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Purification of a new neurotrophic factor from mammalian brain

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We report the purification from pig brain of a factor supporting the survival of, and fibre outgrowth from, cultured embryonic chick sensory neurons. The purified factor migrates as one single band, mol. wt. 12 300, on gel electrophoresis in the presence of sodium dodecylsulphate (SDS) and is a basic molecule (pI ≥10.1). Approximately 1 μg factor was isolated from 1.5 kg brain. The final degree of purification was estimated to be 1.4 x 10^6-fold, and the specific activity 0.4 ng/ml/unit, which is similar to that of nerve growth factor (NGF) using the same assay system. This factor is the first neurotrophic factor to be purified since NGF, from which it is clearly distinguished because it has different antigenic and functional properties.

Key words: neurotrophic factor/sensory neurons/development

Introduction

Considerable evidence from transplantation and ablation experiments indicates that developing neurons require factors they receive from their environment for survival and development (Jacobson, 1978). The most direct demonstration for the involvement of specific factors is that antibodies to a well-characterized protein, nerve growth factor (NGF), when injected into perinatal mammals destroy peripheral sympathetic and sensory neurons; conversely, injection of NGF rescues neurons that are normally eliminated during development (for reviews, see Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). The epigenetic control of neuronal survival can also be demonstrated *in vitro*: NGF or other factors present in tissue extracts and media conditioned by various cells must be present if neurons are to survive in culture (for review, see Varon and Adler, 1981). So far, however, no defined molecule has been isolated from either conditioned media or tissue extracts; indeed, not even NGF has been characterized from a tissue or fluid relevant to the physiology of the developing nervous system (see Thoenen and Barde, 1980 for an extensive discussion). The chemical information about NGF is entirely derived from preparations purified from tissues such as the submandibular gland of the adult male mouse where it is present in large amounts for unknown reasons.

We have previously described the presence of a factor in glioma-conditioned medium which supports the survival of embryonic sensory neurons (Barde *et al.*, 1978) and can be distinguished from NGF by immunological and functional criteria. Furthermore, it was demonstrated that mammalian brain contained an activity similar to that of glioma-conditioned medium (Barde *et al.*, 1980). We now describe the purification of a neuronal survival factor from mammalian brain. Its mol. wt. was determined to be 12 300 with an isoelectric point ≥10.1.

Results

Figure 1 shows a flow chart of the purification procedure that includes six steps.

Steps I–III

To determine the initial activity, the homogenate was centrifuged for 40 min at 20 000 *g* (a step not normally included in the purification procedure) and the supernatant tested for activity. The total activity was lower than in the supernatant after acidification to pH 4.5. This could be due to the removal of inhibitors likely to be present in the initial homogenate since it has been observed that high concentrations of this homogenate are toxic to the neurons. This phenomenon disappears during the course of the purification. After acidification and centrifugation, most of the ac-

