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Purification and characterization of murine beta-nerve growth factor

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Abstract

Beta-nerve growth factor (β-NGF) is a trophic factor in the nervous system. We aimed to isolate and characterize this protein in view of its potential therapeutic use in neurodegenerative diseases. For purification a two-step ion-exchange procedure was followed. The characterization was performed using separation and immunological techniques, as well as a biological assay. These studies showed that the obtained protein consisted of a mixture of β-NGF molecules, intact at their NH_2-terminal extreme, and molecules which have lost the NH_2-terminal octapeptide and exhibit modifications increasing its hydrophobicity. All these molecular species were recognized immunologically and showed biological activity. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: β-NGF; Characterization studies; Separation techniques

1. Introduction

Nerve growth factor (NGF) is the oldest member of the neurotrophin family. These proteins play import roles in neuronal development and survival [1]. NGF is present in the mouse submaxillary gland, the richest natural source of this protein [2], as a complex of three different polypeptides; named alpha (α), beta (β) and gamma (γ) subunits, respectively. The subunits are noncovalent linked, forming pentameric complexes (α_2 β γ_2). However only the β-subunit, a basic protein (isoelectric point, pI=9.3), is responsible for the biological activity. This protein is a dimer of 26 kDa composed by two identical polypeptide chains of 118 amino acids and a known sequence. Individual NGF chains have three intrachain disulfide links and are associated with each other through noncovalent forces [3].

Two methods for isolating the murine β-NGF have been described. In one of them β-NGF is isolated as one of the subunits of the purified 7S complex [4–6]. In the other method, the protein is isolated directly from the gland homogenate without prior separation of the complex [7]. This last method could introduce two specific proteolytic cleavages by exposition to enzymes present in the submaxillary gland extract. One of these cleavages results in the removal of either or both the COOH-terminal arginine residues from the two chains. The second results in the production of chains which are shorter and have lost the NH_2-terminal octapeptide (Ser-Ser-Thr-His-Pro-Val-Phe-His) [3,8,9].

NGF is an essential trophic factor for the survival, development and maintenance of sympathetic and

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sensory neurons in peripheral nervous system, as well as basal forebrain and striatal cholinergic neurons in the central nervous system [10,11]. The specificity and trophic actions of NGF on these neuronal populations and its efficacy preventing neurodegeneration have led to its proposal of evaluation in the treatment of Alzheimer's disease and diabetic neuropathies [12–14]. Clinical studies in patients have been carried out with encoraging results [15–18].

Our purpose in this work was to carry out the physicochemical, immunological and biological characterization of the murine β-NGF obtained in our laboratories. The investigation showed that our protein is similar to the one obtained by Mobley using the same purification method [3,7]. These results support the use of the murine β-NGF obtained in our laboratories in preclinical studies that evaluate its potentiality as therapeutic agent.

2. Experimental

2.1. Reagent

For the different procedures involved in this work the following chemicals used were: Tris, hydrocloride acid and acetic acid (Merck, Darmstadt, Germany); sodium acetate and sodium chloride (BDH Laboratory Supplies Poole, Dorset, UK); acetonitrile (Merck) and trifluoroacetic acid (Pierce, Rockford, IL, USA) with HPLC grade. All reagents used in the electrophoresis were of analytical grade (Merck). Solution Kit and Phast Gels (Pharmacia LKB, Uppsala, Sweden) were used in the isoelectric focusing (IEF). Monoclonal anti-NGF antibody (Boehringer Mannheim Biochemica, Mannheim, Germany) and (Promega, Wisconsin, MD, USA) were used in the two-site enzyme inmunoassay (EIA) and Western Blot, respectively. Mouse immunoglobulin G (Sigma, St Louis, MO, USA) and purified mouse β-NGF (Boehringer Mannheim Biochemica) was used in the EIA. The reagents used in the biological assays were 10×Eagle's basal medium, $NaHCO_3$, fetal calf serum (GIBCO, Scotland, UK), glutamine and NaOH (Merck).

2.2. Equipment

The ion-exchange chromatography system was composed by a low pressure pump and an UV detector (Pharmacia).

