AN ACTIN DEPOLYMERIZING PROTEIN FROM PIG PLASMA

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1. Introduction

Actin filaments are depolymerized by a protein component of plasma and serum [1–3]. This paper describes the isolation from pig plasma of a protein with actin depolymerizing activity (plasma ADF). It consists of a single polypeptide of ~92 000 M_r. It interacts rapidly with actin in a stoichiometry close to 1:1, without affecting the size or charge of the actin polypeptide. An abstract containing some of these data has been presented [4].

2. Materials and methods

Plasma ADF was assayed using the DNase inhibition assay [5] as described in [3]. ADF activity of 1 unit inhibits a DNase activity of 1 A_{260} unit . min^{-1} . cm^{-1} by 50%. Protein concentrations were estimated spectrophotometrically, assuming $\epsilon^{1\%}_{280\ nm}$ = 10 cm^{-1}. Values obtained by this method were within 10% of those determined by the Folin method.

Pig plasma was obtained as a by-product of platelet preparations [6]. Plasma ADF was prepared by one of two alternative procedures (fig.1):

Method A: Serum was prepared from 400 ml plasma by addition of thrombin (2 units/ml, 30 min, 20°C) and the clot removed by centrifugation. PMSF was added to 0.5 mM and 40–60% ammonium sulphate cut prepared, which was applied to a DEAE-cellulose column (Whatman DE-52), 50 X 2.5 cm, in 50 mM imidazole–HCl (pH 6.3), 10^{-4} M $CaCl_2$, 1 mM NaN_3. Activity was recovered in the void volume and further fractionated on a CM-cellulose column (Whatman CM-52), 35 X 2.5 cm, equilibrated in 5 mM succinate (pH 6.0), 10^{-4} M $CaCl_2$, 1 mM NaN_3, and eluted with an NaCl gradient of 0–0.2 M in the same buffer. The activity eluted as a single peak, after the bulk of the protein, at 75–95 mM NaCl. This material was then passed through a con A–Sepharose column (14 X 0.8 cm) in 100 mM Tris–HCl (pH 8.0), 1 M NaCl, 1 mM NaN_3, 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$ to remove glycoproteins and finally residual, contaminating immunoglobulin was removed by passage through a protein A–Sepharose (Sigma) column (1–2 ml) in 0.14 M NaCl, 20 mM imidazole–HCl (pH 7.0), 1 mM NaN_3.

Method B: Plasma, rather than serum, was the starting material for ammonium sulphate fractionation and DEAE-cellulose chromatography (pH 6.3) as in (A). The activity was then fractionated on a DEAE-cellulose column, 50 X 2.5 cm in 20 mM Tris–HCl (pH 8.5), 50 mM NaCl, 2 mM NaN_3 and eluted with a gradient of 50–200 mM NaCl. The active component eluted after all other proteins, at 80–100 mM NaCl.

Con A–Sepharose was prepared by coupling 200 mg con A (Pharmacia) to 5 g BrCN-activated Sepharose (Pharmacia) for 2 h, 20°C in 25 ml 0.5 M NaCl, 0.1 M $NaHCO_3$, 0.1 mM $MnCl_2$. Unreacted groups were blocked with 1 M ethanolamine–HCl (pH 8.1), 1 mM $MnCl_2$, 1 mM $CaCl_2$, 4°C for 24 h and the resin prepared for use in 100 mM Tris–HCl (pH 8.0), 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, 1 mM NaN_3. DNase–Sepharose was prepared by coupling 5 mg DNase (Sigma DN-EP) to 2 g activated Sepharose in 10 ml 5 mM triethanolamine for 2 h at 20°C. Unreacted groups were blocked with ethanolamine and the washed resin stored in 20 mM imidazole–HCl (pH 7.0), 0.1 mM $CaCl_2$, 3 mM NaN_3. The DNase–

Abbreviations: ADF, actin depolymerizing factor; con A, concanavalin A; PMSF, phenylmethyl sulphonyl fluoride; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid

Sepharose was saturated with actin by incubating a sample containing 16 pmol DNase with 27 pmol F-actin overnight, then washing thoroughly with 20 mM imidazole–HCl (pH 7.0). This material is actin–DNase–Sepharose.