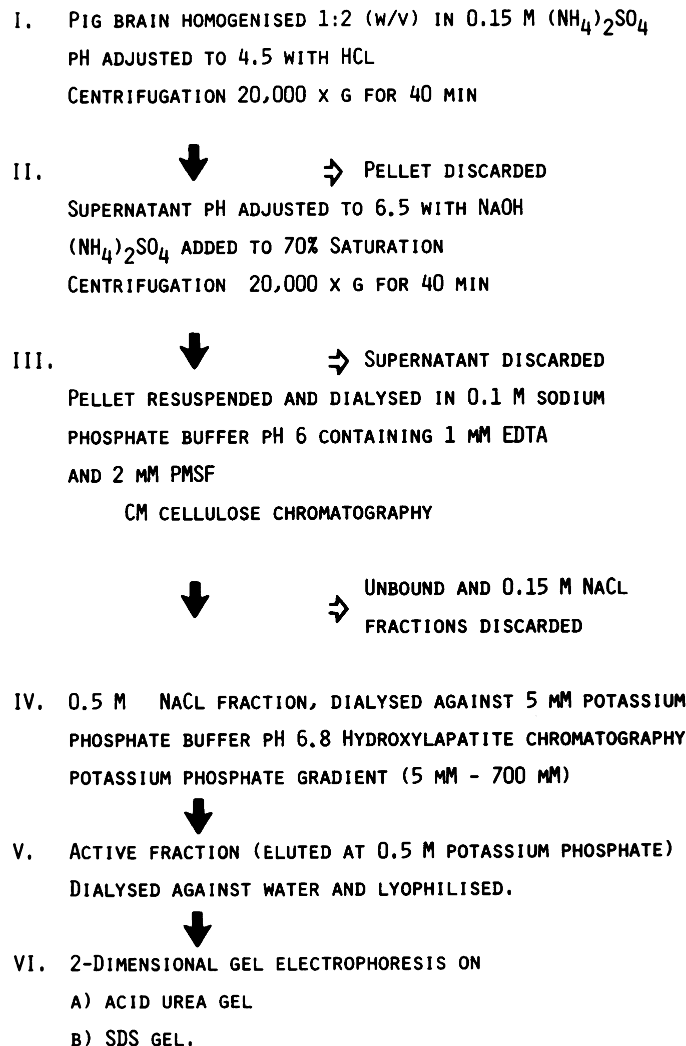

Fig. 1. Flow chart of the purification procedure.

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Table I. Purification stages for the neurotrophic factor from 3 kg pig brain

	Stage	Protein (μg)	Specific activity (ng/ml per U)	Purification factor (fold)	Yield (%)
I	homogenate	5.2×10^7	5×10^5	1	100
II	supernatant	2.8×10^7	1.5×10^5	3.3	179
III	70% $(NH_4)_2SO_4$	1.8×10^7	1.5×10^5	3.3	115
IV	CM-cellulose	3.5×10^4	2.5×10^3	2×10^2	13.5
V	hydroxylapatite	555	100	5×10^3	5.3
VI	two-dimensional gel electrophoresis	2	–	1.4×10^6[a]	–

At each stage the activity was assayed as described in Materials and methods. 1U is defined as the protein concentration (in ng/ml) which supports the survival of half the maximum number of neurons surviving in response to a saturating concentration of factor. For stages I – V, proteins were measured according to the method of Lowry *et al.*, (1951) with bovine serum albumin as standard. The amount of protein after stage VI was determined by re-electrophoresis of an aliquot of the active fraction (see text) and comparison of the intensity of silver-stained bands (Oakley *et al.*, 1980) using different amonts of cytochrome C as standards.
[a]This purification factor is not derived from the measurement of the specific activity at this point but only on protein measurements, and based on the assumption mentioned in the text.

tivity can be precipitated by ammonium sulphate. The pellet was resuspended in a buffer containing EDTA and phenylmethyl sulfonyl fluoride (PMSF) and was stored until all the material to be applied to the CM-cellulose (step IV) could be collected (3 x 1 kg starting material). Large decreases in the specific activity were observed upon storage without protease inhibitors.

Step IV

This chromatography step on CM-cellulose could be performed routinely without testing the fractions for activity, because proteins were eluted step-wise, and the 0.5 M NaCl eluate, a clearly defined peak of optical density at 280 nm (OD_{280}), contained the activity. While a large purification factor (see Table I) was obtained, there was also a considerable decrease in total activity. Part of the explanation for this low yield is unspecific adsorption to the CM-cellulose (often observed for very basic proteins), and re-use of the same material in the course of several purifications progressively increased the yield (data not shown).

Up to this step, it is interesting to note that the procedure has striking similarities to those used for the purification of pituitary fibroblast growth factor (Gospodarowicz, 1975), glial growth factor (Brockes *et al.*, 1980), and the procedure we use to isolate NGF from male mouse submandibular gland (Suda *et al.*, 1978).