The HPLC system was composed of two pumps (model 2248), one gradient controller (model 2252) and a high pressure mixer (Pharmacia LKB). The Biochrom software (CIGB, Havana, Cuba) was used for data acquisition and processing.

The electrophoretic system consisted of an adjustable vertical gel system and a electrophoresis power supply (model EC 135, EC-Apparatus Corporation, St Petersburg, FL, USA). An imaging densitometer (model GS-700, Bio-Rad, Hercules, CA, USA) and Molecular Analyst Software were used for data acquisition and processing.

The system for the determination of the NH_2-terminal sequence used an automatic sequenator (model 810-813, Knauer, Berlin, Germany). For the data acquisition and processing, Chromapack CR-3A automatic integrator (Shimadzu,Tokyo, Japan) was used.

The IEF was performed using the Phast system (Pharmacia LKB).

The EIA was performed using a microplate fluorometer (Fluoroskan II, Labsystems, Helsinki, Finland)

The Western Blot utilized a trans-blot semidry transfer (Bio-Rad).

The biological assay utilized a laminar flow (Gelaire Flow Laboratories, Gruppo Flow, Milan, Italy) and an inverter transmitted light microscope (Leitz Labovert, Wetzlar, Germany).

2.3. Ion exchange chromatography

Two ion-exchange step were performed, first through a CL 6B Sepharose chromatography column (40×1.6 cm) (Pharmacia) balanced with 18 ml/h of Tris–HCl and second step used a CL 6B Sepharose chromatography column (20×1.6 cm) (Pharmacia) balanced with 20 ml/h of sodium acetate for removal of contaminants in the submaxillar glands homogenate and the final separation of the β-NGF subunit. The temperature during the purification procedure was 4°C. The detection was carried out with a UV cell set at 280 nm.

2.4. High-performance liquid chromatography

A C8 column (250×4.6 mm, 5 μm) (The Separations Group, Inc Vydac, Hesperia, CA, USA), using a gradient of 15–45% of acetonitrile (buffer B) in the presence of trifluoroacetic (buffer A), with 0.8 ml/min flow-rate, temperature of 34°C, and UV detection to 214 nm were the runnig conditions. At each chromatographic run 20 μg of NGF at a concentration of 500 μg/ml were injected, using saline solution (0.9% NaCl) as vehicle. The method was validated taking into consideration the selectivity, precision, and detection limit.

2.5. Electrophoresis

Electrophoretic runs (SDS–PAGE) was conducted in 15% polyacrylamide gels and buffer (0.025 *M* Tris–HCl, 0.192 *M* glycine, 0.1% of SDS, pH 8.3), using constant voltage (120 V) and current of 30 mA according to the method described by Laemmli [20]. The NGF samples were applied in reducing conditions using 5% β-mercaptoethanol. The gels were fixed in 20% trichloroacetic acid, stained in G-250 blue coomasie bands and analyzed with the software Molecular Analyst. For the determination of detection limits of this method, quantities of 20, 10, 5 and 1 μg of NGF at a concentration of 500 μg/ml were applied.

2.6. Isoelectric focusing

The IEF was performed through an optimized method as recommended by manufacturers (Phast System, Pharmacia LKB). In brief, the separation consisted of three steps: a prefocusing step in which the pH gradient in the gel is formed, a sample application step, and a focusing step at 2000 V. Phast gels IEF 3-9 were used. Protein lanes were visualized by silver staining of gels. A broad p*I* kit (pH 3–10) of 11 known proteins was simultaneously processed as standards for the calculation of isoelectric point (p*I*).

2.7. Determination of the NH_2-terminal sequence

The Edman degradation method was used for amino acid sequenciation [19]. A C_{18} columm (250×2 mm, 5 μm) (Knauer), with a gradient of 15–98% of acetonitrile (buffer B) in the presence of sodium acetate 15 m*M* and 5% of tetrahidrofurane (buffer A), 280 μl/min flow-rate, temperature of 55°C and UV detection to 269 nm were the runnig conditions. Quantities of 5–100 pmoles of NGF at a concentration 500 μg/ml were required for the analysis.

