immunoblotting, N-terminal amino acid sequence analysis, mass spectrometric analysis, isoelectric point analysis, and biological characterization.

### *Protein quantification by ELISA*

Protein quantification of rmNGF was determined by ELISA (Millipore, America). Briefly, samples were diluted with sample diluent (tenfold dilution) and run down a column of the plate. Standard was diluted with sample diluent (twofold dilution) and run down a column of the plate. Samples or standards were added to the wells (pre-coated with sheep anti-mouse NGF polyclonal antibody) and incubated for 3 h at room temperature. The plate was washed and anti-mouse NGF monoclonal antibody (1:100) were added and incubated for 2 h at room temperature. The plate was then washed and peroxidase conjugated donkey anti-mouse IgG polyclonal antibody (1:1000) were added and incubated for 2 h at room temperature. TMB substrate was added to each well and the plate was incubated for 10 min. The reaction was stopped by the addition of stop solution. The plate was immediately read by a microplate reader at 450 nm.