Step V

As Figure 2a shows, most of the material eluted from the CM-cellulose bound to hydroxylapatite at low ionic strength. The activity was then eluted well after the major protein peak when a linear potassium phosphate gradient was applied to the column. The activity was present in the fractions eluting with ~0.5 M potassium phosphate (see Figure 2a). Because the activity corresponds to only a minor part of the total proteins (see Table I and Figure 2b), it did not elute with any OD_{280} peak, making it necessary to test several fractions for activity. By this stage, when a purification factor of several thousand fold was attained, the material was still very heterogeneous when analysed by SDS-gel electrophoresis (Figure 2b). However, we observed (see below and Materials and methods) that activity could be recovered after electrophoresis in the presence of 0.1% (w/v) SDS, and was present in only one slice corresponding exactly to the mol. wt. of horse heart cytochrome C. This observation led us to use electrophoresis for the final purification (see below, step VI). Isoelectric focusing performed with the material obtained at this stage (see Materials and methods) indicated a pI of 10.1 or greater. Indeed, the activity was obtained in the most alkaline slice of the gel. This estimation is in good agreement with the observation that on the first dimension of the two-dimensional gel system described below, the activity was found to migrate ahead of horse heart cytochrome C (pI = 10.0) and approximately like egg white lysozyme (pI = 10.5).

Step VI

This step is a two-dimensional gel electrophoresis. As a separating system for the first dimension, we used that described by Reisfeld *et al.* (1962) for basic proteins with the addition of 6 M urea to the gel. This system was more satisfactory than isoelectric focusing because at this stage of the purification the material consisted mainly of basic proteins which could not be adequately separated by isoelectric focusing. After electrophoresis and equilibration in the SDS-buffer (see Materials and methods), the lane from the first dimension was re-electrophoresed in the presence of SDS (second dimension). The second gel was then sliced into pieces of area 1.5 x 4 mm and thickness 3 mm at the position of the activity in terms of mol. wt., using cytochrome C as a visible marker (see Materials and methods). The proteins were then electrophoresed out of the slices and aliquots of the solution thus obtained were tested for activity (see Materials and methods). Several slices were tested since there are no visible markers indicating the position of the activity after the separation of the first dimension. The protein content of the active slice was determined by re-running an aliquot on an SDS-gel and comparing the intensity of silver staining with that of known amounts of cytochrome C on the same gel. This procedure further indicated that the final material is homogeneous, using gel electrophoresis in the presence of SDS as a criterion (see Figure 3).

After removal of SDS and urea, the restoration of activity was not complete, thus preventing a direct determination of the specific activity: when the material obtained after step V was subjected to treatment with urea and SDS ~5% of the activity was recovered. In spite of this denaturation, the specific activity is high enough at this stage of the procedure to allow the detection of the activity after the two-dimensional gel electrophoresis. Due to this partial denatura-

