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RAT PANCREAS ACTIN : PURIFICATION AND CHARACTERIZATION

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Isolation of rat pancreas actin was performed with three different technics : polymerization-depolymerization method, affinity chromatography on DNase I-Sepharose 4B or ion exchange chromatography on DEAE-cellulose. Inhibition of DNase I activity, localization by SDS polyacrylamide slab gel electrophoresis and presence of microfilaments allowed its identification. Affinity process led us to obtain actin which kept inhibitory activity (30,000 U per mg) on DNase I when using vacuum dialysis. Actin eluted from DEAE-cellulose associated reversibly in 50-70 Å microfilaments in the presence of phalloidin, was pure at 95 % and had a satisfactory inhibitor activity (77,000 U per mg).

Microfilaments have been shown to be involved in the secretory process of exocrine pancreas (1,2,3), moreover these structures are widely distributed in these cells (2). In non-muscle cells, actin is in a dynamic state forming transitory, localized microfilaments ; in the exocrine pancreas these structures generally occupied the apical part of the acinar cell (2). The most obvious mechanism for mobilization of actin within the cell would be the presence of a pool of non polymerized actin that could be rapidly converted to F-actin (microfilaments) when and where needed. The true mechanism of actin polymerization in the exocrine pancreas is still unknown. Therefore to elucidate the rat pancreas actin properties, we have undertaken its purification by three different technics : polymerization-depolymerization method, affinity chromatography on DNase I-Sepharose 4B and ion exchange chromatography on DEAE-cellulose.

Abbreviations: DNase I, deoxyribonuclease I ; ATP, adenosine 5'-triphosphate ; DTE, dithioerythritol.

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MATERIALS AND METHODS

Polymerization-depolymerization : Rat pancreas was homogenized in 3 vol (W/V) of 2 mM Tris buffer, containing 0.2 mM $CaCl_2$/0.1 mM DTE/0.2 mM ATP, pH 8.0 ; and centrifuged at 100,000 xg for 1 hr at 4°C. The polymerization-depolymerization steps were carried out essentially as described by Spudich and Watt (4).

DNase I-Sepharose 4B chromatography : DNase I (from bovine pancreas, grade II, Boehringer) was further purified to remove any trace of contaminant. The non-retained fraction of DEAE-Sephadex A50 was applied on a CM-Sephadex A-50 which kept the most active fraction of the DNase I(unpublished results). DNase I was coupled to CNBr-Sepharose 4B in our laboratory according to Dhermy et al (5). Homogenization of rat pancreas in 5 vol (W/V) was performed in 10 mM Tris-HCl/5 mM $CaCl_2$/0.2 mM ATP, pH 7.0 (buffer A). After a-100,000 xg, 1 hour, 4°C-centrifugation, the supernatant was applied on the DNase I-Sepharose 4B column previously equilibrated with buffer A. The adsorbed proteins were eluted stepwise with the following buffers : a) buffer A + 0.15 M NaCl ; b) 0.5 M sodium acetate, 10^{-3} M $CaCl_2$ and 30 % glycerol (pH 6.5) containing either 0.75 M guanidine-HCl or c) 3.0 M guanidine-HCl.

DEAE-cellulose chromatography : As described by Gordon et al (6) DEAE-cellulose (Whatman DE 52) column was previously equilibrated with 10 mM imidazole/0.1 mM Ca^{++}/0.5 mM ATP/0.3 mM DTE/pH 6.5 containing 0.1 M KCl (buffer B). Rat pancreas was homogenized in 5 vol (W/V) of buffer B without KCl and centrifuged at 100,000 xg for 1 hour at 4°C. A column volume of buffer B without KCl was applied before and after the sample. The column was washed with buffer B + 0.15 M KCl and eluted with buffer B + 0.25 M KCl. Each 4 ml fraction was assayed for DNase I-inhibitor activity and when successful adjusted to 2 mM $MgCl_2$ and 25 µg/ml phalloidin for a night at 4°C and 3 hours at 25°C. After a 100,000 xg centrifugation at 25°C during 2 hours, the pellets were homogenized in 5 mM imidazole/2 mM $MgCl_2$/0.5 mM ATP, pH 6.5, observed in electron microscopy and dialyzed for 120 hours against 3 mM imidazole/0.1 mM $CaCl_2$/0.2 mM ATP/0.3 mM DTE, pH 6.0 and centrifuged at 100,000 xg for 1 hour at 4°C. The supernatant contained the soluble globular actin.