2.8. Two-site enzyme immunoassay

A two-site EIA for NGF described previously by Söderström was used in this study. The EIA was based on the sandwich of antigen between anti-mouse-β NGF antibody 27/21 and a β-Galactosidase-conjugated antibody 27/21 [21]. Immunoplates (black 96-well multidishes, Dinatech Microfluor, Virginia, USA) were coated with monoclonal antibody 27/21. Control wells were coated with normal mouse immunoglobulin G. Samples of mouse β-NGF (Boehringer) at 0.1–100 ng/ml were also included as standard. The antibody 27/21-β-Galactosidase-conjugate (Boehringer, 4 units of enzyme activity per ml) was added. The accumulation of methyllumbelliferone was followed in a microplate fluorometer.

2.9. Western blot

The Western blot used the semi-dry transfer buffer (48 m*M* Tris, 39 m*M* glycine, 20% methanol, 1.3 m*M* SDS, pH 9.2) and a nitrocellulose transfer membrane (nitro pure, 0.45 μm). The transfer was performed over 1.5 h, the voltage was fixed between 15 and 25 V. The current fixed depends on the area of gels.

2.10. Biological assay

Biological activity was determined according to the methods described by Ebeldal [22]. The best routine in assaying samples of unknown NGF activity is to prepare a series of culture dishes with chains of two to three sympathetic ganglia of chick embryos (White Leghorn) incubated for 9 days in a mixture of collagen, 10×Eagle's basal medium, $NaHCO_3$, L-

glutamine, 1% fetal calf serum, and then add serial dilutions of β-NGF. Incubation is carried out at 37°C under standard cell culture condition. Fibre outgrowth, density, thickness and size of the fiber halos formed around the ganglia can be examined after 1 or 2 days, using dark field or phase-contrast microscopy. Care should be taken to distinguish nerve fibres from spindle-shaped cells which may be numerous under some conditions. Fibre outgrowth is normally scored on a semi-quantitative scale in coded cultures to determine the biological activity unit. ''A biological unit of NGF has been defined as the protein content of the factor per ml of tissue culture medium necessary to obtain a dense halo of nerve fibres within the first 48 h'' [23].

3. Results and discussion

3.1. Purification of β-NGF

As previously mentioned, β-NGF was purified by ion-exchange chromatography according to Mobley's method modified by Ebendal [7,9]. During standarization of the purification process we introduced some modifications: a more exhaustive homogeneization and a supernatant filtration stage by glass fiber (Fig. 1).The average yield obtained was 6.7 mg from 40 g of submaxillary glands, similar to that previously reported. The novelty in the described procedure reside in the improved purity of the final product and the more exhaustive characterization of the purified protein.

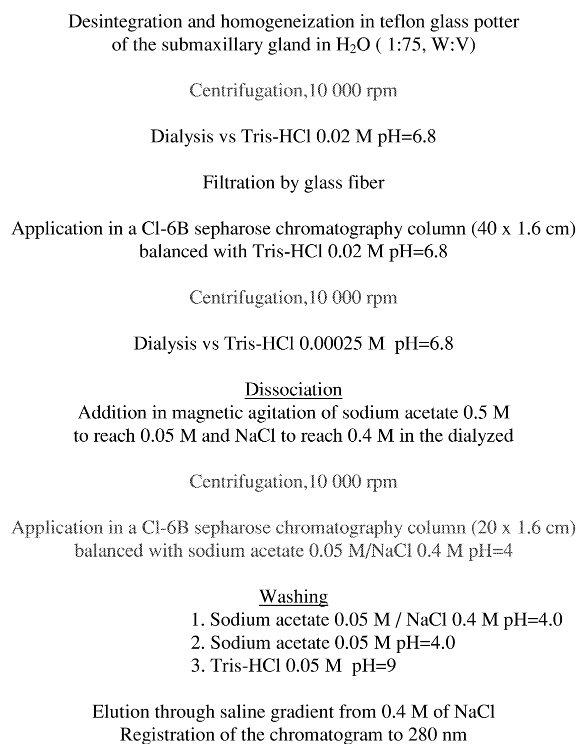

Fig. 1. Technological flow of the murine β-NGF purification process.

