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ISOLATION OF GLIAL FIBRILLARY ACIDIC PROTEIN FROM BOVINE BRAIN WHITE MATTER AND ITS PURIFICATION BY AFFINITY CHROMATOGRAPHY ON SINGLE-STRANDED DNA-CELLULOSE

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SUMMARY: Myelinated axons, which had been prepared from bovine brain white matter employing the flotation method, were extracted with Triton X-100 in a low ionic strength buffer containing 4 mM Mg^{2+}. After delipidation of the detergent-resistant, residual material with chloroform-methanol, glial fibrillary acidic protein (GFAP) was solubilized with 6 M urea. It was enriched by ion exchange chromatography in the presence of 6 M urea on CM-Sepharose CL-6B at pH 5 and on DE52-cellulose at pH 7.6, respectively. The final purification was achieved by affinity chromatography on single-stranded DNA-cellulose in 6 M urea. Employing SDS-polyacrylamide gel electrophoresis, the molecular weight of the purified GFAP was determined to be 51,000. 2D-polyacrylamide gel electrophoresis revealed a major protein constituent of pI 4.7 to 4.8, accompanied by 3 acidic isoelectric variants. Upon incubation at 37° C in the presence of 150 mM KCl, GFAP assembled into 10 nm-filaments.

The study of the biochemical properties of intermediate filament subunit proteins is considerably hampered by their insolubility in buffers of low to physiological ionic strength. We have shown previously that this obstacle can be partially overcome when the proteins are purified to homogeneity. Thus, we were able to demonstrate that vimentin is a nucleic acid-binding protein (1, 2) and we also could characterize its behavior towards the intermediate filament-specific, Ca^{2+}-activated proteinase (3). For our comparative studies on the biochemical properties of intermediate filament subunit proteins, we also

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Abbreviations: GFAP, glial fibrillary acidic protein; ssDNA, single-stranded DNA; IEF, isoelectric focusing; SDS, sodium dodecylsulfate.

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needed a soluble GFAP preparation. GFAP, which is a marker protein for astroglial cells (4), was previously prepared from brain white matter (5) or from spinal cord (6). For the isolation of crude GFAP, we used established procedures including the axon flotation method described by Shelanski et al. (7) and urea extraction of isolated axons (5); however, for final protein purification, we employed affinity chromatography on ssDNA-cellulose. ssDNA-cellulose affinity chromatography has been previously used for the purification of vimentin (8) and desmin (9).

Materials and Methods

Reagent grade chemicals and biochemicals were purchased from Merck AG (Darmstadt, FRG). DE52-cellulose was from Whatman (Maidstone, England), ampholines from LKB (Bromma, Sweden). CM-Sepharose CL-6B was obtained from Pharmacia (Uppsala, Sweden) and the Bio-Rad protein assay kit from Bio-Rad (Richmond, Calif., U.S.A.). ssDNA-cellulose was prepared as described previously (10).

Flotation of axons from bovine brain white matter: If not otherwise specified, all operations were carried out at 0 to 2° C. The flotation method used was a modification of the procedure described by Shelanski et al. (7). Briefly, 150 g of fresh bovine brain white matter was homogenized in 830 ml of Buffer A (0.9 M sucrose, 30 mM Na-phosphate, pH 6.5, 1 mM EGTA) using a Potter-Elvejehm homogenizer with a motor-driven teflon pestle. Centrifugation of the homogenate at 9,500 x g_{av} for 30 min resulted in the flotation of a white layer consisting of myelinated axons. The myelinated axons were collected, washed once with 600 ml of Buffer B (30 mM Na-phosphate, pH 6.5, 1 mM EGTA, 6 mM 2-mercaptoethanol) and recovered by centrifugation at 9,500 x g_{av} for 30 min. Washed axons were resuspended in 770 ml of Buffer B and the suspension was adjusted to 1 M sucrose. After stirring overnight, the axons were separated from myelin by centrifugation at 100,000 x g_{av} for 60 min.

