Purification of a Sensory-Specific Protein of 35 kDa (SSP-35)

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Protein purification is a key technique for the identification of novel sensory nerve-specific proteins. A 35 kDa protein, the sensory neuronal specific protein, was isolated and purified from rabbit spinal ganglia and sensory fibers by homogenizing and deriving soluble extracts, followed by ion exchange chromatography using DEAE-Sephacel and gel filtration HPLC. Western blot analysis showed that the protein was present in spinal sensory ganglia but not in spinal motor neurons with anti-rat 35 kDa protein polyclonal antibody. The sensory-specific protein 35 kDa is termed SSP-35 in this paper. We found that the purified SSP-35 promoted axonal growth of the dorsal root ganglia of chick E8 embryos. Our data reveal that the protocol is an effective method for the purification of SSP-35. The protein may not only be a useful marker for sensory neurons, but also a possible tool to study the regeneration and function of sensory neurons.

Key words purification; sensory-specific protein 35 kDa (SSP-35); neurotrophic effect

Although many morphological, histologic, and electrophysiological studies have examined the distinction between the sensory and motor fasciculi of the peripheral nerves, these methods are time consuming and can not distinguish between both kinds of nerve fibers. Therefore, they are infrequently used clinically. Yet, the accurate and rapid identification of the two different kinds of nerve fibers is one of the most intriguing and important problems in microsurgery. Sensory neurons are a major derivative of the neural crest, for which no definitive molecular markers have been identified in mammals.¹⁾ To identify a novel sensory nerve-specific protein, purification of sensory-specific protein is necessary. In this study, we identified a 35 kDa protein from rabbit spinal ganglia and sensory fibers that is sensory neuron-specific. We examined the purification of 35 kDa protein by combining chromatography, and finally established a reproducible method for purification of the 35 kDa protein. This paper describes the purification and effect of 35 kDa on promoting axonal growth of the dorsal root ganglia (DRG) of chick E8 embryos, and termed this protein SSP-35 in this paper because of its presence in spinal sensory ganglia but not in spinal motor neurons.

MATERIALS AND METHODS

Chemicals and Animals Male New Zealand white rabbits, each weighing 2.5 kg, were obtained from the Academy of Military Sciences, Beijing, China. Phenylmethylsulfonyl fluoride (PMSF) was purchased from E. Merck, U.S.A.; a protein assay kit from Bio-Rad, U.S.A.; Ultrafree-CL with an Ultrafiltration membrane for a protein concentrating device (10-kDa cutoff) from Millipore, U.S.A.; an HPLC TSK G 2000 SW column (7.5×300 mm) from Tosoh, Japan; DEAE-Sephacel and sepharose-4B from Pharmacia, Sweden; Horseradish peroxidase-conjugated goat anti-rat IgG from Boehringer Mannheim, Germany; Duplecco's Modified Eagle's Medium (DMEM/F12) and fetal calf serum (FCS) from Gibco, U.S.A.; collagen type I from Sigma, U.S.A.; Culture bottles were from Nunc, U.S.A.; nerve growth factor (NGF, 2.5 S) from Pepro, U.K.; and Phase contrast microscopy was from Olympus, Japan.

Purification Procedure Step 1. Dissection of Spinal Sensory Ganglia: New Zealand white rabbits were anesthetized with pentobarbital and then sacrificed by exsanguination. Spinal sensory ganglion dorsal root (sensory neurons) and ventral root (motor neurons) were dissected under a dissecting microscope.

Step 2. Preparation of Homogenates: The two pooled samples were placed on ice and homogenized using a Polytron Apparatus (Academy of Military Medical Sciences, Beijing, China) at the setting of 10 for 2×5 min in 10 volumes of 20 mM phosphate buffer (PB) pH 7.4 containing 1 mM EDTA and 0.1 mM PMSF. Homogenates were centrifuged at 8000×*g* for 10 min, and samples of the resulting two supernatants (crude extracts) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)²⁾ and visualized with silver stain.³⁾

Step 3. DEAE-Sephacel Column Chromatography: Sensory neuron supernatant of the crude extracts obtained in Step 2 was applied to a DEAE-Sephacel column (2.0×7.0 cm), and equilibrated with 20 mM PB (pH 7.4) containing 1 mM EDTA. The column was washed extensively with the same buffer to remove the unbound protein, and the adsorbed protein was eluted by a linear gradient of 0.05 to 0.30 M NaCl in the equilibration buffer (total volume 200 ml) at a flow rate of 60 ml/h.

