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IDENTIFICATION OF A FACTOR IN CONVENTIONAL MUSCLE ACTIN PREPARATIONS WHICH INHIBITS ACTIN FILAMENT SELF-ASSOCIATION

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SUMMARY: Gel filtration of depolymerized conventionally purified muscle actin separates from the actin monomers a fraction of minor contaminants with a Stokes' radius of 4.7 nm which has the ability to block actin filament network formation. On the basis of heat and trypsin sensitivity, this inhibitory activity appears to be a protein. The inhibitory activity binds to actin filaments and reduces their low shear viscosity by up to 99% in a concentration dependent fashion while reducing polymerization to only a minor extent.

INTRODUCTION

Actin isolated from skeletal muscle by the method of Spudich and Watt (1) is now widely used in many experiments on muscle contraction and cellular motility. It has been shown repeatedly by gel electrophoresis that the actin purified by this method is more than 95% pure and it is generally assumed to be free of active contaminants. However, in the course of low shear viscosity studies of actin gels prepared with protein crosslinkers and of the mechanism of action of cytochalasin B (2,3), we found that these conventional actin preparations contain a low concentration of a potent inhibitor of actin filament interactions. Although it has not yet been possible to purify the molecule responsible for this inhibition of low shear viscosity, we want to alert users of actin to its existence, to show how it can be separated from pure actin and to describe some of its properties.

METHODS

Actin preparation: Actin was prepared from rabbit or chicken skeletal muscle acetone powders by a modification of the method of Spudich and Watt (1). The acetone powder was extracted with 20 ml of Buffer A (2 mM tris-HCl pH 8, 0.5 mM dithiothreitol, 0.2 mM ATP, 0.2 mM $CaCl_2$) per gm of powder for 30 min at 0°C and clarified by a centrifugation at

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16,000 rpm in a Beckman JA-20 rotor for 30 min at 4°C. To remove the trapped actin, the pellet was resuspended with the same volume of Buffer A and clarified as above. The supernates were combined. Extracted actin was polymerized by the addition of KCl to 50 mM and $MgCl_2$ to 2 mM for 30 min at room temperature. After cooling to 4°C for 30 min, KCl was added to 0.8 M and the solution was stirred for 30 min at 4°C. Actin filaments were pelleted by centrifugation at 100,000 g for 3 h. The surfaces of the pellets were washed with Buffer A; the pellets were gently homogenized in Buffer A to give a solution of 8-10 mg/ml which was then dialyzed for 48 h. vs several changes of Buffer A to depolymerize the actin. Finally the actin was clarified by centrifugation at 100,000 g for 1 to 2 h. at 4°C.

Biochemical techniques: Methods for gel electrophoresis (4), viscometry (2) and electron microscopy (5) have been described. Actin concentration was measured by absorbance using $E_{290} = 0.65\ cm^2 \cdot mg^{-1}$. The Stokes' radius was calculated by the method of Ackers (6) using blue dextran, catalase, bovine serum albumin, myoglobin and ^3H_2O to calibrate the Sephadex G-150 column.

RESULTS

Actin filament solutions have a much higher viscosity at low shear rates than high shear rates (2,3,7,8 and Table 1) presumably due to formation of networks by weak interaction of filaments. It seemed possible to us that this network formation is promoted by traces of cross-linking molecules contaminating the actin, so we attempted to purify the actin further by gel filtration (Figure 1). This procedure separates actin oligomers in the void volume from the bulk of the monomeric actin (1,9). Using a miniature falling ball viscometer (2) with a low shear rate to detect associations between the filaments, we found, to our surprise, that

TABLE 1. COMPARISON OF ACTIN FILAMENT VISCOSITIES

	Ostwald	Falling Ball	
Actin	2 mM $MgCl_2$	2 mM $MgCl_2$	2 mM $MgCl_2$, 100 mM KCl
Conventional	0.40 cs	12 cp	10 cp
Gel filtered	0.52 cs	88 cp	654 cp

Conditions: For Ostwald viscometry 0.5 mg/ml actin was polymerized for 10 min at 25°C in 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.25 mM DTT, 0.1 mM ATP, 10 mM imidazole pH 7.0. For falling ball viscometry 1 mg/ml actin was polymerized for 10 min at 25°C in 10 mM imidazole pH 7 and 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA ± 100 mM KCl.