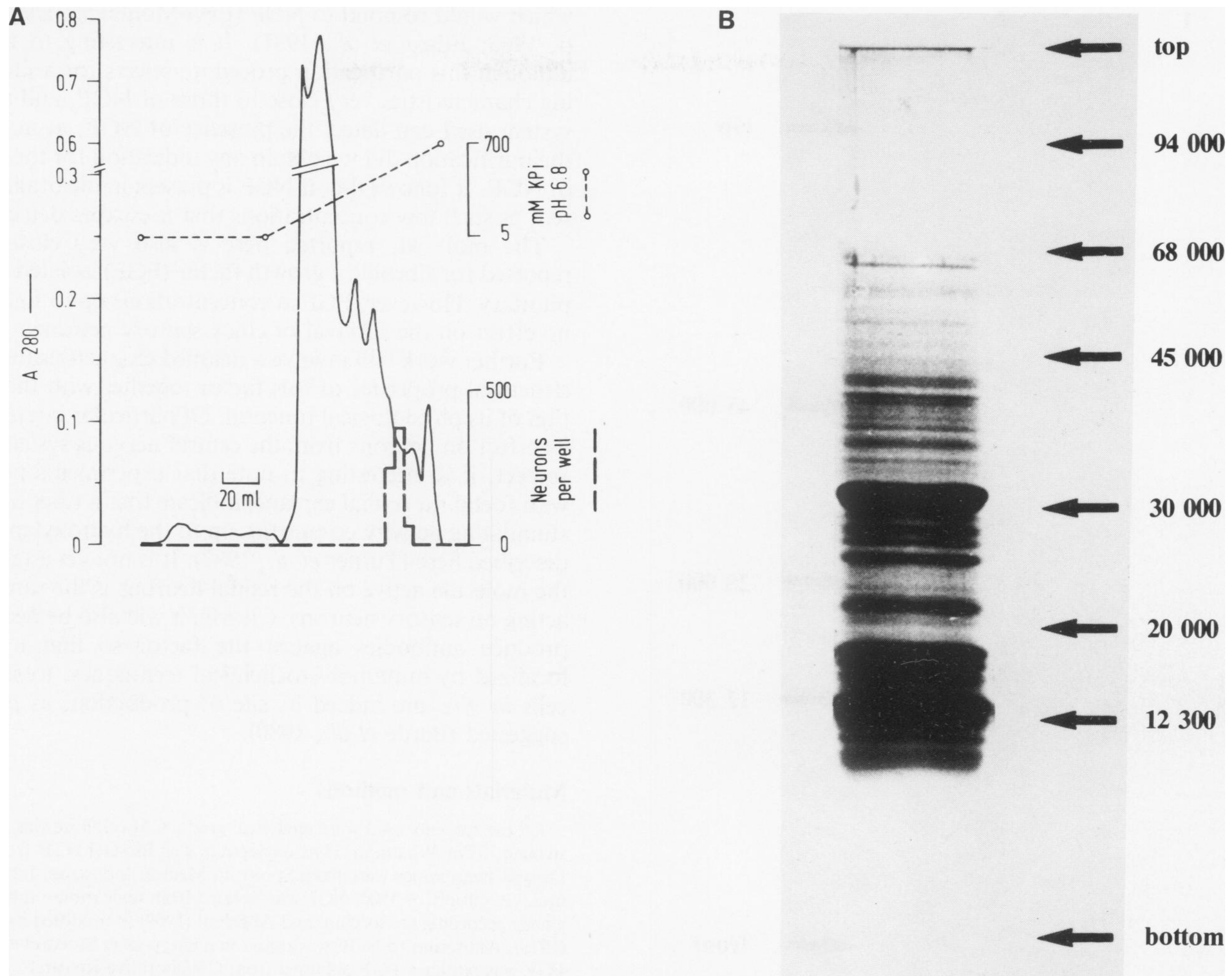

Fig. 2. **(A)** Elution profile from the hydroxylapatite column (stage V). Conditions are as described in Materials and methods. All the fractions were tested for activity at a protein concentration of 1 μg/ml. No activity was detected in the main protein peak even at 10 μg/ml. KPi: potassium phosphate buffer. **(B)** SDS-gel profile of the most active fraction eluted from the hydroxylapatite column. 50 μg protein was dissolved in Laemmli (1970) sample buffer (containing no mercaptoethanol) and applied on a 10–25% exponential polyacrylamide gel gradient. To increase the resolution in the low mol. wt. region, this gel was run for ~40 additional min after the bromophenol blue front had reached the bottom of the gel. The mol. wt. markers used were: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor and cytochrome C. The gel was stained with Coomassie blue.

tion, the overall degree of purification cannot be calculated from the final specific activity. However, if it is assumed that all the activity would be represented by the band obtained after re-electrophoresis, then the protein content of that band indicates a purification factor of ~ 1.4×10^6 fold at the end of the procedure. Using the same assumption, a final specific activity of 0.4 ng/ml per U may also be calculated.

Biological activity of the purified factor

Table II shows the effect of the purified factor on the survival of sensory neurons. It can be seen that the factor alone supports the survival of 20% of the cells plated (counted as neurons after 2 days of culture). When the factor, used at saturating concentrations, was combined with NGF (also used at saturating concentrations), their effect was additive. It is interesting to note that the properties of the purified factor are thus similar to those reported for extracts of rat brain (Barde *et al.*, 1980). The effect of the purified factor was not blocked by the addition of antiserum to NGF (Table II).

