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ISOLATION, PURIFICATION AND CHARACTERIZATION OF THE INTERMEDIATE FILAMENT PROTEIN DESMIN FROM PORCINE SMOOTH MUSCLE

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ABSTRACT

Desmin was isolated from porcine stomach smooth muscle, which had been treated with 0.6 M KCl in the presence of Triton X-100, by extraction with 6 M urea and chromatographed on CM-Sepharose CL-6B at pH 5. After delipidation with chloroform-methanol, the protein was purified by affinity chromatography on arginine methyl-ester Sepharose 4B and single-stranded DNA-cellulose, respectively. Chromatography on single-stranded DNA-cellulose removed a considerable amount of vimentin which had been extracted and enriched together with desmin. The molecular weight of the purified desmin was 55,000 as determined by polyacrylamide gradient slab gel electrophoresis in the presence of Na-dodecylsulfate. Two-dimensional polyacrylamide gel electrophoresis revealed one major po-

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lypeptide of pI 5.3 to 5.4 accompanied by two to three acidic, isoelectric variants. During incubation in the presence of 150 mM KCl, desmin assembled into 10 nm filaments. This method allows the isolation of large amounts of pure desmin in a relatively short time with only minimal denaturation of the protein.

INTRODUCTION

For our studies on the nucleic acid-binding properties of intermediate filament subunit proteins[1,2] and their behaviour towards the intermediate filament-specific, Ca^{2+}-activated proteinase[3,4], we needed a pure desmin preparation which is soluble in buffers of low ionic strength. Desmin, which is found predominantly in smooth, skeletal and cardiac muscle[5] has been first isolated from chicken gizzard smooth muscle[5]. Subsequently, various procedures for the isolation and purification of desmin from various sources have been described[6-9]. They are all based on the preparation of the detergent-resistant cytoskeletal fraction in the presence of high salt which is then extracted under denaturing conditions, for instance with 6 to 8 M urea or 1 M acetic acid. The crude filament protein can be purified either by ion exchange chromatography or by several polymerization-depolymerization cycles. We here describe a procedure for desmin purification which takes advantage of the property of desmin to bind to arginine methylester Sepharose 4B[10] and to single-stranded (ss)DNA-cellulose under conditions described previously for the purification of vimentin[11].

The method is a modification of the procedure described by Geisler and Weber[9] in that the first steps of desmin extraction from porcine stomach smooth muscle were essentially the same, but the purification of crude desmin was carried out by affinity chromatography.

MATERIALS AND METHODS

Reagent grade chemicals and biochemicals were purchased from Merck AG (Darmstadt, FRG). CF-11 cellulose was from Whatman (Maidstone, U.K.), ampholines from LKB (Bromma, Sweden), calf thymus DNA from Sigma (St. Louis, MO. U.S.A.), CM-Sepharose CL-6B and CNBr-activated Sepharose 4B from Pharmacia (Uppsala, Sweden). L-arginine methylester hydrochloride was from Serva (Heidelberg, FRG) and the Bio-Rad protein assay kit from Bio-Rad (Richmond, CA., U.S.A.). L-arginine methylester Sepharose 4B was prepared according to the procedure described by Unkeless et al.[12], except that 0.1 M $NaHCO_3$, pH 8.9 was used as coupling buffer; ssDNA-cellulose was prepared as described previously[13].

Extraction of porcine stomach smooth muscle

All steps were performed at 0 to 2° C if not specified otherwise. Smooth muscle was taken from stomachs of pigs immediately after they were killed. After removal of nonmuscle tissue, the muscle was cut into small pieces, frozen in liquid N_2 and stored at -80° C. 100 g of frozen tissue were homogenized in 500 ml 40 mM imidazole-HCl, pH 6.9, 0.6 M KCl, 1 mM EGTA, 1 mM 2-mercaptoethanol, 0.5 % (w/v) Triton X-100 (Buffer I) using a Waring Blender.

