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Nerve growth factor from *Vipera lebetina* venom☆

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ABSTRACT

Nerve growth factor was isolated from the *Vipera lebetina* venom by a four-step procedure including gel filtration, ion exchange, heparin and hydrophobic chromatography. The purified protein is a glycosylated non-covalently bound homodimer with monomeric molecular mass of 14,380 Da. The cDNA encoding NGF is cloned and sequenced. The amino acid sequence translated from the cDNA comprises 117 or 119 amino acids depending on the N-terminus (truncated or not). The recombinant NGF (expressed in *Escherichia coli*) was used to prepare the anti-NGF antiserum. The antiserum interacted with the wild-type NGF and enabled to localize NGF during the purification procedure in parallel with MALDI-TOF analysis of tryptic peptides. The isolated NGF caused neurite outgrowth from PC12 cells in concentrations beginning from 2.5 ng/ml.

1. Introduction

Nerve growth factor (NGF) belongs to neurotrophin family. The neurotrophin family includes four members: NGF, BDNF, NT-3 and NT-4/5 (Götz and Schartl, 1994). Nerve growth factor is a protein with well-established survival effects, differentiation and maintenance function of sympathetic and embryonic sensory neurons (Harper and Thoenen, 1980). NGF was first discovered in mouse sarcoma 180 and 37 (Bueker, 1948; Levi-Montalcini, 1952), then in snake venom (Cohen and Levi-Montalcini, 1956) and male mouse submaxillary gland (Cohen, 1960). NGF is produced in the murine submaxillary gland as a precursor complex of about 130 kDa (7S-NGF). This complex comprises alpha, beta and gamma subunits. Alpha and gamma subunits are members of the kallikrein family of serine proteases and are involved in the cleavage of the 7S-complex and releasing beta-NGF. Although both the 7S-NGF and beta-NGF (2.5S) induce neurite outgrowth in PC12 cells, there are differences in the potencies of these compounds. The majority of research on NGF has been performed using NGF isolated from the male mouse submaxillary gland. Mouse NGF is very similar in its biological activities to human NGF (Ullrich et al., 1983). Interest in NGF and in its receptors has increased in the last decade due to the discovery of the therapeutic potential of human NGF in neurodegenerative disorders (Gao et al., 1997; Lazarovici et al., 2006).

Many NGFs have been isolated and characterized from snake venoms that are considered to be a rich source of NGF. NGF was detected in three families of poisonous snakes: Elapidae, Viperidae, Crotalidae (Hogue-Angeletti and Bradshaw, 1979). The majority of snake venom NGFs are non-covalently bound dimers with molecular mass of about 25 kDa containing two identical (or very similar)

Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; PC12, pheochromocytoma 12; IPTG, isopropyl-β-D-thio-galactopyranoside; DHB, 2,5-dihydroxybenzoic acid; α-CHCA, α-cyano-4-hydroxycinnamic acid; ACN, acetonitrile; TFA, trifluoroacetic acid; PNGase F, peptide: N-glycosidase F; UPLC, ultra performance liquid chromatography; PBS, phosphate-buffered saline.

☆ The nucleotide sequence of NGF was deposited to GenBank with the accession number of AY740013.

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subunits with molecular mass of about 13 kDa (Smith et al., 1992). NGFs from *Bitis arietans* (Smith et al., 1992) and *Bungarus multicinctus* (Furukawa and Hayashi, 1978) are covalently bound dimers. Several venom NGFs have been identified as glycoproteins: *Bothrops atrox* (Glass and Banthorpe, 1975), *Vipera russelli* (Pearce et al., 1972), *Vipera lebetina* (Siigur et al., 1985) nerve growth factors contain carbohydrates. At present, many NGFs have been cloned and sequenced and their sequences are highly homologous (Oda et al., 1989; Inoue et al., 1991; Koyama et al., 1992; Danse and Garnier, 1993; Selby et al., 1987; Kashima et al., 2002). Koh et al. (2004) isolated two cDNAs encoding isoforms of NGF from *Naja sputatrix*, and also purified NGF from *N. sputatrix* venom. From the studies with this NGF they conclude that this, sputaNGF, can substitute mouse 7S-beta-NGF in studies on nervous system. NGF preparation isolated from the venom of *V. lebetina* had molecular mass of 32.5 kDa both in reduced and non-reduced form and exhibited arginine esterase activity (Siigur et al., 1985). Another example of esterase with nerve growth promoting activity is the protein isolated from the venom of *Agkistrodon halys* Pallas (Guo et al., 1998) that is named NGF-like protease (NLP) while its N-terminal amino acid sequence is homologous to serine proteases.

The aim of this study is to isolate the individual NGF from the preparation with esterase, to clone and sequence the cDNA encoding *V. lebetina* NGF, and to express the recombinant protein for preparation of antiserum.

