Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

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Gel filtration of the Nerve Growth Factor at different stages of purification

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **48** (1970), n.4, p. 446–450.

Accademia Nazionale dei Lincei

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Chimica biologica. — *Gel filtration of the Nerve Growth Factor at different stages of purification* (*). Nota di VINCENZO BOCCHINI, presentata (**) dal Corrisp. F. CEDRANGOLO.

RIASSUNTO. — Il Nerve Growth Factor (NGF) è una proteina specifica per la crescita e la differenziazione del sistema nervoso simpatico. Essa è stata purificata 120 volte dalle ghiandole salivari sottomascellari di topo maschio adulto e caratterizzata nella sua composizione in aminoacidi e in alcune proprietà chimico–fisiche. Il peso molecolare della proteina pura, misurato alla ultracentrifuga è risultato essere 30.000. Tale valore è notevolmente più basso rispetto a quello di 90.000 calcolato per la proteina nativa. I risultati di esperimenti di filtrazione su gel di Sephadex G–100 riportati nel presente lavoro indicano che tale variazione di peso molecolare è da attribuirsi ad una rottura della molecola nativa che si verifica in seguito a cromatografia a scambio ionico su carbossi–metil–cellulosa a pH 5.0; tale cromatografia è l'ultima tappa della procedura di purificazione. Le proprietà biologiche del NGF a peso molecolare 30.000 sono identiche a quelle della proteina nativa.

The Nerve Growth Factor (NGF) is a protein which specifically promotes the growth and differentiation of sensory and sympathetic nerve cells [1, 2]. When injected into newborn mice it produces an increase in size of the superior cervical ganglia; its effect *in vitro* can be demonstrated by a nerve fibers outgrowth from sympathetic or sensory ganglia explanted from chick embryos. The NGF was recently purified from adult mice salivary glands [3] and characterized for the amino acid composition and physicochemical properties [4]. The molecular weight of the pure protein, determined at the ultracentrifuge, was established as 30,000 [3]. This value was considerably lower compared with the 90,000 m.w. estimated by gel filtration experiments for the native protein [3]. In the present study gel filtration experiments have been performed in order to investigate which step of the purification procedure could account for the splitting of the native protein which resulted in such a change in the molecular size.

MATERIALS AND METHODS.

The NGF was purified according to the procedure previously described [3]. Table I reports briefly the purification procedure which involved the following steps: i) homogenate in distilled water of the salivary gland followed by addition of streptomycin sulfate and centrifugation; ii) gel filtration on

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(**) Nella seduta dell'11 aprile 1970.

Sephadex G–100 at *p*H 7.5 of the total extract; iii) dialysis at *p*H 5.0 of the G–100 pool; iv) ion-exchange chromatography on carboxy-methyl-cellulose of the G–100 pool at *p*H 5.0. In this last step elution was performed by salt gradient (0–1.0M NaCl) at a constant *p*H 5.0: under these conditions all the NGF activity was associated with the last protein peak. This protein fraction did not show any heterogeneity when examined by several analytical tests [3, 4].

Gel filtration experiments were performed on Sephadex G–100 (Pharmacia, Uppsala, Sweeden). Swelling of the resin and packing of columns were done according to the instructions given by the manufacturer; calibration of columns and evaluation of the molecular size were done as indicated by Andrews [5].

The NGF activity was assayed *in vitro* according to the technique of Levi–Montalcini *et al.* [6]. Proteins were estimated by Lowry's method [7].

TABLE I.

Relative values of biological activity and protein recovery at different steps of the NGF purification.

FRACTION	NGF biological activity	Protein	Extent of purification
Gland extract	100	100	1
G–100 pool, *p*H 7.5	80	13	6
G–100 pool, *p*H 5.0	64	10	6
CM pool, *p*H 5.0 (pure NGF)	45	0.4	120

RESULTS AND DISCUSSION.

Samples from each purification step indicated in Table I were separately loaded on Sephadex G–100 columns equilibrated at the proper *p*H. Results of these experiments are reported in fig. 1. The total extract, not reported in fig. 1, behaved like the G–100 pool at *p*H 7.5 regarding to NGF activity distribution along the elution pattern. The native NGF in the G–100 pool either at *p*H 7.5 or 5.0 emerged from the column considerably earlier that the purified NGF. An approximate estimation indicated as 95,000 and 70,000 the molecular size of the native NGF at *p*H 7.5 and 5.0 respectively. Both these values were considerably higher compared with that of about 24,000 calculated for the pure NGF collected after chromatography on carboxy-methyl-cellulose at *p*H 5.0. Therefore, these results clearly indicated that a complete splitting of the native NGF molecule occurred only after ion-exchange

chromatography on carboxy-methyl-cellulose at *p*H 5.0. Similar result was obtained by Varon *et al.* [8, 9] under somewhat different conditions. Lowering the *p*H from 7.5 to 5.0 apparently partially reduced the molecular size.