3.2. Physicochemical characterization

3.2.1. Chromatography profile

The validation of the RP–HPLC method showed its selectivity: (1) impurities or other unrelated species do not absorb at other wave lenghts (210, 226 and 280 nm); (2) the absorbance of vehicle (0.9% NaCl) is null; (3) impurities do not coelute with the main peak in the electrophoretic analysis; and (4) the degradation of the sample by thermolisis does not generate additional interfering species (data not shown).

The method is precise because the variation coefficient, in the statistical analysis of the retention times and percent of areas of the mean peaks (Table 1), was lower than 4.2%, for $n=5$, with an acceptance interval between 95 and 105% [24].

The chromatography profile of β-NGF obtained by RP–HPLC showed two main peaks (II and III) that constitute approximately 98%, and one minoritary peak that constitutes only 2%. This profile (Fig. 2) and the retention times of the main peaks (Table 1) were similar to the ones reported by Mobley [8].

3.2.2. Determination of the molecular mass of the species present in the β-NGF samples

The electrophoretic run of a β-NGF sample (lane 1) by SDS–PAGE developed three bands: an intense band of 10 kDa, another band of 9 kDa and a less intense band of 13 kDa. Fractions I, II and III obtained by RP–HPLC were also applied in lanes 3–5, respectively. Fraction I showed a band coincident with 13 kDa band observed in lane 1, which constitutes the minoritary species by both techniques (RP–HPLC and SDS–PAGE). Fraction II coincided

Table 1
The statistical analysis of the retention times and percent of area for the mean peaks obtained by RP–HPLC, showed that all variation coefficients were lower than 4.2% ($n=5$)[a]

	Retention times			Percent of area		
Peaks obtained by RP–HPLC	I	II	III	I	II	III
Mean	21.28	22.19	23.7	3.11	56.8	40.8
Standard deviation	0.28	0.29	0.33	0.10	2.14	0.54
Variation coefficient (%)	1.3	1.3	1.4	3.2	3.7	1.3

[a] This result demonstrated that the RP–HPLC method employed is precise for an acceptance interval between 95 and 105%.

with the intense band of 10 kDa observed in lane 1. Fraction III, composed of two molecular species, showed two bands of 10 and 9 kDa, respectively (Fig. 3).

3.2.3. Determination of the NH_2-terminal sequence

The NH_2-terminal sequence analysis demonstrated that fractions I and II contained only one NH_2-terminal sequence, that coincided with the one reported for β-NGF. Fraction III contained two molecular β-NGF species: one with intact NH_2-terminal sequence and another one that has lost the NH_2-terminal octapeptide (Ser-Ser-Thr-His-Pro-Val-Phe-His). These results justify the difference of 1 kDa obtained in the bands of fraction III, equivalent to the loss of eight amino acids (Table 2).

The electrophoretic run under reducing conditions revealed that the intact specie at its NH_2-terminal extreme had 13 kDa, which suggest that β-NGF dimer would have 26 kDa.

The difference in hydrophobicity between the species of 10 kDa of the fractions II and III suggests a modification in the species of 10 kDa of fraction III for the COOH-terminal extreme, as could be the loss of one or two hydrophilic amino acids or other modification that increase its hydrophobicity.

3.2.4. Detection of impurities

Prior reports indicate that murine β-NGF can be contaminated by renin or by other proteins, notably gamma globulin [3,25]. These impurities were not detected during the electroforetic analysis of our final