Step 4. HPLC TSK G 2000 SW Column Chromatography: 35 kDa protein fractions were pooled and concentrated using an Ultrafree-CL with an ultrafiltration membrane. The concentrated sample was then gel-filtrated on a TSK G 2000 SW column (7.5×300 mm) equilibrated at a flow rate of 1 ml/min with 40 mM PB (pH 6.0) containing 0.1 M NaCl and 0.01% NaN₃. Fractions containing 35 kDa protein were pooled and used as purified SSP-35 in further studies.

Identification of SSP-35 SSP-35 protein elution was determined by the following two methods: (i) Aliquots of fraction were resolved by SDS-PAGE and stained with silver. A 35 kDa band, which appeared in spinal sensory ganglia, was compared to the motor neuron. (ii) Using polyclonal antibodies to 35 kDa protein, elution of the 35 kDa protein was directly monitored by means of Ouchterlony immunodiffusion⁴⁾ and Western blot.⁵⁾

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Preparation of Anti-rabbit SSP-35 Polyclonal antibodies to 35 kDa protein were raised in rats by subcutaneous injections of purified 35 kDa (50 μ g/rat) emulsified with complete Freund's adjuvant and purified by column chromatography using 35 kDa-Sepharose 4B.

Protein Determination Protein was quantified using a protein assay kit according to the manufacturer's instructions using bovine serum albumin as the standard.

Neurotrophic Effect DRG were dissected from E8 chicken under a dissecting microscope. The ganglia were cultured in DMEM/F12 medium containing 10% FCS on a collagen-coated tissue culture bottle. Cultures were maintained in a 5% CO₂ 95% air atmosphere at 37 °C. After a 2-h incubation, NGF (2.5 S) as positive control, purified 35 kDa protein and no 35 kDa peak fraction by gel filtration chromatography were added to final concentrations of 30, 60, and 60 ng/ml, respectively; as above, the same volume of DMEM/F12 medium also was added as a native control. The cultures were then incubated 24 h. Observation and photography were performed under phase contrast microscopy.

Western Blot Reduced samples were resolved by SDS-PAGE using the method of Laemmli,²⁾ and were Western blotted using polyclonal antibodies to SSP-35 by the method of Towbin *et al.*⁵⁾

RESULTS

A 35 kDa Protein in Sensory Ganglia The homogenized soluble extract of spinal sensory ganglia contained 35 kDa protein that was absent from the motor neuron extract (Fig. 1). The 35 kDa protein also was detected in extracts of spinal sensory ganglia but it was not detected in extracts of motor neurons by Western blot using polyclonal antibodies raised against the 35 kDa protein (Fig. 2). The 35 kDa protein was eluted at about 0.20 M NaCl in DEAE-Sepharose column chromatography (Fig. 3). Gel filtration finally yielded a puri-

fied 35 kDa band (Fig. 4). About 30 μ g of 35 kDa protein were reproducibly obtained from 500 mg of rabbit spinal sensory ganglia and their fibers.

Neurotrophic Effect of 35 kDa Protein The effect of 35 kDa on the cultured DRG of the E8 chicken was examined. Freshly prepared DRG were cultured in DMEM/F12

Fig. 2. Detection of SSP-35 by Western Blotting

Aliquots (10 μ l) of homogenized soluble extracts of spinal sensory ganglia and motor neuron were resolved by SDS-PAGE using 12.5% polyacrylamide gel. The gel was Western blotted using rat anti-rabbit SSP-35. Lane 1, protein molecular weight markers; lane 2, homogenized spinal motor neurons; lane 3, homogenized spinal sensory ganglia.

Fig. 1. A Comparison between Extracts of Spinal Sensory Ganglia and Motor Neurons

Aliquots (10 μ l) of homogenized soluble extracts of spinal sensory ganglia and motor neuron were resolved by SDS-PAGE using 12.5% polyacrylamide gel. Lane 1, protein molecular weight markers; lane 2, spinal motor neurons; lane 3, spinal sensory ganglia and roots.