The homogenate was centrifuged at 9,250 x g_{av} for 10 min. The pellets were reextracted twice, with the omission of Triton X-100. The resulting fibrous white pellet was extracted with 150 ml 10 mM Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol, 6 M urea (Buffer II), using an Ultra-Turrax (Janke und Kunkel KG, Staufen, FRG) for homogenization. Insoluble material was pelleted by centrifugation at 31,000 x g_{av} for 30 min. This step was repeated twice and the combined supernatants were dialysed against 10 mM Na-acetate, pH 5, 3 mM EDTA, 6 mM 2-mercaptoethanol, 6 M urea (Buffer III) overnight.

CM-Sepharose CL-6B column chromatography and delipidation of protein

The dialysed solution was applied to a 21 x 3.5 cm column of CM-Sepharose CL-6B previously equilibrated with Buffer III. The column was washed with Buffer III at a flow rate of 90 ml/h and bound material was eluted in one step with Buffer III containing 1 M KCl. The eluted material was extensively dialysed against dist. H_2O and lyophilized. The residual material was delipidated by 3 extractions with 100 ml portions of chloroform:methanol = 2:1. Homogenization was achieved by means of an Ultra-Turrax. Interval centrifugation was at 31,000 x g_{av} for 10 min. The final pellet was dried in vacuo at room temperature and dissolved in 150 ml Buffer II with an Ultra-Turrax.

Arginine methylester Sepharose 4B affinity chromatography

The protein solution was applied to a 35.5 x 3 cm

column of arginine methylester Sepharose 4B previously equilibrated with Buffer II, at a flow rate of 45 ml/h. 10 ml fractions were collected. The column was washed with 200 ml portions of the following buffers: (1) 10 mM Tris-acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercapto-ethanol (Buffer IV), (2) Buffer IV containing 0.5 M KCl, (3) Buffer II containing 1 M KCl. 50 µl of each fraction were mixed with 10 µl of a solution containing 45 ml 10 % Na-dodecylsulfate, 12.5 ml 2-mercaptoethanol, 100 mg bromophenol blue. Twenty µl of each sample were analysed by Na-dodecylsulfate-polyacrylamide gel electrophoresis. The desmin-containing fractions were pooled and dialysed against Buffer II overnight.

ssDNA-cellulose affinity chromatography

A 35 x 1.8 cm column of ssDNA-cellulose was loaded with the dialysed protein solution at a flow rate of 40 ml/h and washed with Buffer II. The desmin was eluted with a linear 0 to 200 mM KCl gradient in Buffer II programmed by an LKB 1300 Ultrograd (Bromma, Sweden). The protein elution was monitored with an LKB Uvicord S at 280 nm and the conductivity of each 10 ml fraction was determined with a Digitalmeter Digi 610 (Wissenschaftlich-Technische Werkstätten, Weilheim, FRG). The salt concentration was estimated by reference to a calibration curve. After Na-dodecylsulfate-polyacrylamide gel electrophoretic analysis, desmin-containing fractions were combined, dialysed against dist. H_2O and lyophilized. The protein was redissolved in Buffer II at a concentration of approxi-

mately 2 mg/ml and dialysed against 10 mM Tris-acetate, pH 7.6, 6 mM 2-mercaptoethanol. One ml aliquots were frozen in liquid N_2 and stored at -80° C.

Polyacrylamide gel electrophoresis

Na-dodecylsulfate-polyacrylamide gel electrophoresis was performed as described previously[14], 2-D gel electrophoresis as described by Shoeman and Schweiger[15]. Na-dodecylsulfate-polyacrylamide gels were stained with Coomassie Brilliant Blue G-250, destained in 10 % acetic acid and scanned at 590 nm in a Gilford 2400S spectrophotometer connected to a 7225-A Hewlett-Packard plotter. The relative amounts of desmin were computed by integration of the optical density peaks.

Filament assembly and electron microscopy

Purified desmin was incubated at 37° C in 10 mM Tris-acetate, pH 7.6, 150 mM KCl, 6 mM 2-mercaptoethanol for 1 h at a concentration of 0.3 mg/ml. The filaments were negatively stained[16] and viewed in a Zeiss EM 9 electron microscope.