. Inhibition of DNase I

Measurements of DNase I activity have been previously described by Kunitz (7). For the assay of DNase inhibitor activity, 900 µl of DNA substrate (100 mM Tris-HCl/4 mM $MgSO_4$/1.8 mM $CaCl_2$, pH = 7.5) was mixed with 50 µl of DNase I solution with or without actin sample (Blikstad et al (8)). The hyperchromicity at 260 nm was recorded on a Zeiss DMR 21 spectrophotometer.

. Electron Microscopy

A drop of solution containing separated filaments was applied on a carbon coated grid for 3 mn. The grid was then rinsed with 0.1 M KCl. Negative staining was performed with a drop of 2 % uranyl acetate aqueous solution for 3 mn followed by a drop of water. The negatively stained grid was examined with a Philips EM 300 electron microscope at 80 kV.

. Electrophoresis

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was performed on 7.5 % slab gel according to Launay et al (9).

RESULTS

Inclusion of 2 mM $MgCl_2$ and 50 mM KCl followed by 0.6 M KCl in rat pancreas supernatant gives a transparent pellet after ultracentrifugation. No

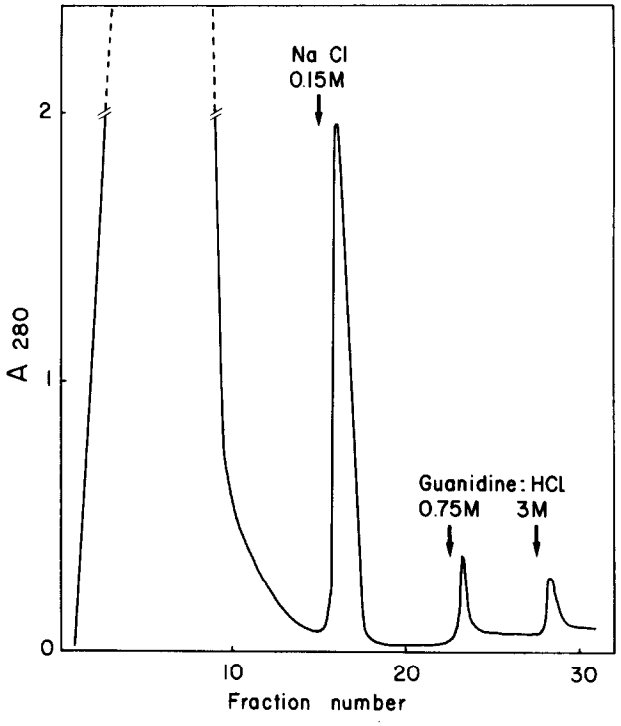

Fig. 1 : Elution profile of a rat pancreas supernatant from Sepharose bound DNase I column (2 x 1 cm). For details see "Materials and Methods".

depolymerization occurs after dialyzing this resuspended pellet and no material appears in electrophoresis, but in the front, with the non-sedimented part of the ultracentrifuged dialyzate (data not shown). In the same way inhibition effect on DNase I activity failed.

Figure 1 gives the elution profile of a rat pancreas supernatant applied on the affinity column. After vacuum dialysis, the 3M guanidine-HCl peak, consists of a major band (85 %) comigrating with rabbit skeletal muscle actin on SDS polyacrylamide slab gel (Figure 3B) ; at the same level a minor band is found in the 0.75 M guanidine-HCl peak (Figure 3A). The calculated specific inhibitor activity of actin (8) in the 3M guanidine-HCl peak is 30,000 U/mg.

Elution profile of the DEAE-cellulose is presented in Figure 2. Actin fractions identified by DNase I inhibition assay are indicated by the stippled area. This was confirmed by gel electrophoresis ; Figure 3D shows the importance of the 42,000 band (11 % of the total protein) in the first 0.25 M KCl

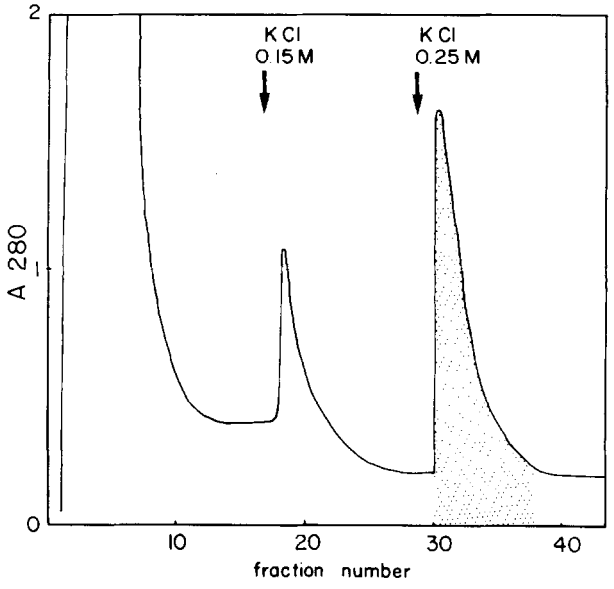

Fig. 2 : DEAE-cellulose chromatography of a rat pancreas supernatant. The eluted actin is indicated by the stippled area. More details are given under "Materials and Methods".

