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ACTIN ORGANIZATION IN CHICK EMBRYO FIBROBLASTS AFTER INFLUENZA VIRUS INFECTION. I. ISOLATION AND CHARACTERIZATION OF ACTIN FROM CHICK EMBRYO CELLS

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Summary. — Comparison of two starting materials for actin purification has shown that preparation of actin from aceton-dried cytoskeleton was more effective than from native chick embryos (CE). The isolated actin formed a single band of Mr=42-43 000 in SDS-PAGE; less purified samples revealed additional faint bands. G form of actin (non-polymerized) inhibited the activity of DNase I, electron microscopy showed actin filaments and bundles formed upon its polymerization. The freshly purified homogeneous actin has not lost its DNase I-inhibiting activity when incubated for 60 min at 35° or 45°C. Older or less purified actin samples kept under similar conditions showed 18-25% decrease of their DNase I-inhibiting activity and a loss of their polymerization ability. Digestion with trypsin caused a decrease of DNase I-inhibiting activity of fresh as well as for older actin samples.

Key words: actin; chick embryo; DNase inhibition test; trypsin

Introduction

Also non-muscle cells contain contractile proteins forming microfilaments (MF). These participate in several cell functions as locomotion, division, endo- and exocytosis, mobility of cellular organelles and maintenance of cell shape. They may correlate with transformation, differentiation and transmembrane control of cell functions. In accordance with these diverse functions, MF do not form permanent structures, but their organization changes depending on the functional state of cells (Mimura and Asano, 1982).

Changes in the organization of MF and their bundles were described after certain virus infections (Graessmann *et al.*, 1980; Pautrat *et al.*, 1980; Heeg *et al.*, 1981; Meyer *et al.*, 1981; Bussereau and Perrin, 1982; Losse *et al.*, 1982; Bedows *et al.*, 1983; Murti and Goorha, 1983). The greatest changes were observed in paramyxovirus-infected cells revealing reorganization of MF since 30 min post-infection (p.i.).

Actin is the main component of MF. Its isolation and characterization has been described by several investigators. The following starting materials

were recommended: acetone powder (Spudich and Watt, 1971; Kuczmarski and Rosenbaum, 1979), actomyosin (Kobayashi *et al.*, 1977; Fechheimer and Cebra, 1979), native tissues (Gordon *et al.*, 1977; Nagata *et al.*, 1982) or cytoskeleton (Rosenberg *et al.*, 1981; Kobayashi *et al.*, 1982). To investigate the possible role of actin and the changes in its organization in the course of virus infection we purified actin from CE cells. This report describes the efficiency of purification process and the properties of actin obtained from two starting materials, namely native CE and cytoskeleton containing acetone-dried powder.

Materials and Methods

Chemicals. EGTA (aethylenglycol-bis-(2-aminoaethyl) tetraacetic acid and 2-mercaptoethanol (2-MCE) were obtained from Fluka, Switzerland; ATP sodium salt (NaATP) and dithiothreitol (DTT) from Calbiochem; phenylmethyl sulphonyl fluorid (PMSF) and DNase I from bovine pancreas from Serva, Heidelberg, F. R. G.; DEAE-cellulose DE52 from Whatman (England); DNA type 1 highly polymerized from calf thymus was coming from Sigma, U. S. A.; Sephadex G-100 was a product of Pharmacia (Sweden) and trypsin was from Difco Laboratories.

Preparation of starting material. 11-day-old CE were cleaned, washed with 0.14 mol/l NaCl solution or with phosphate buffered saline (PBS) pH 7.2 and stored at —20 °C. The cytoskeleton was prepared from a part of native CE by the technique of Rosenberg et al. (1981). It was sedimented at 3000 rev/min but unlike to Rosenberg's procedure it was dried with aceton (aceton-dried cytoskeleton powder).

Actin isolation. Actin was extracted from either native CE or from the aceton-dried cytoskeleton according to the method of Nagata et al. (1982) with the exception that DEAE cellulose DE-52 was used instead of DEAE-Sephadex A-50 and gel filtration was performed on Sephadex G-100 in G buffer instead of Sephadex G-150 (G buffer: 3 mmol/l imizadol buffer pH 7.5, 0.1 mmol/l $CaCl_2$, 0.5 mmol/l ATP, 0.75 mmol/l 2-MCE).