Protein determination

Protein concentrations were measured using the Bio-Rad protein assay kit according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Porcine smooth muscle was extracted with Triton X-100 in a buffer of pH 6.9 containing 0.6 M KCl yielding a cytoskeletal fraction in which myosin, actin and desmin were major protein constituents (Fig. 1, lanes 2 and 3).

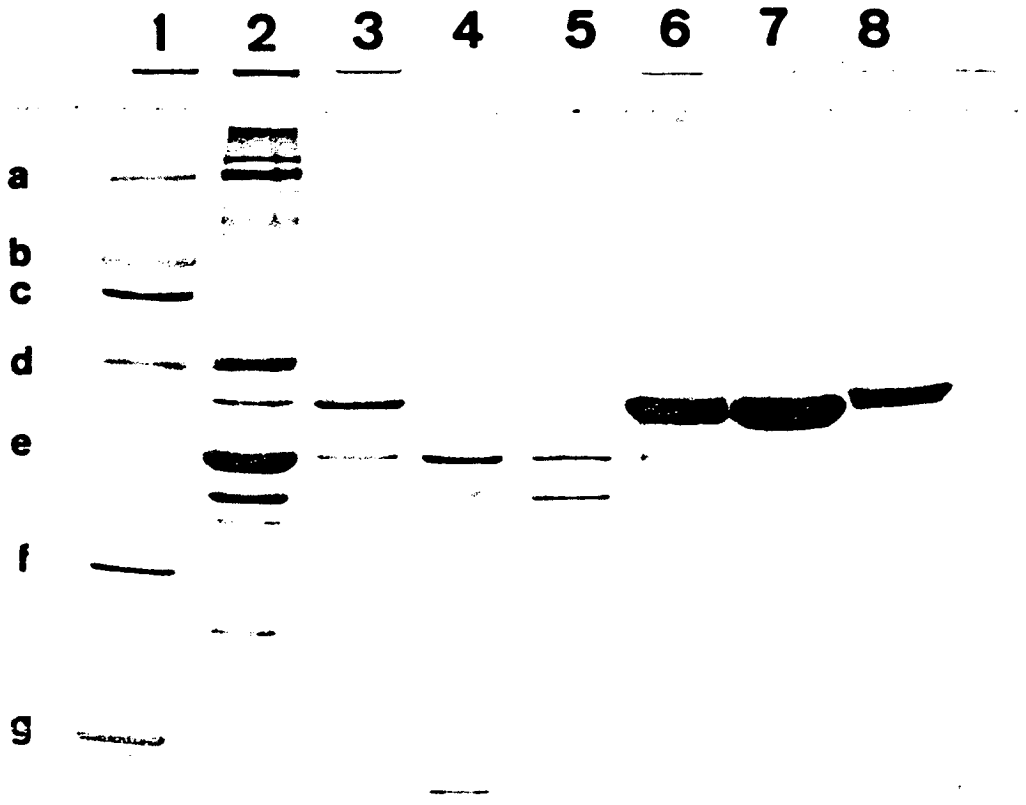

FIGURE 1

Na-dodecylsulfate-polyacrylamide gel electrophoresis of protein samples from different stages of the purification of desmin. Lane 1, molecular weight markers: (a) myosin (Mr = 205,000), (b) ß-galactosidase (Mr = 116,000), (c) phosphorylase a (Mr = 92,500), (d) bovine serum albumin (Mr = 68,000), (e) ovalbumin (Mr = 45,000), (f) carboanhydrase (Mr = 31,500), (g) myoglobin (Mr = 17,800); Lane 2, combined 0.6 M KCl supernatants; Lane 3, 6 M urea extract; Lane 4, flow through of arginine methylester Sepharose 4B chromatography; Lane 5, 0.5 M KCl eluate of arginine methylester Sepharose 4B chromatography; Lane 6, 1 M KCl/6 M urea eluate of arginine methylester Sepharose 4B chromatography; Lane 7, desmin after ssDNA-cellulose chromatography; Lane 8, vimentin after ssDNA-cellulose chromatography.