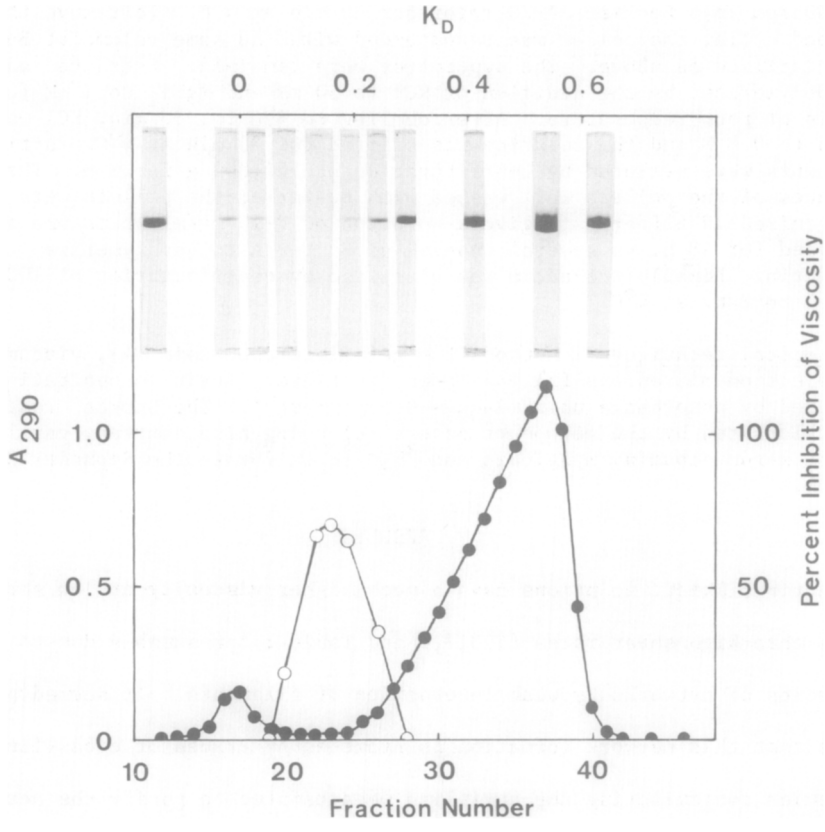

Figure 1. Gel filtration chromatography of depolymerized conventional actin on Sephadex G-150 in Buffer A. Forty mg of actin in 5 ml were applied to a 2.5 x 51 cm column and 5 ml fractions were collected. Absorbance at 290 nm (●). Inhibition of the low shear viscosity of the purified actin expressed as a percent of the viscosity of actin alone was measured by mixing an equal volume of the peak actin fraction with other fractions in 10 mM imidazole pH 7, 2 mM $MgCl_2$ at 0^oC, followed by incubation at 25^oC for 10 minutes and measurement of low shear viscosity (o). The protein composition of the starting sample (on the left) and several column fractions (above the corresponding fractions) was assessed by polyacrylamide gel electrophoresis in sodium dodecylsulfate.

gel filtered actin has a higher viscosity at low shear than the starting sample (Table 1). This difference is amplified by KCl (Table 1) or K-glutamate (not shown). These observations suggested 1) that network formation might be due to self-association of actin filaments rather than to cross-linking by minor contaminating molecules, and 2) that conventional actin preparations might contain an inhibitor of these self-associations.