Extraction and delipidation of axons: Isolated axons were extracted once with Buffer C (10 mM Tris-acetate, pH 7.6, 1 mM EGTA, 4 mM Mg-acetate, 6 mM 2-mercaptoethanol, 0.1 % Triton X-100) and pelleted by centrifugation at 30,000 x g_{av} for 10 min. This step was repeated twice in the absence of Triton X-100. The final pellet was lyophilized. The dried material was delipidated by 3 extractions with 200 ml portions of chloroform/methanol (2:1) using an Ultraturax (Janke and Kunkel, Staufen, FRG) for homogenization. The dried, delipidated material was then extracted 3 times with Buffer D (10 mM Tris-acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol, 6 M urea). After centrifugation at 30,000 x g_{av} for 10 min, the extract was dialysed against Buffer E (10 mM Na-acetate, pH 5, 3 mM EDTA, 6 mM 2-mercaptoethanol, 6 M urea) overnight.

Ion exchange chromatography on CM-Sepharose CL-6B and DE52-cellulose: The dialysed protein solution was applied to a 17 cm x 2.5 cm CM-Sepharose CL-6B column previously equilibrated with Buffer E. Bound material was eluted with a 1 l linear 0 to 300 mM KCl gradient in Buffer E at a flow rate of 50 ml/h. Appropriate fractions were combined and dialysed against Buffer D. A 10 cm x 2.5 cm DE52-cellulose column equilibrated with Buffer D was loaded with the dialysed protein solution and the chromatogram was developed with a 500 ml linear 0 to 200 mM KCl gradient in Buffer D at a flow rate of 60 ml/h. The elution of GFAP was followed by SDS-polyacrylamide gel electrophoresis. Fractions containing GFAP were pooled and dialysed against Buffer D.

ssDNA-cellulose affinity chromatography: The dialysed protein solution was applied to a 20 cm x 2.6 cm column of ssDNA-cellulose previously equilibrated with Buffer D. After washing with Buffer D, bound GFAP was eluted with a 1 l linear 0 to 200 mM KCl gradient in Buffer D at a flow rate of 30 ml/h. GFAP-containing fractions were combined, dialysed against dist. H_2O and lyophilized. The residue was dissolved in 6 ml of Buffer D and dialysed extensively against 10 mM Tris-acetate, pH 7.6, 6 mM 2-mercaptoethanol. The protein concentration was 1 mg/ml. 8 1 ml aliquots were frozen in liquid N_2 and stored at -80° C.

Polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis was performed as described previously (11), 2D-gel electrophoresis as described in (12). Protein concentrations were measured using a Bio-Rad protein assay kit according to the manufacturer's instructions.

Intermediate filament formation and electron microscopy: Purified GFAP (0.3 mg/ml) was incubated at 37° C in 10 mM Tris-acetate, pH 7.6, 6 mM 2-mercaptoethanol, 150 mM KCl for 1 h. The filaments were negatively stained (13) and viewed in a Zeiss EM 9 electron microscope.

Results and Discussion

In this study, bovine brain white matter was used as a relatively rich source for GFAP. In a first step, myelinated axons were prepared employing the flotation method described by Shelanski et al. (7). In order to be able to handle a large amount of brain white matter at a given rotor capacity and within a reasonably short period of time, the myelinated axons were floated only once. Following the separation of myelin and axons by high speed centrifugation, the latter were extracted with Triton X-100 in a low ionic strength buffer containing 4 mM Mg^{2+}. Under these conditions, the GAP is retained in detergent-resistant, residual cell structures, whereas a considerable amount of cellular protein is solubilized.

In the course of the isolation of vimentin from Ehrlich ascites tumor cells (14) and of desmin from porcine smooth muscle (9), we noticed that despite of Triton X-100 extraction of cells or tissue the intermediate filament proteins were still complexed with substantial amounts of lipids. This contamination with lipids caused tailing of the filament proteins during their purification on ion exchange and affinity chromatography columns. Since GFAP is structurally closely related to vimentin and desmin (15, 16), its association with residual lipids was expected and, therefore, the detergent-insoluble material was delipidated with chloroform/methanol. After removal of lipids, the GFAP was almost quantitatively solubilized upon extraction of the detergent-insoluble material with 6 M urea. Only a small amount of GFAP remained associated with the nuclear material (see Table I).