The protein content of the extracts was determined according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. In the purified actin sample the protein content was calculated from $OD_{290\ nm}$ (Gordon, 1977; Burridge *et al.*, 1982). The actin concentration in each fractionation step was estimated by DNase-inhibition test (Blickstad *et al.*, 1978).

Polyacrylamide gel electrophoresis (PAGE) of actin was made in 4.5 and 10% gels containing 0.1% SDS (sodium dodecylsulphate) according to Laemmli (1970). The polymerization capacity of G actin was estimated by measuring the OD at 232 nm (Kuczmarski and Rosenbaum, 1979).

The morphology of actin filaments was examined in Philips 300 electron microscope at 80 kV. The samples were spread over formvar-coated grids fastened with a carbon membrane. Negative staining was performed with 2% phosphotungstic acid (pH 6.9—7.0).

Results

Comparison of the isolation techniques

Actin was purified from either native CE or acetone-dried cytoskeleton (obtained by the method of Rosenberg et al., 1981 and acetone-dried) by the same procedure: extraction into G buffer, clarification at 100 000 g for 1 hr at 0 °C, chromatography on DEAE cellulose in D buffer (the same as G buffer except of 10 mmol/l imidazol) containing 0.1 mol/l KCl. For elution from the column 0.1—0.5 mol/l linear KCl gradient was used (Fig. 1). After concentration of the actin-containing fraction by ultrafiltration through YM-10 membrane, 2 mmol/l $MgCl_2$ was added to induce polymerization. F actin was sedimented at 100,000 g for 3 hr at 25 °C, resuspended in a small vol

of G buffer and depolymerized by dialysis against 50—100 vol of the same buffer for 3 days. G actin was clarified at 100,000 g as above. If necessary, further purification was performed on Sephadex G-100 in G buffer (Fig. 2).

Comparing the purity of actin in each fractionation step Figs (3-I, 3-II; Table 1) the usage of cytoskeleton powder as starting material seemed to be more favourable. The G buffer extract from the cytoskeleton powder was not only richer for actin but also less viscous than the extract from native CE. This may be the reason why DEAE-cellulose chromatography was more efficient. Actin fraction obtained from DEAE-cellulose after polymerization and ultracentrifugation showed 92—97% purity when starting with acetone-