TABLE 1. PURIFICATION OF DESMIN FROM 100 g OF PORCINE SMOOTH MUSCLE

	Volume(ml)	Total protein(mg)	Desmin (%)	Total Desmin (mg)	Recovery (%)
High salt supernatant	1865	1678	5	83.9	100
6 M urea extract	430	580.5	30.7	178.2	
CM-Sepharose CL-6B chromatography	250	335	41.7	139.7	53.3
Arginine methylester Sepharose 4B chromatography	180	185.4	55.4	102.7	39.2
ssDNA-cellulose chromatography	194	101.5	95	100.9	38.5

Treatment of this fraction with 6 M urea resulted in the solubilization of desmin; it accounted for 31 % of the solubilized protein (Table I). Although desmin is insoluble in buffer of high salt concentration[9], we found a considerable amount, about 32 % of the total, recovered desmin, in the high salt extract (Table I, Fig. 1).

We experienced some difficulties in handling the 6 M urea extract in the further purification of desmin, since a certain amount of DNA was solubilized and, consequently, the extract was rather viscous. These difficulties were overcome by chromatography of the protein extract on CM-Sepharose CL-6B in 6 M urea at pH 5. DNA did not bind to the column under these conditions, so that desmin was eluted in one step with 1 M KCl in 6 M urea. However, only little purification of desmin could

be achieved in this step (Table I).

Intermediate filaments and their subunit proteins are usually highly contaminated with lipids because of the high affinity of these components for each other (Nelson and Traub, unpublished data). Even after extraction with Triton X-100 from cells or tissues and purification by ion exchange or affinity chromatography in the presence of 6 M urea, the intermediate filament proteins retain a substantial amount of lipids. They contribute considerably to the insolubility of the filament proteins and also cause tailing in the course of protein purification on ion exchange and affinity columns. To avoid these effects, we delipidated the crude desmin preparation with chloroform-methanol before further purification.

Principally, at this stage the desmin-enriched protein solution could be directly applied to an ssDNA-cellulose column. We noticed, however, that under these circumstances the desmin eluted from the ssDNA-cellulose column contained relatively large amounts of high molecular weight proteins. We therefore included an arginine methylester Sepharose 4B affinity chromatography step (Fig. 2). As we recently showed, all subunit proteins which are able to assemble into intermediate filaments in vitro have a high affinity for arginine-containing polymers at high ionic strength[10].

When the protein was applied to the affinity matrix column in 6 M urea and at low ionic strength, a substantial fraction of the contaminating proteins passed

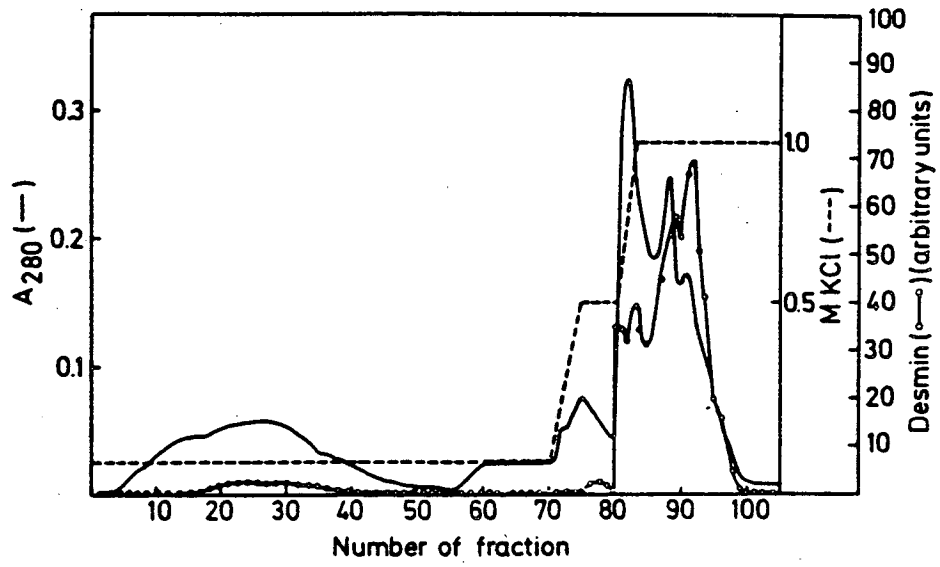

FIGURE 2

Arginine methylester Sepharose 4B chromatography of desmin. For experimental details, see Materials and Methods.