During 6 M urea extraction of the detergent-resistant cell residues, a substantial amount of DNA was dissolved. It was removed by chromatography of the extract on CM-Sepharose CL-6B at pH 5. Bound GFAP was eluted at 150 mM KCl in the presence of 6 M urea (Fig. 1). Whereas cation exchange chromatography

TABLE 1. Purification of GFAP from 150 g bovine brain white matter

	Volume (ml)	Total protein (mg)	GFAP %	Total GFAP (mg)	Recovery %
Triton X-100, 4 mM Mg^{2+} extract of axons	258	312.2	0	0	-
6 M urea extract of delipidated axons	130	273	10	27.3	100
HCl extract of residue	24	26.4	1.3	0.36	
CM-Sepharose CL-6B chromatography	148	37.5	30	11.25	41
DE52-cellulose chromatography	63.5	15.3	68.9	10.50	38
ssDNA-cellulose chromatography	47	8.6	93	8	29

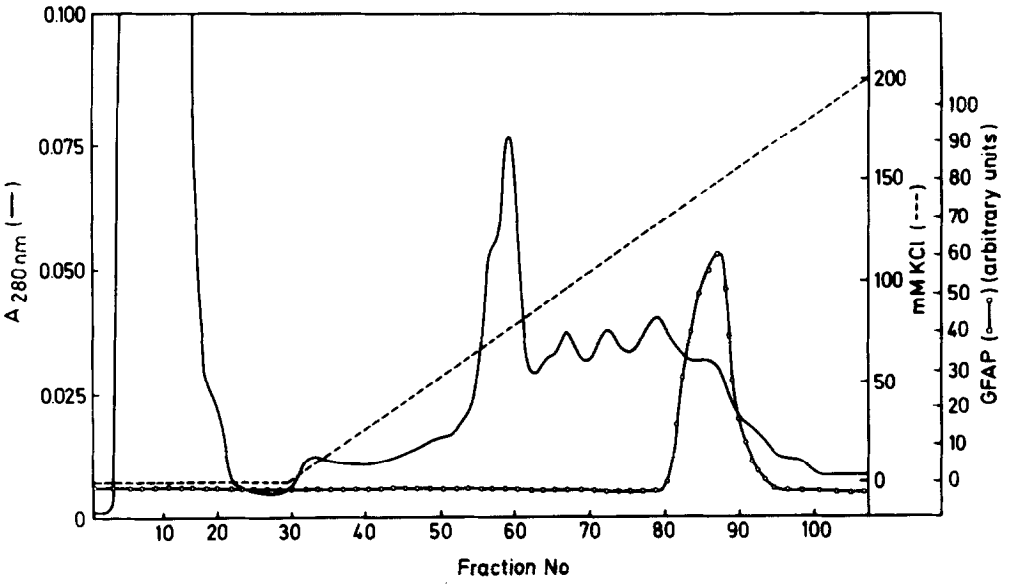

Fig. 1 SDS-polyacrylamide gel electrophoresis of protein samples from different stages of the purification procedure. Lanes 1, total white matter extract; 2, Triton X-100, 4 mM Mg^{2+} supernatant; 3, 6 M urea extract of delipidated axons; 4, HCl-soluble material from the 6 M urea pellet; 5, CM-Sepharose CL-6B GFAP peak fraction; 6, DE52-cellulose GFAP peak fraction; 7, GFAP after ssDNA-cellulose affinity chromatography; 8, molecular weight markers.

brought about only modest protein purification (from 10 % to 30 % purity, Table I), anion exchange chromatography on DE52-cellulose in the presence of 6 M urea resulted in a considerable enrichment for GFAP (from 30 % to nearly 70 % purity, Table I). The filament protein was eluted at 30 mM KCl (Fig. 2).