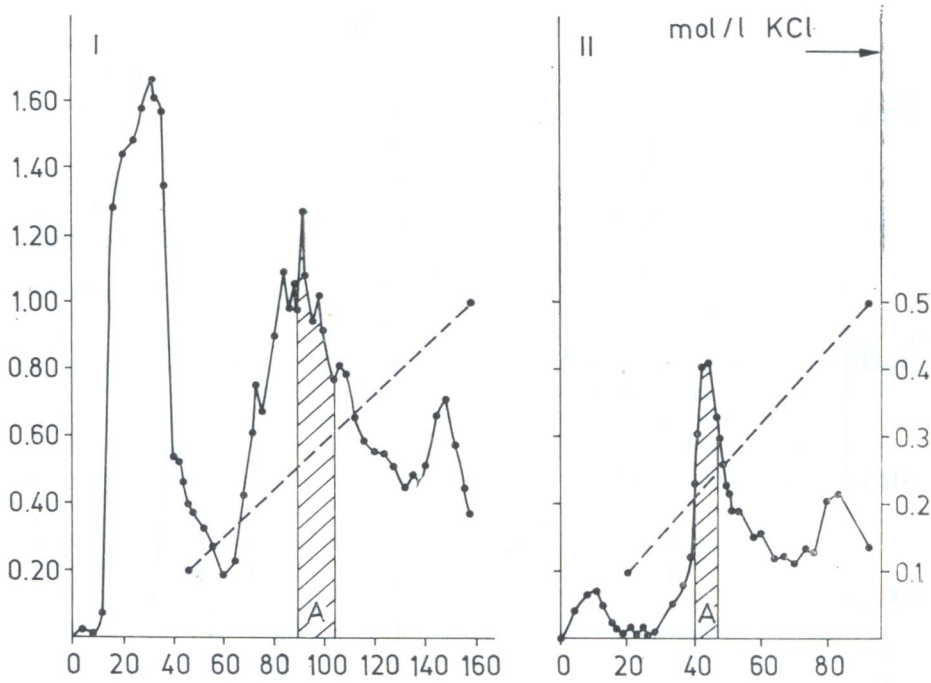

Fig. 1.
Chromatography of actin on DEAE-cellulose
I — extract from native CE
II — extract from aceton-dried cytoskeleton powder
Extracts clarified at 100 000 g for 1 hr were dialyzed against D buffer (10 mmol/l imidazol pH 7.5, 0.1 mmol/l $CaCl_2$, 0.5 mmol/l ATP, 0.75 mmol/l 2-ME, 0.1 mol/ KCl) and applied on the column equilibrated with the same buffer. After washing with D buffer, the column was eluted in a linear 0.1—0.5 mol/l KCl gradient. Actin (A) was present in fractions of 0.25—0.3 and of 0.23—0.26 mol/l KCl, respecitely, as detected with DNase I-inhibition test.
Abscissae: fraction number; left ordinates: $OD_{290\ nm}$; right ordinate: mol/l KCl gradient.

-dried cytoskeleton. In contrast, the same purification product when started with native CE contained 66—71% actin only. To gain actin of > 90% purity, the sample from native CE should have been further purified by gel filtration on Sephadex G-100.

Figs 2 and 3 show that gel filtration on Sephadex G-100 removes the high--molecular impurities and F actin in the first peak. The second peak consisted of practically homogeneous G-actin. The actin yield from acetone-dried cytoskeleton powder was similar to that from native CE (35—60 mg actin from 10 g acetone powder), although during preparation of the cytoskeleton