the column, actin being the most prominent component (Fig. 1, lane 4). A second fraction of non-specifically adsorbed protein could be eluted with 0.5 M KCl (Fig. 1, lane 5). Desmin was then quantitatively desorbed with a buffer containing 1 M KCl and 6 M urea (Fig. 1, lane 6). The first few desmin-containing fractions showed a strong Tyndall effect and, after standing for some time, attained a gel-like consistency. A possible explanation for this observation is that the elution conditions allow a certain amount of filamentous aggregates to form which elute in the exclusion volume, before the urea concentration becomes high enough to solubilize them. This explanation would be consistent with the observation of Geisler and Weber[9] who showed that desmin is not fully denatured in the presence of 6 M urea. The eluted protein

fraction contained 55 % of desmin.

We routinely detected substantial amounts of vimentin in crude desmin preparations obtained from porcine stomach smooth muscle. Irrespective of the question whether desmin and vimentin are coexpressed in the same cell type or originate from different cell types of the starting material, both proteins remain associated with the cytoskeleton after extraction of the tissue with Triton X-100 in the presence of 0.6 M KCl[17,18]. Consequently, they are coextracted when the detergent-resistant residual cell structures are treated with 6 M urea. Since desmin and vimentin have very similar isoelectric points (desmin: pI = 5.35; vimentin: pI = 5.25; see below) and molecular weights (desmin: M_r = 55,000; vimentin: M_r = 58,000; see below), they cannot be separated at a large scale with conventional separation methods. Also, several polymerisation-depolymerisation cycles are ineffective in separating vimentin from desmin because both proteins coassemble into the same intermediate filament[19,20]. However, in the course of the biochemical characterization of desmin (our unpublished results), we could show that, like vimentin[2], desmin is a nucleic acid-binding protein, with, however, an affinity for ssDNA that is somewhat lower than that of vimentin[11]. As illustrated in Fig. 3, desmin is eluted from ssDNA-cellulose at approximately 55 mM KCl, whereas vimentin is eluted at 80 mM KCl in the presence of 6 M urea. It should be noted here that desmin and vimentin show the same affinity for arginine methylester Sepharose 4B; both proteins are eluted from