GFAP shows a similar affinity for nucleic acids at low ionic strength and in the absence of divalent cations as vimentin (1, 2) and desmin (our unpublished results) and the neurofilament triplet proteins (our unpublished results). This property of GFAP was exploited for its final purification by affinity chromatography on ssDNA-cellulose. In the presence of 6 M urea, it eluted at 30 mM KCl (Fig. 3). As shown in Fig. 4 (slots 6 and 7), ssDNA-cellulose chromatography removed particularly high molecular weight proteins.

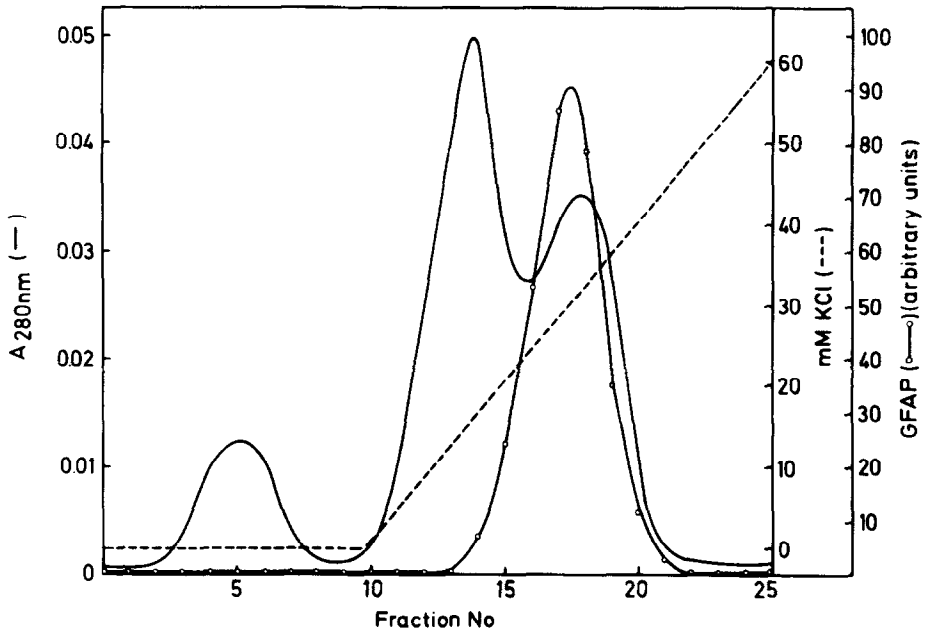

Fig. 2 CM-Sepharose CL-6B chromatography of GFAP. For experimental details, see Materials and Methods.

The purification of GFAP was followed by SDS-polyacrylamide gel electrophoresis and scanning of the Coomassie Brilliant Blue-stained gels at 590 nm (Fig. 4). In general, the final purity was higher than 93 %; the recovery of GFAP was approxi-

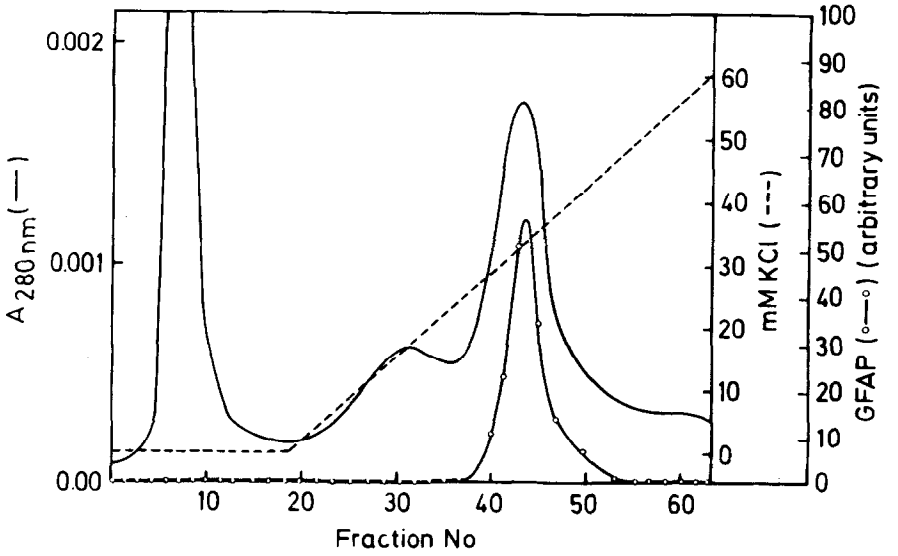

Fig. 3 DE52-cellulose chromatography of GFAP after CM-Sepharose CL-6B chromatography (see Materials and Methods).