B 25 26 35 36 39 41 43 45 kDa

Fig. 3. DEAE-Sepharose Column Chromatography (A) and SDS-PAGE (B)

A. Soluble extracts of spinal sensory ganglia were chromatographed on a column of DEAE-Sepharose as described in materials and methods. The 35 kDa protein was eluted with 200 mM NaCl. The fractions indicated with the black bar were collected. B. Aliquots (10 μ l) of the fractions 25, 26, 35, 36, 39, 41, 43, 45 were resolved by SDS-PAGE, using a 12.5% polyacrylamide gel and visualized with silver stain.

Table 1. Effect of SSP-35 on the Axonal Growth of DRG of Chick E8 Embryos

Treatment	Concentration	DRG number	With neurites
DMEM/F12		15	0
NGF	30 ng/ml	15	15
SSP-35	60 ng/ml	15	10
No 35 kDa fraction	60 ng/ml	15	0

The ganglia were cultured in DMEM/F12 medium containing 10% FCS on a collagen-coated tissue culture bottle as described in materials and methods. Data presented are from a representative example of three similar experiments.

Fig. 4. SDS-PAGE after Gel Filtration Chromatography

The 35 kDa protein fractions were obtained by TSK G2000 gel filtration chromatography. Aliquots (10 μ l) of protein molecular weight markers and fraction containing SSP-35 were resolved by SDS-PAGE, using 12.5% polyacrylamide gel, and were visualized with silver stain. Lane 1, protein molecular weight marker; lane 2, an aliquot (10 μ l) of fraction containing SSP-35.

medium in the presence or absence of purified 35 kDa. The number of DRG with neurites were determined (Table 1). We found distinct morphological differences between the DRG treated with 35 kDa (60 ng/ml) and the untreated DRG (Fig. 5). In cultures treated with 35 kDa, a number of DRGs contained neurite processes of varying lengths (Fig. 5b) (Table 1). In contrast, the untreated DRG cultures contained significantly fewer neurite-bearing DRG (Fig. 5a). Neurite processes of DRG cultured in the presence of 35 kDa protein were longer and denser than those of the controls. Our data show 35 kDa could promote neurite outgrowth but eluted no 35 kDa peak fraction by gel filtration chromatography (Table 1).

DISCUSSION

The study of cellular diversification during neurogenesis requires markers to identify different neural cell types. Recently it has become clear that transcription factors can serve as useful markers of neuronal identity. For example, the protein MASH1 identifies autonomic progenitors in the peripheral nervous system.⁶⁾ Similarly, Islet-1 and additional recently characterized proteins in the Lim homeodomain family mark subsets of functionally distinct motor neurons.^{7,8)}

Fig. 5. Phase-Contrast Photomicrographs of Culture of DRG
A. no SSP-35. B. SSP-35 (60 ng/ml) ($\times 100$).

Using reverse transcriptase (RT)-PCR, Saito *et al.*¹⁾ identified a novel 29 kDa paired homeodomain protein specifically expressed in sensory neurons and a subset of their central nervous system (CNS) targets. Our study was based on a difference in protein components of spinal sensory neurons and motor neurons. We isolated and purified a protein from rabbit spinal ganglia and sensory fibers that was not present in motor neurons (Figs. 1 and 2). These data are consistent with 35 kDa as a special antigen in spinal sensory neurons (Fig. 2). In addition, we found that 35 kDa protein promoted axonal growth in DRG of chick E8 embryos (Fig. 5b, Table 1).

We suggest that 35 kDa protein may be a novel protein with a neurotrophic effect. N-Terminal analysis of it is now in progress. The protein may be not only as a useful marker for sensory neurons, but also as a possible tool to study the regeneration and function of sensory neurons.

REFERENCES

- 1) Saito T., Greenwood A., Sun Q., Anderson D., *J. Mol. Cell. Neurosci.*, **6**, 280 (1995).
- 2) Laemmli U. K., *Nature (London)*, **227**, 680 (1970).
- 3) Daniel M. B., Stuart J. E., "Protein Methods," Wiley-Liss, London, 1991, p. 117.
- 4) Ouchterlony O., *Ark. Kemi*, **1**, 43 (1949).
- 5) Towbin H., Staehelin T., Gordon J., *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350 (1979).
- 6) Lo L., Guillemot F., Juyner A. L., Anderson D. J., *Perspect. Dev. Neurol.*, **2**, 191 (1994).
- 7) Ericson J., Thor S., Edlund T., Jessell T. M., Yamada T., *Science*, **256**, 1555 (1992).
- 8) Matsumoto S. G., *Dev. Brain Res.*, **83**, 17 (1994).