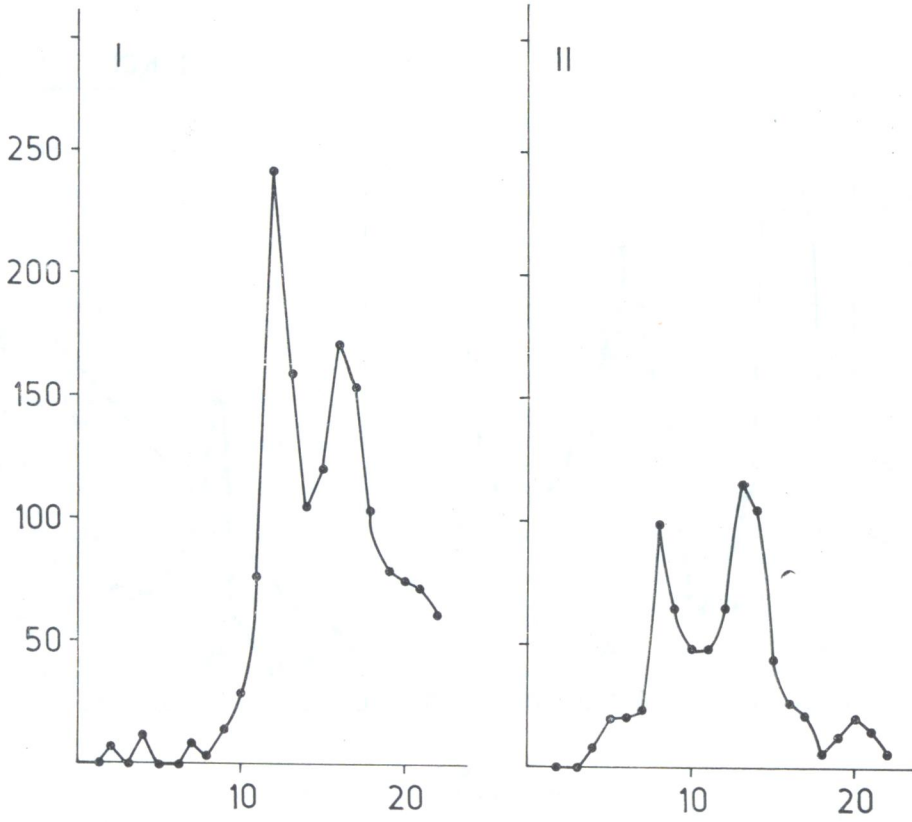

Fig. 2.
Gel filtration of actin on Sephadex G—100
I — extract from native CE
II — extract from acetone-dried cytoskeleton powder
Actin was obtained by polymerization-depolymerization procedure after DEAE--cellulose chromatography and applied on the column. The column was washed and eluted with G-buffer; nearly homogeneous actin is present in the 2nd peak. Abscissae: fraction number; ordinates: $OD_{290\ nm}$

Table 1. Comparison of actin content in fractions obtained at actin isolation

Isolation step	Percentage of actin (out of total protein)	
	I	II
G buffer extract (clarified)	4.85	11
Fraction eluted from DEAE cellulose	17.5	41
Supernatant after polymerization of the above fraction	8.5	13.2
Polymerized material after depolymerization and clarification	66 – 71	92 – 97.5
Sephadex G-100 (2nd peak)	85 – 100	n.d.

I – starting material: material CE
II – starting material: cytoskeleton powder
Protein content was determined according to Lowry; actin content by DNase I-inhibition test.
N.d. = not done.
For further explanations see Materials and Methods.

a considerable amount of actin (probably G actin) had got into the Triton X-100 extract. Purification of the actin from the acetone-dried cytoskeleton powder was quick and well reproducible.

Properties of actin

Purified actin from acetone-dried cytoskeleton powder as well as actin from native CE formed a single main band in SDS-PAGE revealing Mr 42—43 000. Both samples at the same concentration in solution (0.4—0.5 mg/ /ml) had a similar polymerization velocity (as determined due to OD_{232} change according to Kuczmarski and Rosenbaum, 1979). Electron microscopic examination of actin polymers confirmed the presence of actin filaments and bundles (Fig. 4).

Stability of actin

Purified actin, stored in lyophilized form at —20 °C after solubilization in water has preserved its original DNase I-inhibition activity. The actin samples stored in G buffer at 4 °C showed after 1—2 weeks a decreased capability to inhibit the DNase I activity as well as an impaired polymerization.

A loss of actin activity might occur due to inactivation or proteloytic cleavage. The thermal inactivation was followed at 35 °C, 41 °C and 45 °C according to the method of Heacock *et al.* (1982). The results have shown that DNase I-inhibiting activity of fresh homogeneous actin even increases after 60 min incubation at given temperatures. In contrast, the DNase I-inhibiting activity of partially purified actin has decreased by 16% or 27% when incubated at 41° or 45 °C, respectively; the aggregation of this material was confirmed in SDS-PAGE as well as by electron microscopy (Figs 5-II, 7).

Digestion of actin with trypsin was followed according to Burtnick and Chan (1980). The actin was incubated with trypsin (Difco) by an actin to trypsin molar ratio of 12 : 1 at 32 °C for 0—60 min. At given intervals, the

samples were removed, mixed with the same vol of denaturing SDS solution, heated for 3 min to 100 °C and analysed in SDS-PAGE. As seen from Fig. 6, trypsin cleaved the actin quickly forming different lower mol. mass peptides. Similar results observed by Burtnick and Chan (1980) when subtilisine was used for hydrolysis of actin.

The DNase I-inhibiting activity of fresh homogeneous actin decreased by 29% after 60 min incubation with trypsin. Trypsin digestion of actin was more extensive when older or less purified samples were used. After 60 min incubation with trypsin only 10% of original DNase I-inhibiting activity remained; when polymerization had been induced in such samples no actin filaments were seen by electron microscopy (Fig. 5-III).

Discussion

The method of actin isolation from rabbit muscles as described by Spudich and Watt (1971) is unsuitable for purification of actin from non-muscle cells. The actin originating from non-muscle cell extracts shows a decreased ability for polymerization (its critical concentration is enhanced) and the actin yield is low. Extracts from certain non-muscle cells contain an actin polymerization inhibitor (Nagata *et al.*, 1982) or alternatively, F actin may interact with certain actin-binding proteins forming complexes sedimenting already at lower centrifugation speed (Rosenberg *et al.*, 1981). The resulting firm structures are hardly soluble.