2. Materials and methods

2.1. Materials

V. lebetina venom was collected in Central Asia and purchased from Tashkent Integrated Zoo Plant (Uzbekistan). Sephadex G-100 superfine was from Pharmacia (Sweden), CM52 cellulose from Whatman Biochemicals, Heparin–agarose from Kemotex (Tallinn, Estonia). Ni-NTA – Qiagen, USA, PNGase F was from New England Biolabs, Inc (USA). All other reagents were of analytical grade.

2.2. Purification of the nerve growth factor

NGF was isolated from the crude venom by a four-step procedure. Crude *V. lebetina* venom (1.5 g) was dissolved in 10 ml of 0.2 M ammonium acetate, pH 6.7. Insoluble material was removed by centrifugation (5000×*g* for 15 min) and the supernatant was applied to the column (2.2 × 140 cm) of Sephadex G-100 superfine equilibrated with 0.2 M ammonium acetate. The elution was carried out with the same solution at a flow rate 6.8 ml/h and fractions were collected at 1 h intervals at 4 °C. The fractions containing NGF were lyophilised, dissolved in 0.2 M ammonium acetate pH 6.7 and loaded onto a Whatman CM52 cellulose column (2.8 × 21 cm) equilibrated with the same solution. After washing the non-adsorbed material, the adsorbed NGF was eluted with 0.6 M ammonium acetate pH 6.7, lyophilised and applied onto a heparin–agarose column (1.7 × 10 cm) in 0.2 M ammonium bicarbonate pH 8.3. The column was washed with 0.2, 0.5 and 2 M ammonium bicarbonate pH 8.3. The eluted NGF fractions were lyophilised and applied to a UPLC C4 column (Waters Acquity BEH300 C4, 2.1 × 100 mm). Elution was performed at 0.5 ml/min using a linear gradient of acetonitrile (5–60% in 20 min) followed by an isocratic elution with 60% acetonitrile in 0.1% trifluoroacetic acid for 5 min. The NGF-containing fraction was collected and lyophilised.

2.3. Construction and screening of the venom gland cDNA library

Poly(A) mRNA was prepared from a venom gland of a *V. lebetina* snake using poly $(A)^+$ Quick mRNA Isolation Kit (Stratagene, La Jolla, USA). The cDNA library was constructed using Uni-ZAP XR vector Kit and Uni-ZAP XR Gigapack Cloning Kit (Stratagene, USA) according to the manufacturer's instructions and as described previously (Siigur et al., 1996).

Based on the published sequence of the genomic DNA segment encoding NGF core fragment from *V. lebetina* (Hallböök et al., 1991) a pair of primers, P1 and P2, were designed to generate a specific probe for cDNA library screening. Using the total cDNA as a template, PCR was performed with the primers P1 (5′-TCCAAGTCCAGTATCAGGTGG-3′) and P2 (5′-CCAAGATGCCTGATTGCCTTC -3′) corresponding to nucleotides 552–572 and 649–669 in preproNGF cDNA sequence, respectively. The purified PCR product with the expected size (117 nt) was subcloned in pUC57/T vector (Fermentas) and sequenced. The verified PCR product was ^{32}P labeled by random priming with Klenow fragment (Promega) and ^{32}P ATP and used to screen the cDNA library.

About 10^5 plaques were spread onto NZY plates, transferred onto Hybond–N nylon membranes (Amersham, UK) and hybridized to ^{32}P probe at a concentration 5×10^5 cpm/ml. In vivo excision procedure was done to obtain phagemid clones from positive plaques. Several plasmid DNAs were isolated, inserts were removed by digestion with NotI/XhoI and the clone with the longest insert was mapped with restrictases. Insert DNA was subcloned in three restriction fragments into p-Bluescript SK(+) and sequenced along both strands in ABI Prism 310 Genetic Analyzer using ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Perkin–Elmer Biosystems). The full-length cDNA sequence and predicted amino acid sequence were compared with sequences from the GeneBank database (NCBI).

2.4. Expression and purification of the His-tagged protein

The cDNA encoding *V. lebetina* mature NGF protein was amplified using a forward primer, 5′-CACGGATCCGCAACTCATCCTGTGCATAAC-3′, and a reverse primer, 5′-CACGTCGACCTATCCGAAGTTGTCATTTTTC-3′. The PCR product was double digested with BamHI/SalI and ligated into BamHI/SalI linearized pQE30 expression vector (Qiagen, USA). The expression host *Escherichia coli* TG-1 was transformed with the plasmid construct and expression of protein was induced with 1 mM IPTG. Protein purification was performed under denaturing conditions on a Ni-NTA resin (Qiagen) column according to the manufacturer's protocol. The 6×-His- tagged NGF protein was eluted in 8 M urea,