Discussion

The purification procedure described here leads to the isolation of ~2 μg protein from 3 kg pig brain. The activity migrates as one band on SDS-polyacrylamide gel electrophoresis, where its mol. wt. was determined to be 12 300. This value corresponds to the mol. wt. determined by gel filtration (on Bio-gel P30 or P60, or by h.p.l.c.) in the absence of SDS (data not shown). The active molecule is thus likely to be a monomer (in contrast to NGF), but further work is needed to establish this point firmly, when more of the purified material is available. A very large overall degree of purification was necessary to obtain homogeneity as shown by SDS-gel electrophoresis: a purification factor of 1.4×10^6-fold may be estimated (see Results and Table II). This is because the specific activity of the factor is high: the calculated value of 0.4 ng/ml per U (3×10^{-11} M) is essentially identical to that of NGF using the same assay system.

Although the factor characterized here has striking similarities in terms of mol. wt. and isoelectric point to the monomeric chain of NGF from mouse, guinea pig, or bull (see Server and Shooter, 1977; Chapman *et al.*, 1979; Harper *et al.*, 1982), it is clearly a different entity: at no stage of the purification was it possible to block or reduce its activity by the addition of antibodies to mouse NGF (see Table II), whereas all mammalian NGFs can be blocked by antibodies to mouse NGF (Harper and Thoenen, 1981). Furthermore,

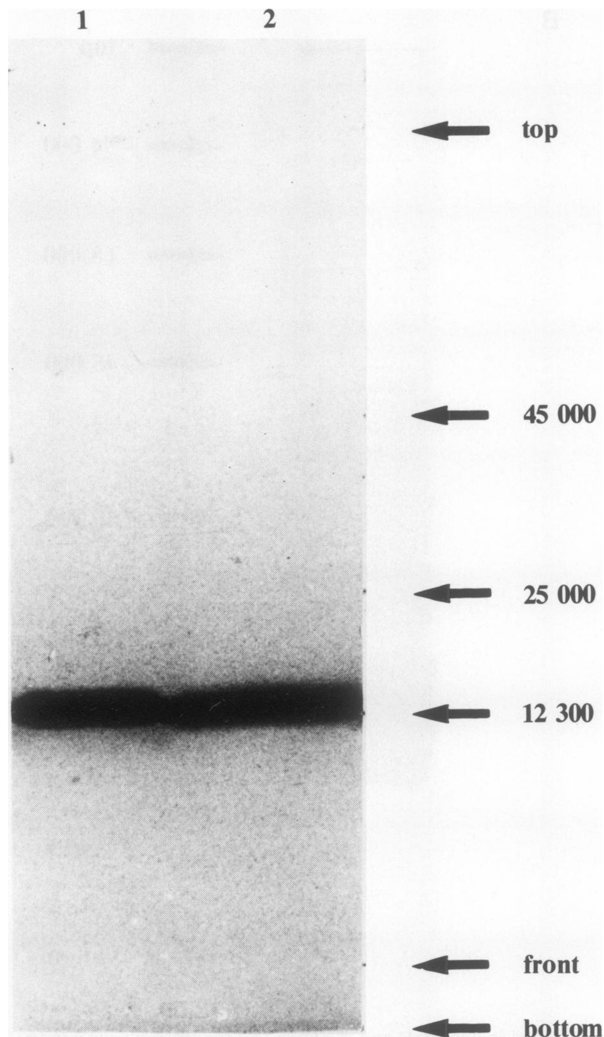

Fig. 3. SDS-gel electrophoresis of the purified factor. **Lane 1** is 0.5 μg of horse heart cytochrome C. **Lane 2** is the purified factor as obtained after stage VI. Top and bottom of the separating gel are indicated by the arrows, as well as the bromophenol blue dye front. The mol. wt. standards used were ovalbumin, chymotrypsinogen A, and cytochrome C. Conditions are as in Figure 2 except that this gel was stained with silver (Oakley *et al.*, 1980).