Gordon *et al.* (1977) recommended to prepurify actin by DEAE-cellulose chromatography removing the majority of undesirable components. The actin-containing fraction then polymerizes alike to the action from rabbit muscle tissue. The majority of cellular proteins can be removed with 1% Triton X-100 (Rosenberg *et al.*, 1981) Kobayashi *et al.*, 1982). Cytoskeleton prepared under these conditions is insoluble.

Comparing the actin isolation procedures from native CE and from the cytoskeleton, the latter has turned out more suitable for actin preparation. It is quicker as already after DEAE-cellulose chromatography by simple polymerization and depolymerization actin sample of considerable purity of 92—97% may be obtained. In contrast, from native CE actin of comparable purity can be obtained only after gel filtration on Sephadex G-100.

Some purified actin samples showed in addition to the actin band an another zone of Mr = 36 000. A ccordingto Nagata *et al.* (1984) as well as due to Burtnick and Chan (1980) this zone may be a proteolytic cleavage product. Incubation of purified actin with trypsin (Difco 1 : 250) resulted in a quick digestion to low molecular peptides. The trypsin-resistant core described by Burtnick and Chan (1980) was found in our experiments only as a part of proteolysis products. It is unclear whether this discrepancy was caused by different properties of trypsin, or by different behaviour of actin prepared from CE in contrast to actin coming from rabbit muscle.

Certain actin samples (especially the older ones) although homogeneous in PAGE, revealed a lower DNase I-inhibiting activity as expected. Heacock

et al. (1982) described the thermal inactivation of actin along with its decreased DNase inhibition activity and impaired polymerization ability. Experiments in which actin purified to 80—90% was incubated at 41 °C showed after 60 min about 80% of the original DNase I-inhibiting capacity.

Based on the presented results we suggest that purification of actin from cytoskeleton powder is quicker and more effective. Therefore, the possibility of inactivation or proteolytic cleavage of the actin is lower.

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Explanation of Figures (Plates XXVII—XXXI):

Fig. 3. Analysis of fractions obtained during actin purification in SDS-PAGE
I — from native CE
II — from aceton-dried cytoskeleton
1, 6: extract from G buffer clarified at 100 000 g for 1 hr
2, 7: actin fraction from DEAE-cellulose
3, 8: actin purified by polymerization-depolymerization after DEAE-cellulose chromatography and clarified by centrifugation
4: actin further purified on Sephadex G-100 (first peak)
5: actin further purified on Sephadex G-100 (second peak)

Fig. 4. Electron micrograph of nefatively stained F actin fibres (diameter 7 μm). Magn. × 56,000.

Fig. 5. Electron micrograph of negatively stained actin. Contrasted with PTA. F actin was obtained by polymerization of:
5-I: Native G actin (magn. × 99,000)
5-II: G actin inactivated for 60 min at 45 °C (magn. × 99,000)
5-III: G actin incubated for 60 min at 30 °C with trypsin (magn. × 99,000).

Figs. 6—7. Actin digestion with trypsin and its thermal inactivation.
Actin was incubated with trypsin at 32 °C (tracks 2—5 and 1′, 3′) or inactivated at 45 °C (tracks 7, 8 and 5′, 6′) as indicated in the Results. At intervals, samples were removed for SDS-PAGE.
Fig. 6. Homogeneous actin (0.92 mg/ml)
tracks 1, 6: no trypsin (controls); incubation of 0 and 60 min;
2—5: trypsin digestion for 0, 20, 40 and 60 min;
7 and 8: inactivation at 45 °C for 30 and 60 min, respectively
Fig. 7. Non-homogeneous actin, lyophilized and solubilized (1.23 mg/ml)
1′ and 3′: incubated without trypsin for 30 and 60 min, respectively;
2′ and 4′: incubated with trypsin for 30 and 60 min, respectively
5′ and 6′: inactivated at 45 °C for 30 and 60 min, respectively