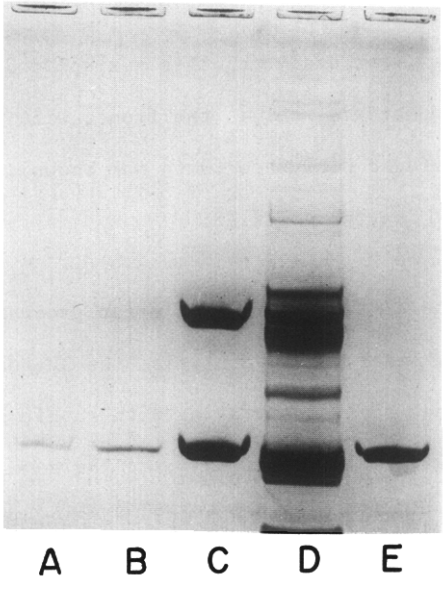

Fig. 3 :
SDS polyacrylamide slab gel electrophoresis.
A : 0.75 M guanidine-HCl peak (18 µg)
B : 3 M guanidine-HCl peak (16 µg)
C : Molecular weight markers ; from the top to the bottom : bovine serum albumine (67,000 daltons), 5 µg ; rabbit skeletal muscle actin (42,000 daltons), 10 µg.
D : 0.25 M KCl peak (60 µg).
E : Purified rat pancreas actin (13 µg) after DEAE-cellulose chromatography and polymerization-depolymerization step with phalloidin.

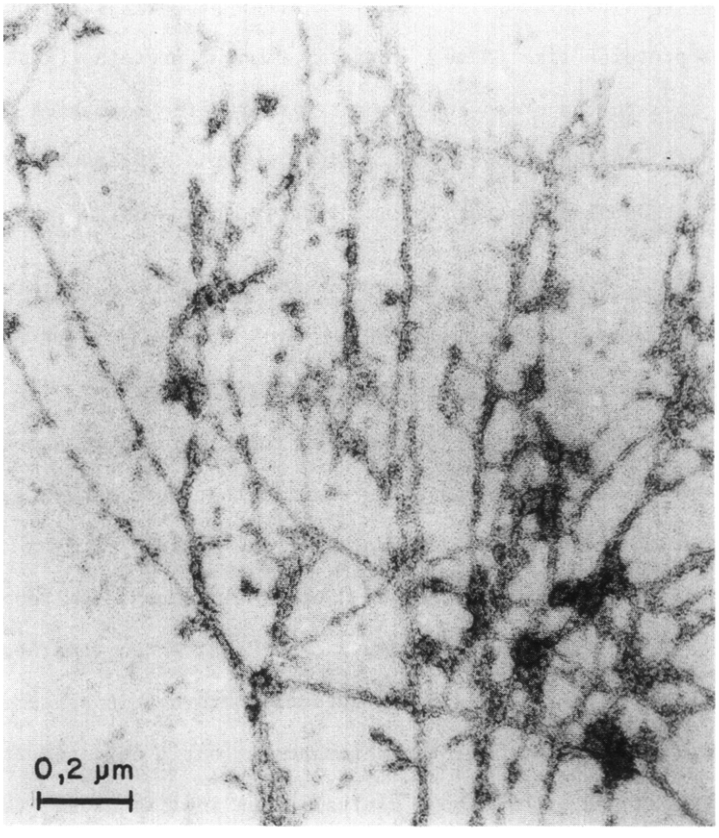

Fig. 4 : Electron microscopic observation of the pellet obtained by Spudich and Watt's technic.
Magnification : x 60,900.

fraction, the intensity of this band decreasing gradually from the fourth fraction (data not shown). After adjusting selected eluted fractions to 2 mM $MgCl_2$ + 25 µg/ml phalloidin and incubation, the depolymerized ultracentrifuged pellet contains in its soluble part the purified pancreas actin ; this obtained extract is at 95 % pure at least as shown by gel electrophoresis (Figure 3E). DNase I inhibitor activity corroborates this observation and shows that 98 % of these proteins are pure actin (77,000 U/mg).