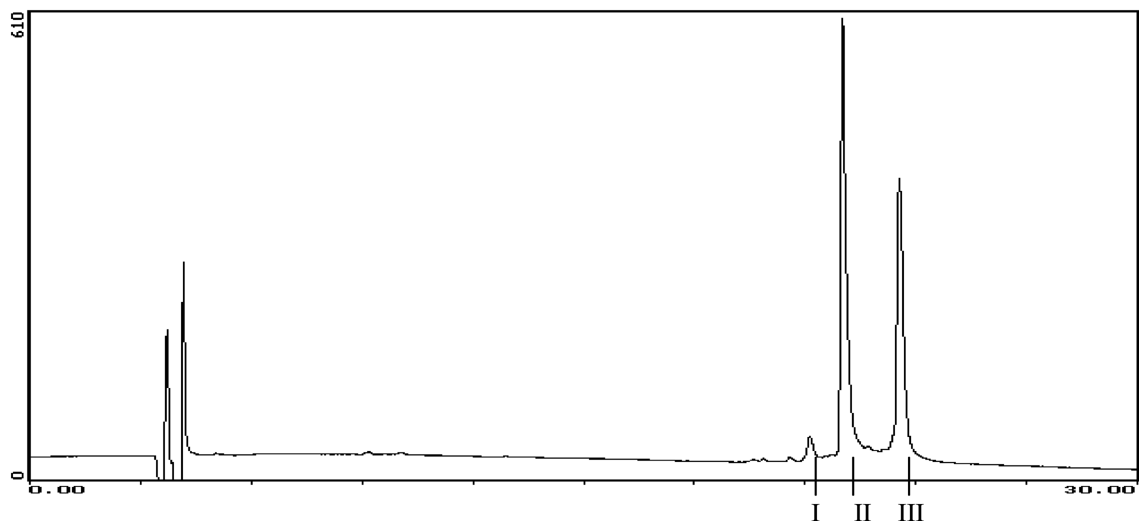

Fig. 2. The chromatography profile obtained from the murine β-NGF samples applied by RP–HPLC, showed two main peaks (II and III). This profile coincided with the one described by Mobley for the purification method employed.

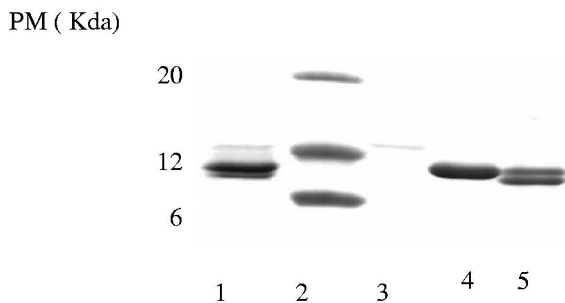

Fig. 3. Electrophoresis was conducted under reducing conditions in 15% polyacrylamide gels. Lane 1: murine β-NGF, lane 2: molecular mass patron (20, 12 and 6 kDa), lanes 3–5: I, II and III fractions obtained by RP–HPLC. Lane 1 showed three molecular species with molecular mass (MW) of 13, 10 and 9 kDa. Fraction I (lane 3) coincides with the species of 13 kDa (lane 1), fraction II (lane 4) coincides with the species of 10 kDa (lane 1) and fraction III (lane 5), composed of two molecular species showed two bands with MW of 10 and 9 kDa.

product. The detection level of renin and gamma globulin in samples obtained in the washing steps of the initial ion-exchange column in the purification process were very low (Table 4).

Table 2
The amino acid sequence of the fractions obtained by RP–HPLC coincide with the sequence reported for murine β-NGF[a]

Fractions	Sequence[b]
I	SSTXPVFXMXEFXVX
II	SSTHPVFHMGEFSVX
III-1	MGEFSVXDSVSVWVG
III-2	SSTHPVFHMGEFSVX

[a] Fraction III is composed of two molecular β-NGF species: one with intact NH_2-terminal sequence and other one that has lost the NH_2-terminal octapeptide.

[b] X represents the cycle of the amino acid sequence where it was not possible to determine the correspondent amino acid.

Table 4
The electrophoretic analysis reported low detection levels of renin and gammaglobuline in obtained samples of washing steps of the initial ion-exchange column[a]

Detection levels of impurities (μg)	
Total	0.584
Renin	0.130
IgG	0.110

[a] These impurities were not detected by SDS–PAGE in samples of our final product.