Flat-bed isoelectric focussing (18 X 18 X 0.1 cm) of native ADF was performed using a slurry of 10 g G-25 superfine Sephadex mixed with 45 ml 16.7% (v/v) glycerol containing 2 ml (pH 3.5–10) and 0.5 ml (pH 5–8) LKB ampholine. The ends of the slab were connected by paper wicks to reservoirs containing 1.6% ethanolamine (cathode) or 0.08% phosphoric acid (anode). Sample (2 ml containing 3 mg protein) was applied to the surface of the slab 3–4 cm from the anode and focussed for ~1500 V . h. 16 fractions were scraped from the slab and eluted with water. pH and ADF activity were measured.

SDS–PAGE was performed in slab gels using the Tris–glycine buffer system [7]. Denaturing isoelectric focussing slab gels were performed as in [8].

3. Results

3.1. *Properties of pig plasma ADF*

Fig.1 shows the analysis by SDS–PAGE of successive stages in the purification of ADF, by methods A and B. In the initial steps, much of the immunoglobulin is removed by ammonium sulphate fractionation and all the albumin on DEAE-cellulose at pH 6.3. CM-cellulose chromatography at pH 6.0 (fig.2A) yields an active fraction containing 3 major components of 90 000, 70 000 and 50 000 M_r. The 70 000 M_r protein and some minor components are removed by con A–Sepharose. Residual immunoglobulin is removed by protein A–Sepharose leaving a nearly pure preparation of the 90 000 M_r component. This polypeptide is identified as ADF because it is always present in active preparations and it binds to actin (see section 3.2). The overall purification (245-fold) in the preparation illustrated in fig.1A is high; 150–180-fold is more typical. Fig.1B shows the same protein, obtained from plasma by method B, using DEAE-cellulose chromatography at pH 8.5 (fig.2B). This method is less reproducible than A; a variable amount of activity may fail to bind to the DEAE-cellulose, but the reason for this is not clear.

Plasma ADF has a polypeptide chain weight of 92 000 ± 2000 as determined by SDS–PAGE and 98 000–110 000 on Sephadex G-150 in 20 mM Tris–HCl (pH 8.5), 50 mM NaCl. No other polypeptides

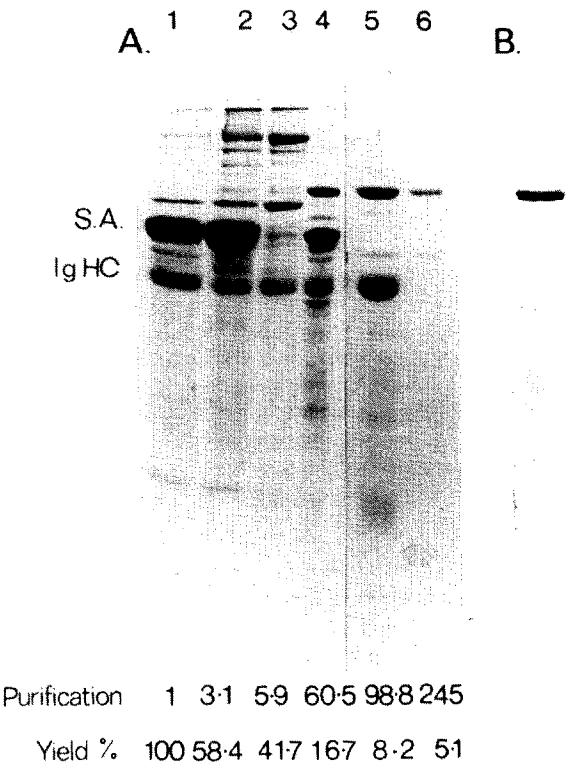

Fig.1. SDS–PAGE, 12.5% gel, of ADF purification. Method A: (1) serum; (2) 40–60% ammonium sulphate fraction; (3) activity from DEAE-cellulose (pH 6.3); (4) activity from CM-cellulose (pH 6.0); (5) protein not bound to con A–Sepharose; (6) protein not bound to protein A–Sepharose. Method B: activity from DEAE-cellulose (pH 8.5).

are consistently found in purified preparations. The pI of native ADF is ~6–6.5. This is consistent with its behavior on DEAE-cellulose, but not with its relatively high affinity for CM-cellulose at pH 6.0.