TABLE 2. LOW SHEAR VISCOSITY OF ACTIN MIXED WITH G-150 COLUMN FRACTIONS

	Pure actin		
Conditions	Alone	+ Void volume	+ Inhibitor
2 mM $MgCl_2$	71 cp	137 cp	7 cp
2 mM $MgCl_2$, 100 mM KCl	154 cp	328 cp	9 cp

Conditions: 500 µg/ml of gel filtered actin alone or with 26 µg/ml void volume fraction or with 7 µg/ml inhibitor fraction was polymerized in a measuring capillary tube for 10 min at 25°C in 10 mM imidazole pH7, 1.8 mM tris, 0.45 mM DTT, 0.18 mM ATP plus $MgCl_2$ and KCl as indicated.

To establish the existence of such an inhibitor of actin filament interactions, we assayed all of the fractions from the gel filtration column for their effect on the low shear viscosity of the gel filtered actin. The void volume fractions increase the low shear viscosity to a small extent (Table 2) while the fractions eluting at K_d = 0.15 reduce the low shear viscosity in both $MgCl_2$ and $MgCl_2$ plus KCl (Figure 1, Table 2). The extent of the inhibition depends on the concentration of inhibitor fraction added at the outset of polymerization (Figure 2). The inhibitor fractions also reduce the low shear viscosity of fully polymerized actin. For example, 7 µg/ml of inhibitor fraction mixed with 500 µg/ml of polymerized actin prevents the recovery of the low shear viscosity so that after 30 minutes the viscosity is only 11% as high as a parallel sample mixed with buffer.

Since the inhibitor activity copurifies with actin during cycles of polymerization and depolymerization and is separated from monomeric actin by gel filtration chromatography, the inhibitor activity appears to bind tightly to actin filaments but not to actin monomers. Binding to actin filaments was demonstrated directly by showing that the inhibitor activity is removed from the supernate after mixing inhibitor with pure actin,

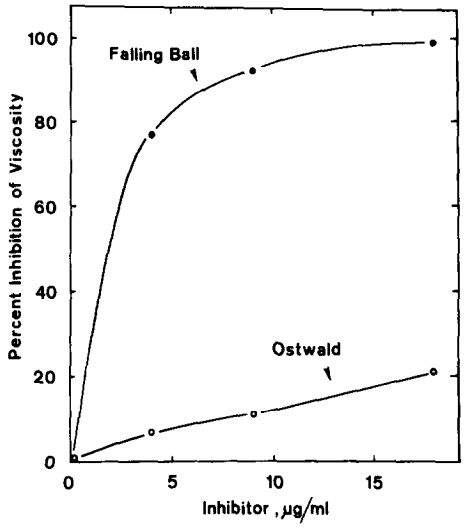

Figure 2. Inhibition of pure actin viscosity by G-150 inhibitor fractions. Using 500 µg/ml of G-150 purified actin and the conditions described in Figure 1, the low shear (falling ball) viscosity (•) and high shear (Ostwald) viscosity (o) were measured with several concentrations of inhibitor.

polymerizing the actin and pelleting the actin filaments. The inhibitor activity does not pellet in the absence of actin.

We used a conventional ("Ostwald") capillary viscometer to examine the effect of the inhibitor fractions on the rate and extent of actin polymerization. These viscometers have a high shear rate which largely disrupts any weak associations between the filaments so that the observed viscosity depends primarily on polymer concentration and size distribution. In 20 mM KCl, where polymerization is slow, the void volume fractions reduce the lag prior to rapid polymerization, while the inhibitor fractions decrease the maximum rate of the viscosity change (Figure 3). In the 2 mM $MgCl_2$ buffer used for the low shear viscosity experiments, the inhibitor fractions reduce to a small extent both the rate of the viscosity change and the steady state high shear viscosity (Figure 3). The inhibition of steady state high shear viscosity depends on the concentration of inhibitor fraction (Figure 2). Thus, the presence of the inhibitor activity may account for the slightly lower Ostwald viscosity of conventional actin compared with gel filtered actin (Table 1).