Table II. Survival of cultured spinal sensory neurons

Addition	Concentration (ng/ml)	Number of surviving neurons (total/well ± s.d.)
control	–	0
NGF	5	1779 ± 205 (n = 3)
factor	40	1195 ± 180 (n = 3)
factor + antiserum (1:1000)	40	1043 ± 54 (n = 2)
factor + NGF	40 + 5	2981 ± 107 (n = 3)

6000 cells were plated and surviving sensory neurons counted after 2 days of culture. The concentrations of NGF and the purified factor (obtained after stage VI) were saturating. The NGF sheep antiserum blocks the effect of 50 ng/ml NGF at the dilution used. It does not block the effect of the factor even if its concentration is increased 5-fold (1:200).

when saturating concentrations of NGF and the factor described here were combined, an additive effect on neuronal survival was obtained (Table II). Finally, this factor does not support the survival of 12-day-old chick sympathetic neurons, which would respond to NGF (Levi-Montalcini and Angeletti, 1968; Edgar *et al.*, 1981). It is interesting to note that although this purification procedure selects for a factor having characteristics very close to those of NGF, and the assay system used can detect the presence of NGF, at no stage of the purification did we obtain any indication for the presence of NGF. It follows that if NGF is present in the brain it is present at such low concentrations that it escapes detection.

The mol. wt. reported here is also very close to that reported for fibroblast growth factor (FGF) isolated from the pituitary. However, FGF at concentrations up to 1 μg/ml had no effect on the survival of chick sensory neurons.

Further work will involve a detailed characterization of the structural properties of this factor together with the delineation of its physiological function. Of particular interest will be its effect on neurons from the central nervous system. In this respect, it is interesting to note that experiments performed with foetal rat retinal explants indicate that a fiber outgrowth stimulating activity co-purifies up to the hydroxylapatite step described here (Turner *et al.*, 1982). It is not yet established if the molecule active on the retinal neurons is the same as that acting on sensory neurons. Clearly, it will also be necessary to produce antibodies against the factor so that it may be localized by immunohistochemical techniques, to see if glial cells *in vivo* are indeed its site of production, as previously suggested (Barde *et al.*, 1980).

Materials and methods

All the reagents used were analytical grade. CM-cellulose was CM-52 preswollen, from Whatman. Hydroxylapatite was Bio-Gel HTP from BioRad. Dialysis membranes were from Spectrum Medical Industries, Inc. and had a mol. wt. cut-off of 3500. NGF was isolated from male mouse submandibular glands according to Bocchini and Angeletti (1969) as modified by Suda *et al.* (1978). Antiserum to NGF was raised in a sheep as in Stoeckel *et al.* (1976). FGF was pituitary FGF obtained from Collaborative Research.

Culture of neurons

The assay system used to determine the biological activity estimates the survival of cultured spinal sensory neurons from 10-day-old chick embryos, as described by Barde *et al.* (1980). 3000 – 6000 cells were plated and surviving neurons counted after 48 h of culture. As described, under control conditions (F14 medium + 10% v/v horse serum) no neurons survived after 48 h. The activity of each sample was determined by making serial dilutions, a unit being defined as that protein concentration (ng/ml) which supports the survival of half the maximum number of neurons that would be supported by a saturating concentration of factor, which at this stage of development is ~2/3 of that observed with NGF (see Barde *et al.*, 1980 and Table II).

Purification procedure

Whole pig brains obtained fresh from a local slaughter house were stored frozen at −70°C until required, when they were warmed overnight to 4°C. All subsequent steps were performed at 4°C unless otherwise stated. 1 kg brain was homogenized in 2 l of 0.15 M ammonium sulphate using a Waring blender, the pH was brought to 4.5 by the addition of 1 M HCl and the homogenate stirred for 2 h. After centrifugation (20 000 *g*, 40 min), the pH of the supernatant was brought to 6.5 with 1 M NaOH and solid ammonium sulphate was added over a period of 2 h to a saturation of 70%. After centrifugation (20 000 *g*, 40 min), the pellet was resuspended with 80 ml 0.1 M sodium phosphate buffer pH 6.0, containing 1 mM EDTA and 2 mM PMSF (freshly diluted from a 200 mM stock solution in ethanol). The suspensions were stored until those corresponding to 3 kg starting material could be pooled and then dialysed against 0.1 M sodium phosphate buffer, pH 6.0, (3 x 10 l over 20 h). All dialyses utilised bags with a mol. wt. cut-off of 3500. The resulting solution (~600 ml) was applied to a CM-cellulose column (600 ml bed volume, diameter 4.5 cm, flow rate 120 ml/h) equilibrated with the phosphate buffer. After washing until the OD_{280} was <0.1, a first step of 150 mM NaCl was included in the buffer and the column eluted until the OD_{280} was <0.05. The fraction eluted with a subsequent step of 500 mM NaCl was then collected.