During the early stages of purification, ADF activity is stable, but more highly purified material (e.g, after CM-cellulose chromatography, or DEAE-cellulose, at pH 8.5) is labile. This lability may sometimes be accounted for by proteolysis, but other preparations lose activity without apparent degradation of the polypeptide chain. The purified factor, like the actin depolymerizing activity of whole plasma [3] is highly heat-labile. ADF prepared by method B had a half-life of 18 min at 50°C in 20 mM Tris–HCl (pH 8.5), 50 mM NaCl, 0.8 mM EGTA. Calcium stabilizes the protein; no activity loss was detected over 40 min in 0.8 mM $CaCl_2$. In neither case did heating promote proteolytic degradation of the polypeptide chain.

3.2. *The interaction of plasma ADF with actin*

Purified ADF was mixed with slightly less than an equivalent amount of F-actin at 20°C. All the actin is depolymerized within 1 min (see below). Samples analyzed by gel electrophoresis and electrofocussing showed no change in the actin mobility (fig.3). Thus, ADF does not affect either the size or charge of actin monomer.

The reaction of ADF with actin is complete within 15 s. In an experiment in which 60 pmol ADF and 240 pmol F-actin were mixed in 150 μl total vol. and 60 pmol DNase added (60 μl) after 15 s, 90% inhibi-