3.2.5. Determination of the purity grade in the purification of β-NGF

The percent of purity by SDS–PAGE in the evaluated β-NGF purifications was greater than 97% (Table 3), the same reported by Boehringer Mannheim for this protein and higher than reported

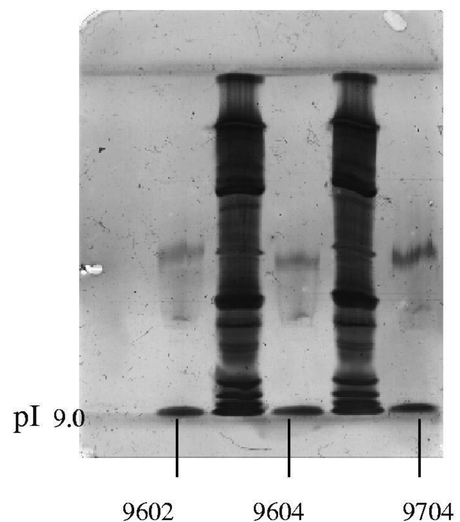

Fig. 4. The analysis by isoelectric focusing of evaluated murine β-NGF samples, demonstrated an isoelectric point of 9.0, similar to the one reported by Mobley.

Table 3
The percent of purity by SDS–PAGE in the evaluated β-NGF purifications was greater than 97%, higher than the reported (≥92%), because renin and gammaglobuline main impurities reported for the employed purification method were not detected in the electrophoretic analysis

Purifications of murine β-NGF	9602	9604	9704	Average
Mean	97.852	98.975	97.345	98.079
Standard deviation	1.413	2.050	0.615	1.552

(≥92%) by Mobley using the same purification method [3].

3.2.6. Determination of the isoelectric point of β-NGF

All β-NGF purifications evaluated by IEF (Fig. 4), showed that this protein has p*I* of 9.0, similar to the one reported by Mobley [7].

3.3. Immunological characterization

The EIA results showed the ability of the method employed to detect the β-NGF molecule and effectively block, using the monoclonal anti-mouse-β-NGF 27/21 antibody, the activity of the molecule in a biological assay. Fractions I, II and III obtained by RP–HPLC were recognized as β-NGF. These results showed that all molecular species present in the evaluated samples were inmunoreactives and keep their inmunogenic properties, in spite of modifications in their amino acid sequence.

The Western blot results also showed immunological recognition of the three molecular species in the β-NGF sample (Fig. 5)

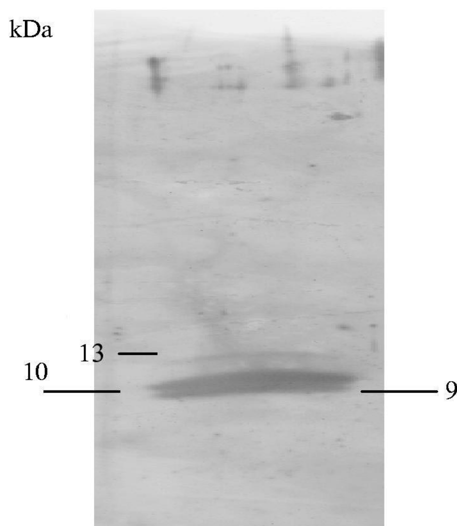

Fig. 5. The three molecular species present in the murine β-NGF samples, identified by RP–HPLC were inmunologically recognized by Western blot.

3.4. Biological characterization

The biological assay performed with the β-NGF samples reported that the biological activity unit was in the 1.5–3 ng/ml β-NGF range. Fractions I, II and III obtained by RP–HPLC showed biological activity.

4. Conclusions

The NH_2-terminal sequence of the murine β-NGF obtained in our laboratories coincides with the one previously reported for this protein. The obtained protein consists of a mixture of molecules of β-NGF which are intact at their NH_2-terminal extreme, and molecules which have lost the NH_2-terminal octapeptide and show some modification increasing its hydrophobicity. All these molecular species were recognized immunologically and showed biological activity. The results obtained in the characterization studies of our protein are similar to the ones reported by Mobley for the purification method employed. These results support the use of murine β-NGF obtained in our laboratories in preclinical studies that evaluate its potentiality as a therapeutic agent.

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