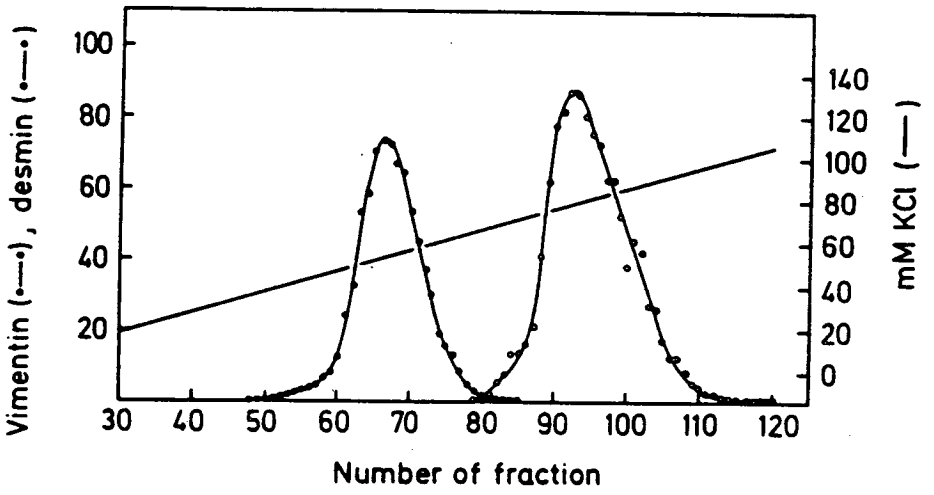

FIGURE 3

Separation of desmin and vimentin by affinity chromatography on ssDNA-cellulose in the presence of 6 M urea. 1 mg desmin and 1.2 mg vimentin in Buffer II were applied to a 7 x 1 cm ssDNA-cellulose column previously equilibrated with Buffer II. The proteins were eluted with a 200 ml linear 0 to 200 mM KCl gradient in Buffer II. 1 ml fractions were collected at a flow rate of 16 ml/h. The protein elution was followed by Na-dodecylsulfate-polyacrylamide gel electrophoresis as described in Materials and Methods.

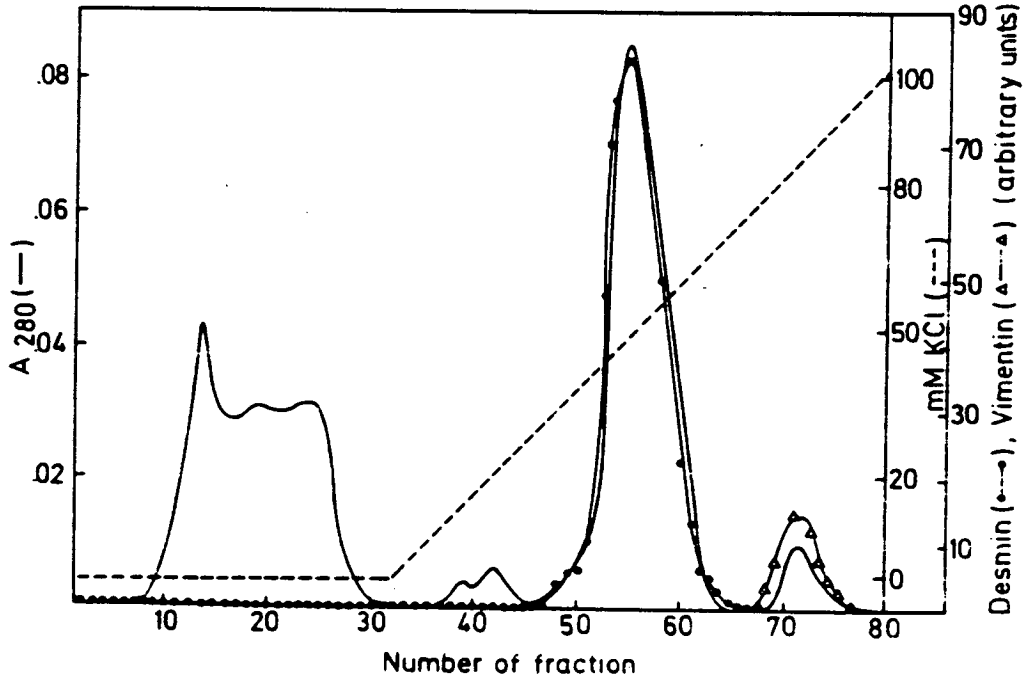

FIGURE 4

ssDNA-cellulose chromatography of desmin after [illegible] ginine methylester Sepharose 4B chromatography. Desmin (o——o); vimentin (▲——▲). For experimental details, see Materials and Methods.

the affinity column with 320 mM arginine[10].

Thus, the final purification of desmin was performed by affinity chromatography on ssDNA-cellulose in 6 M urea under conditions which had been used for the large scale purification of vimentin[11]. As shown in Fig. 4, a considerable amount of vimentin that was coisolated and copurified with desmin during tissue extraction and the previous chromatography steps, respectively, was clearly separated from desmin in the course of KCl gradient elution. Desmin was eluted at a KCl concentration of 50 mM, whereas vimentin was eluted at 85 mM KCl. In Fig. 1 (lanes 3 and 6), vimentin can be seen as a weak band just above the strong desmin band.