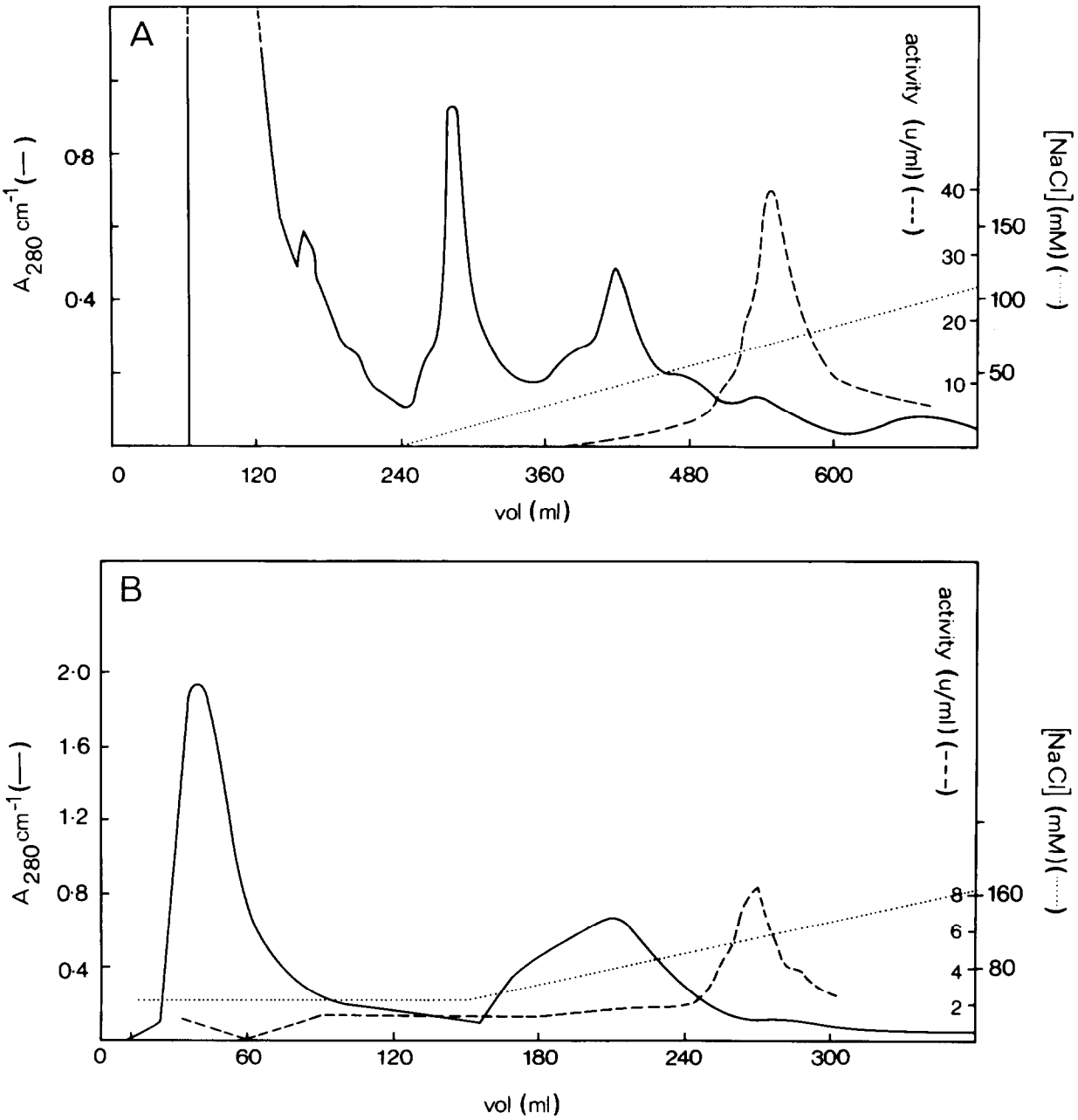

Fig.2. Ion-exchange chromatography of plasma ADF. (A) CM-cellulose chromatography (pH 6.0) on a 35 × 2.5 cm column, according to method A. (B) DEAE-cellulose chromatography (pH 8.5) on a 1.5 × 25 cm column, according to method B.

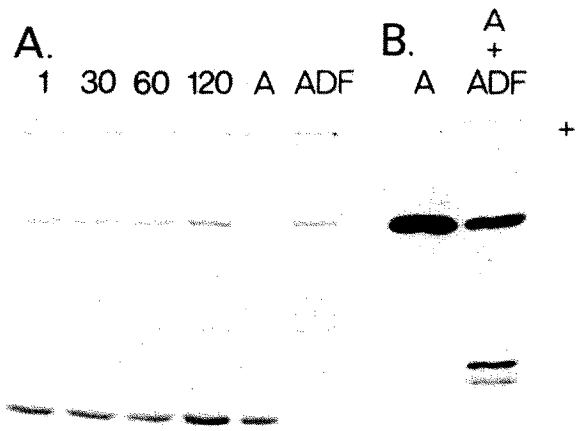

Fig.3. (A) SDS–PAGE (10% gel) of rabbit skeletal muscle actin incubated at 20°C with purified ADF. Depolymerizing capacity of ADF, 33 μg actin/ml; 30 μg actin/ml. Samples taken at 1, 30, 60 and 120 min. (A) control actin; ADF, control ADF. (B) Isoelectric focussing under denaturing conditions of: (A) rabbit skeletal muscle actin; (A+) ADF, actin–ADF mixture incubated 60 min at room temperature. ADF was partially purified as in fig.1(A,4) and had the capacity to depolymerize 240 μg/ml actin; 250 μg actin/ml.