The 500 mM NaCl eluate from the CM-cellulose column (~150 ml) was dialysed against 5 mM potassium phosphate buffer pH 6.8 (2 x 5 l, overnight) and applied to a hydroxylapatite column (12 ml bed volume, diameter 9 mm,

flow rate 20 ml/h). Proteins were eluted with a 100 ml linear gradient of potassium phosphate buffer pH 6.8 (5–700 mM), and 5-ml fractions were collected. The activity recovered was present in the fractions eluted with ~500 mM potassium phosphate. The active material was exhaustively dialysed against H_2O and lyophilized. 250 μg protein (corresponding to about half the total material obtained at this stage) was dissolved in 200 μl, 0.25 M potassium acetate buffer, pH 5.8, containing 6 M urea and Pyronin Yellow as tracking dye, and applied in one slot of a 10% (w/v) polyacrylamide slab gel, 14 x 7 x 0.3 cm, (overlayed with a 1.5-cm stacking gel) at pH 4.5 containing 6 M urea, as described by Reisfeld *et al.* (1962). After electrophoresis at 35 mA for ~5.5 h at room temperature, when the dye front was ~7 mm from the gel bottom, the entire lane (without stacking gel) was cut out and equilibrated at room temperature for 1 h with sample buffer (Laemmli, 1970), modified so that the SDS concentration was reduced to 0.1% (w/v) and no mercaptoethanol (which completely destroys the activity) was added. The lane was then placed horizontally onto an exponential gradient, (10–25%) polyacrylamide SDS slab gel, 14 x 7 x 0.3 cm, overlayed with a stacking gel as described by Laemmli (1970). 60 μg of horse heart cytochrome C (Serva) dissolved in the modified Laemmli buffer was added on both sides of the excised lane and electrophoresis was carried out at room temperature overnight at 12 mA. The gel was sliced at the level of cytochrome C to give pieces of area 1.5 x 4 mm, thickness 3 mm. The proteins were then electrophoresed out of the slices (see below) and aliquots of the solutions thus obtained were re-electrophoresed to determine the protein content of the active slice. To determine the location of the biological activity, horse serum and urea were added to final concentrations of 5% (v/v) and 6 M, respectively, and SDS quantiatively removed by ion exchange according to the method of Weber and Kuter (1971), except that mercaptoethanol was omitted. The samples were then dialysed at 4°C firstly overnight against 5 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, and then for 6 h against the F-14 tissue culture medium used in the bioassay. As for all tests of biological activity, horse serum was then added to 10% (v/v), and the samples sterile-filtered before being diluted serially and used to culture spinal sensory neurons.

Recovery of the proteins from the gel

Proteins were electrophoresed out of the gel slices according to the following procedure: 10% (w/v) polyacrylamide gels prepared in upper buffer (Laemmli, 1970) were cast in glass tubes 4 mm in diameter and 12 cm in length. The gels were 5 cm long overlayed on a 150 μl 1.5 M sucrose solution in water. After polymerization, the sucrose solution was removed and replaced by SDS electrophoresis buffer (Laemmli, 1970). The tubes were closed tightly at the bottom by a piece of dialysis membrane with a mol. wt. cut-off of 3500. The gel pieces (after a brief incubation in a drop of 0.01% v/v bromophenol blue solution) were placed on top of the gel. Electrophoresis was carried out at 2 mA/tube for ~3 h and was stopped after the bromophenol blue had been electrophoresed from the gel. On such gels, proteins with a mol. wt. corresponding to that of cytochrome C are not separated from bromophenol blue. The eluted material was collected in a syringe by penetrating the dialysis membrane. Aliquots were either reapplied to an SDS gel or tested for biological activity after removal of SDS as described above.

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