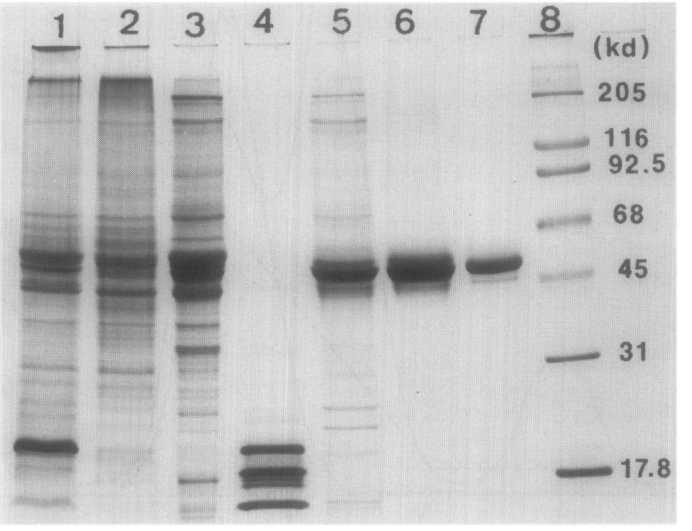

Fig. 4 ssDNA-cellulose chromatography of GFAP after DE52-cellulose chromatography (see Materials and Methods).

mately 30 %. The filament protein is often contaminated with a polypeptide of slightly higher electrophoretic mobility; it is presumably a proteolytic breakdown product of GFAP.

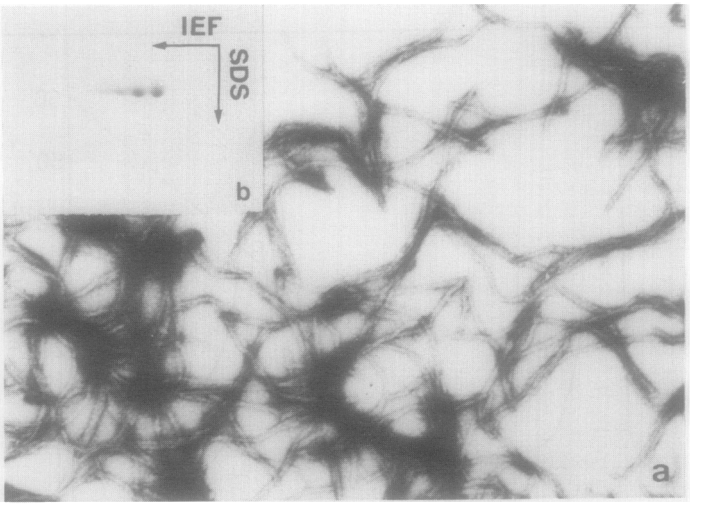

Fig. 5 (a) Electron micrograph of a negatively stained preparation of glial filaments reassembled from purified GFAP (x 26,560). (b) 2D-polyacrylamide gel electrophoresis profile of purified GFAP.

The purified GFAP was further characterized by (1) molecular weight determination on SDS-polyacrylamide gels (M_r = 51,000; Fig. 4), (2) isoelectric focusing (pI = 4.7 to 4.8; Fig. 5b; note the presence of 3 to 4 acidic isoelectric variants), (3) intermediate filament formation upon incubation at 37° C in the presence of 150 mM KCl (Fig. 5a), and (4) degradation by the intermediate filament-specific, Ca^{2+}-activated, neutral thiol proteinase (17, data presented elsewhere).

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