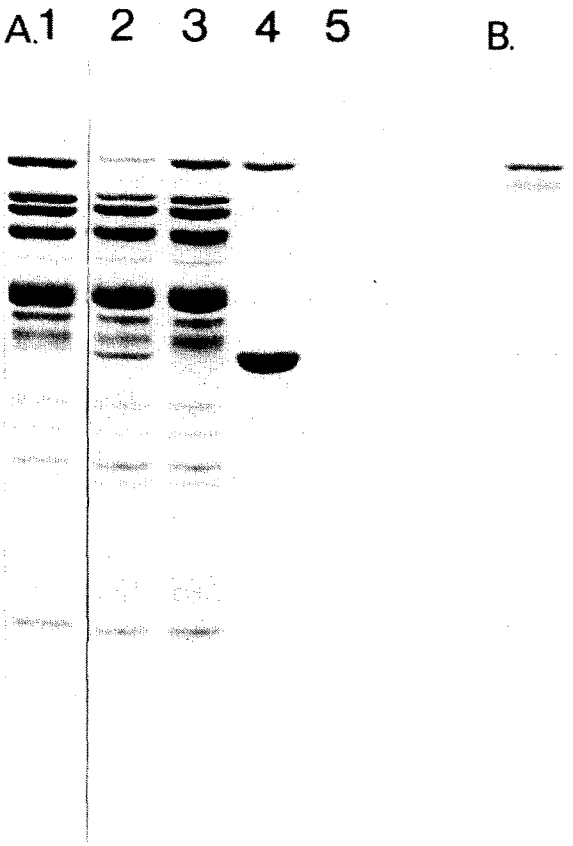

Fig.4. Specific binding of pig plasma ADF to actin–DNase–Sepharose. (A) Partially purified ADF, 1.2 mg (a similar preparation to fig.1(A,4) passed over 0.5 ml columns of actin–DNase–Sepharose or DNase–Sepharose and washed with 50 mM imidazole–HCl (pH 6.3). Tightly bound protein then eluted with 8 M urea. (1) crude ADF; (2) protein not bound to actin–DNase; (3) protein not bound to DNase; (4) urea eluate from actin–DNase; (5) urea eluate from DNase. (B) protein bound to actin–DNase when 3 ml (169 mg protein) whole plasma passed over actin–DNase–Sepharose.

tion of the DNase was achieved and there was no further change over the next several minutes. This result was independent of $CaCl_2$ concentration (1 mM $CaCl_2$, 5×10^{-7} M $CaCl_2$ and 1 mM EGTA were used). Even at a 10-fold lower ADF concentration the reaction was complete within 15 s, although total inhibition was <10%.

Fig.4A shows that the 92 000 M_r protein binds to actin–DNase–Sepharose but not to DNase–Sepharose. Elution with 8 M urea released only actin and the 92 000 M_r polypeptide. Fig.4B shows a similar experiment in which whole plasma was passed over actin–DNase–Sepharose. ADF is 0.1–0.2% of plasma protein and cannot be detected in gels of whole plasma. 59% of the ADF activity bound to the affinity column and the gel shows that the 92 000 M_r polypeptide is the major one eluted with 8 M urea. Thus, ADF forms a relatively stable complex with actin–DNase.

Activity measurements suggest that the ratio of ADF to actin monomers in an excess of F-actin is ~1. In an experiment to calibrate activity units [3], 81.3 units were equivalent to 3.15 nmol actin; therefore assuming M_r 92 000 to ADF and a stoichiometry of 1:1, the specific activity of pure ADF would be 280 units/mg. The highest activity measured in purified ADF was 205 units/mg which corresponds to 73% maximal purity.

4. Discussion

This evidence indicates that a 92 000 M_r polypeptide which binds specifically to actin on an affinity column is the actin depolymerizing factor of pig plasma [3]. Reaction of ADF with an excess of F-actin is complete with 15 s and proportional to the ADF concentration. The maximal activity achieved in highly purified preparations is >70% of theoretical based on a 1:1 stoichiometry of the 92 000 M_r component to G-actin. Gel densitometry of purified preparations combined with activity measurements support the conclusion that ADF acts by combining in a stoichiometry between 0.5:1 and 1:1 with actin monomer, assuming equal staining with Coomassie blue.

The amount of actin depolymerized by plasma and serum is ~100 μg/ml [3]. A 1:1 stoichiometry would thus indicate a concentration of ADF in plasma and serum of 220 μg/ml. The plasma protein concentration of ~60 mg/ml implies a purification of 273-fold would be required to obtain pure ADF. The value obtained here (fig.1) indicates a preparation of 90% has been achieved which is consistent with the stoichiometry discussed above.

The reaction of pig plasma ADF with actin is independent of Ca^{2+} concentration but the heat stability of the protein is markedly enhanced by Ca^{2+}. By contrast the ADF activity of human serum is reported to be abolished by Ca^{2+} chelators [2]; this may be due to an effect on factor stability rather than on the depolymerizing reaction itself.

Norberg et al. [2] suggest that human serum ADF acts catalytically because serum initially saturated with F-actin can carry out further depolymerization after several hours. Our results indicate that the mechanism involves the formation of a stoichiometric complex with G-actin. This mechanism would be consistent with [2] only if the complex dissociated slowly, releasing ADF competent to carry out further depolymerization after several hours.

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