The enrichment of desmin in the above purification procedure was followed by quantitative densitometry of Na-dodecylsulfate-polyacrylamide gels. The results are summarized in Table I. An overall recovery of desmin of 38.5 % was achieved; the purity of desmin was greater than 95 %. In a routine preparation, from 100 g porcine stomach smooth muscle, approximately 100 mg pure desmin and 12 mg vimentin were obtained.

The purified desmin and vimentin were further characterized and identified by (1) molecular weight determination on Na-dodecylsulfate-polyacrylamide gels (desmin: M_r = 55,000; vimentin: M_r = 58,000), (2) isoelectric focussing (desmin: pI = 5.3 to 5.4; vimentin: pI = 5.2 to 5.3; Fig. 5), (3) formation of 10 nm filaments by incubation at 37° C and pH 7.6 in the presence of

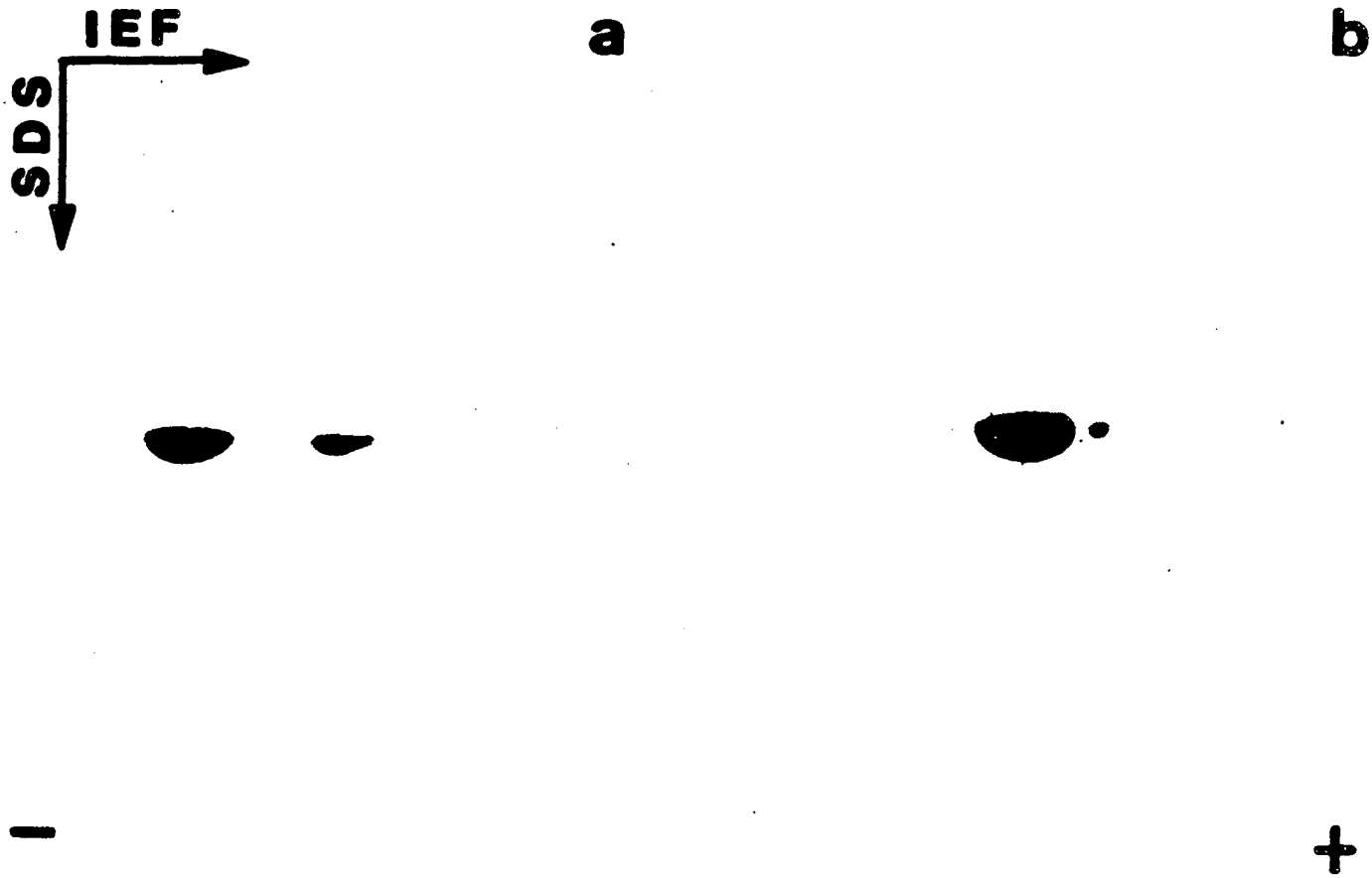

FIGURE 5

Two-dimensional polyacrylamide gel electrophoresis of (a) purified desmin, (b) copurified vimentin.

150 mM KCl (Fig. 6), (4) degradation by the intermediate filament-specific, Ca^{2+}-activated, neutral thiol proteinase (data not shown) and (6) cross-reactivity with the monoclonal antibody α-IFA which is directed against a common antigenic site shared by all intermediate filament proteins[21] (data not shown).