DISCUSSION

Polymerization pellet obtained by Spudich and Watt's technic led us to obtain some structures which seemed closely to microfilaments by electron microscopy (Figure 4). These were frequently associated in bundles like described by Harwell et al (10) for rabbit skeletal muscle actin and Spudich and

Cooke (11) for Dictyostelium discoideum. It seems probable that the presence of additional proteins like DNase I or actin binding proteins (Rosenberg et al (12)(13)) in a rat pancreas supernatant disturbs the formation of actin filaments. These considerations could explain why the aggregated forms obtained did not depolymerize and became inactive proteins.

With the affinity chromatography on Sepharose-bound DNase I we get a protein with other characteristics. As a matter of fact as no salt with polymerizing effect on actin was present during the purification procedure, the protein stayed in the G-form. After vacuum dialysis, the clear solution obtained contains actin only globular. It was shown earlier that guanidine-HCl concentrations higher than 2 M were deletorious for the DNase I inhibitor activity (Lindberg et al (14)(15)), less than 10 % of this one could be recovered ; in the same way, Rohr and Mannherz (16) thought that actin remained denatured after 3 M guanidine-HCl treatment. These results are not in agreement with our study : we find that 40 % of the rat pancreas actin obtained from the 3 M guanidine-HCl peak and after vacuum dialysis, can inhibit DNase I again. When studied by SDS gel electrophoresis this preparation is pure at 85 % but an aggregated fraction of the sample does not penetrate in the gel - the intensity of the Coomassie Blue coloured band no reflecting the protein concentration. The affinity chromatography yield (0.15 % of soluble proteins) of a rat pancreas supernatant could be increased twice by another technic consisting in a prepurification on a DEAE column followed by one cycle of polymerization-depolymerization. Because of the low actin content and presumably the presence of contaminant proteins, magnesium was not sufficient to cause actin association and an accurate reversibility. Then phalloidin was added to induce microfilaments (17,18) ; electron microscopy (Figure 5) shows the high density of the obtained structures. Lengsfeld et al (17) reported that phalloidin polymerizes actin irreversibly. The pellet obtained in our technic depolymerizes after extent dialysis and ultracentrifugation ; the dialyzate lead us to get a supernatant containing globular actin electrophoretically pure (95 %) with a specific inhibitor activity of 77,000 U/mg.

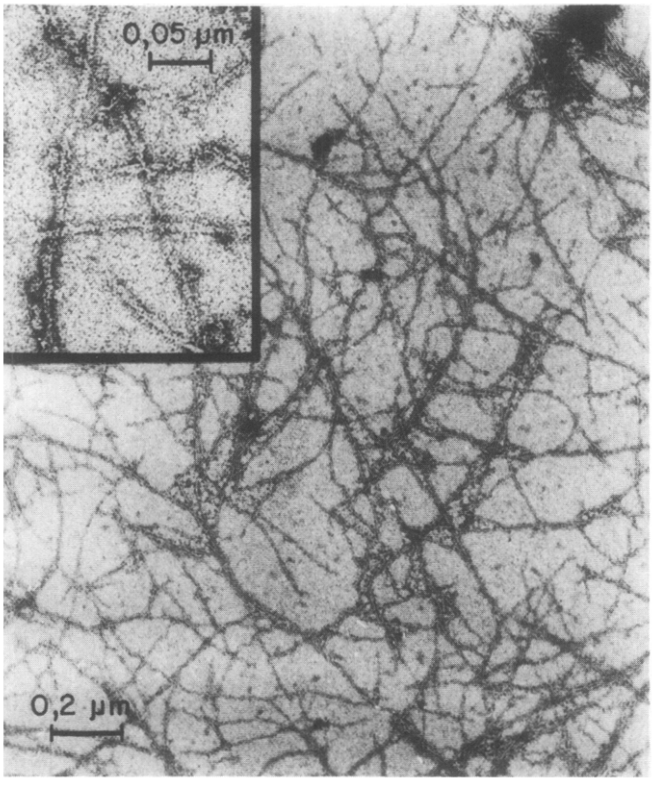

Fig. 5 : Resuspended F-actin pellet obtain after phalloidin treatment. The actin forms filaments of indefinite length but constant width 50 to 70 Å. Magnification : x 48,700. Inset:higher magnification (x 162,400) of the same structures.

We think that the pure actin obtained will allow to specify the existence and the role of DNase I and other acting binding proteins and thus to explain the dynamic equilibrium regulation between polymerized and non-polymerized actin in secretory cells.

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