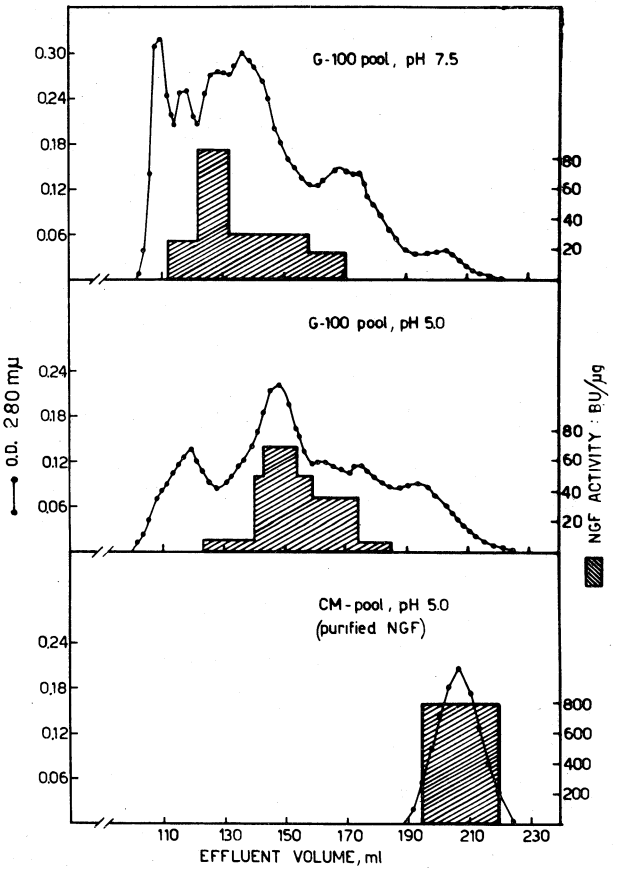

Fig. 1. – Gel filtration on Sephadex G–100 of the NGF at different stages of purification. Protein loaded was 23 mg for the G–100 pool at *p*H 7.5, 15 mg for the G–100 pool at *p*H 5.0 and about 2 mg for the CM–pool. Column dimensions 2 × 115 cm. See text for other details.

Analytical ultracentrifugation studies by Varon and co-workers [8] have demonstrated that the sedimentation properties of the native NGF did not change in the *p*H range 5 to 8. Therefore it is possible that the difference in the elution volume of the NGF activity from the Sephadex column observed between the G–100 pool at *p*H 7.5 and 5.0 (see fig. 1) could reflect a difference in shape, due to the *p*H change, rather than a true difference in molecular size.

It has been previously reported [3] that lowering the *p*H of the G–100 pool from 7.5 to 5.0 caused a precipitate which contained 20–25 % of the

original amount of both protein and NGF activity. The precipitate formed during dialysis from *p*H 7.5 to *p*H 5.0 of the G–100 pool used for the experiments described in fig. 1 was resuspended in 5×10^{-2} M Tris. HCl *p*H 7.5, dialyzed for 24 hours vs. the same buffer and then loaded on a Sephadex G–100 column. Fig. 2 shows that the NGF activity was mostly collected along

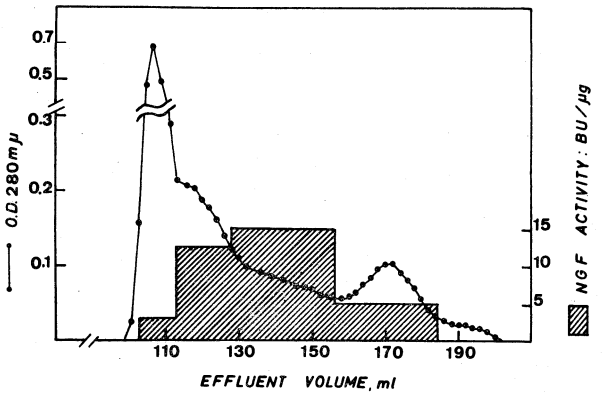

Fig. 2. – Gel filtration on Sephadex G–100 of the precipitate formed during dialysis of the G-100 pool from *p*H 7.5 to 5.0. The precipitate was resuspended in 5×10^{-2}M Tris. HC buffer *p*H 7.5 and then dialyzed vs. the same buffer. Protein loaded 8 mg. Column dimensions 2×115 cm.

the elution pattern at a position which was approximately the same as that of the native NGF at *p*H 7.5 (see fig. 1). Therefore it seems very likely that the precipitate formed during dialysis of the G–100 pool from *p*H 7.5 to 5.0 contained some of the native NGF form; this possibility is also supported by recent data which indicated as 4.6 the isoelectric point of the native NGF [10].

The 30,000 m.w. NGF has biological properties which are identical to those of the native NGF [3]. Studies performed by Shooter *et al.* [8, 9, 11, 12] have demonstrated that the native NGF molecule is an aggregate made of different protein species called α–, β– and γ–subunits. The same Authors have also reported under which conditions these subunits can be separated one from the other and then recombined to form again the native NGF molecule [8, 9]. The 30,000 m.w. NGF, purified as above indicated, has physicochemical properties which are very similar to those described for the β–subunit, claiming for a substantial identity of the two proteins [3, 4].

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