The method described includes purification steps which employ affinity chromatography on arginine methylester Sepharose 4B and ssDNA-cellulose. We have successfully used arginine methylester Sepharose 4B affinity chromatography also for the partial purification of

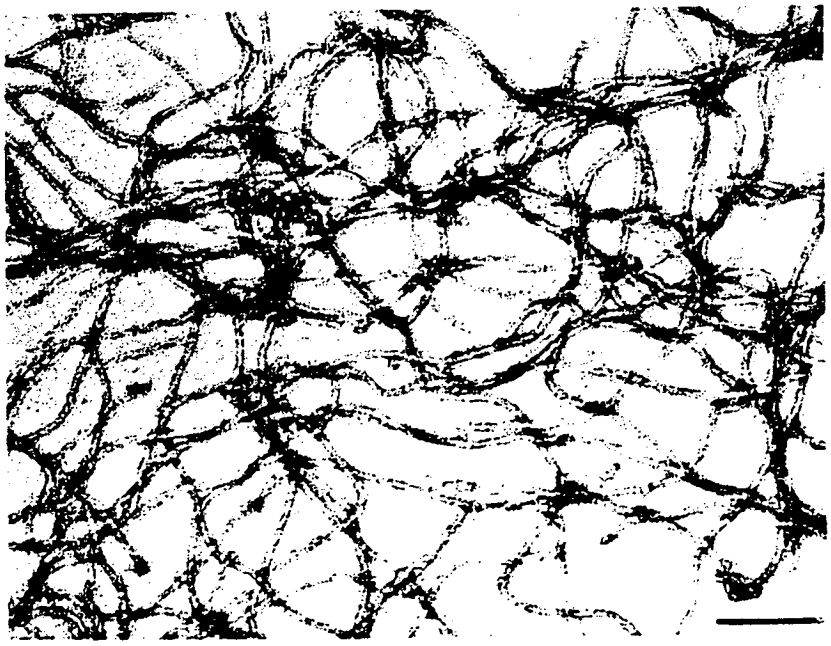

FIGURE 6

Assembly of purified desmin into 10 nm filaments. Desmin was incubated at 37° C for 1 h in 10 mM Tris-acetate, pH 7.6, 150 mM KCl, 6 mM 2-mercaptoethanol. The protein concentration was 0.3 mg/ml. The filaments were negatively stained and viewed in a Zeiss EM 9 electron microscope (x 48,000). (Bar: 0.2 µm)

cytokeratins isolated from newborn rat skin (Traub and Vorgias, unpublished results) and ssDNA-cellulose for the purification of vimentin from Ehrlich ascites tumor cells[11] and of glial fibrillary acidic protein from bovine brain white matter (Vorgias and Traub, unpublished results).

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