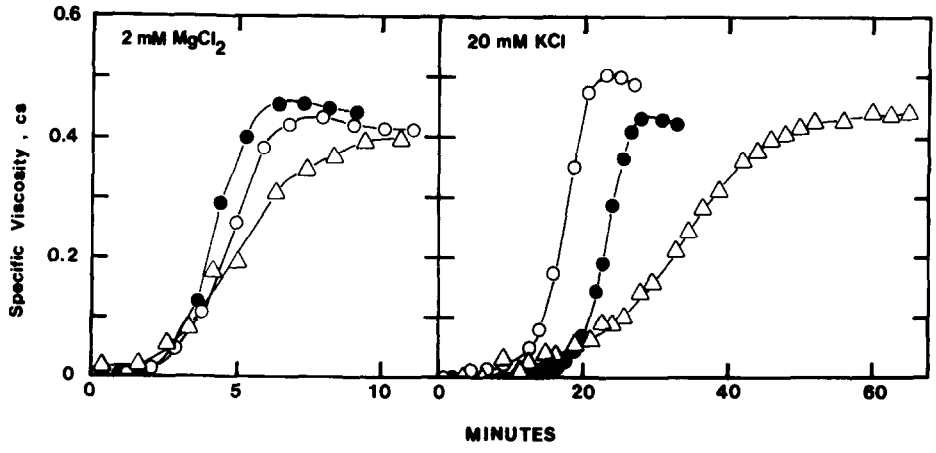

Figure 3. Influence of G-150 fractions on the time course of actin polymerization. Ostwald viscosity was measured at 25°C. 500 µg/ml of purified actin was polymerized in 10 mM imidazole pH 7, 1.8 mM tris, 0.45 mM DTT, 0.18 mM ATP, 0.2 mM $CaCl_2$ with 2 mM $MgCl_2$ or 20 mM KCl as noted. Pure actin (●), plus 26 µg/ml void volume fraction (o) or plus 7 µg/ml G-150 inhibitor fraction (Δ).

The inhibitor fractions reduce the low shear viscosity of actin filaments much more than the high shear viscosity (Figure 2). At a ratio of 15 µg inhibitor fraction to 500 µg actin the low shear viscosity of actin is reduced by 99% (to buffer levels) and the high shear viscosity is reduced by 18% (Figure 2). This high concentration of inhibitor reduces the fraction of polymerized actin by 10% as determined by pelleting. By electron microscopy of negatively stained specimens, the actin filaments formed in the presence of the inhibitor activity are indistinguishable in size and morphology from filaments of pure actin.

Conventional actin preparations from both rabbit and chicken skeletal muscle contain this inhibitor activity. Different acetone powders vary in their content of inhibitor activity, accounting for the variability observed in the low shear viscosity of different conventional actin preparations.

The viscosity inhibiting activity eluted from the gel filtration column at the position expected for a molecule with a Stokes' radius of 4.7 nm. The ability of these fractions to inhibit the low shear viscosity of actin filaments was destroyed by boiling or by trypsin digestion but not by RNase,

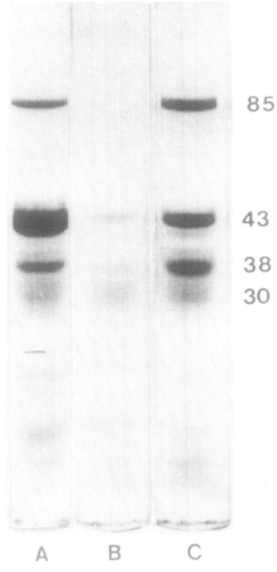

Figure 4. DEAE-cellulose chromatography of the pooled G-150 inhibitor fractions. The low shear viscosity of actin was inhibited by the samples to the following extent: (A) Pooled G-150 peak, 98% inhibition; (B) 125 mM KCl DEAE fraction, >99% inhibition, (C) 250 mM KCl DEAE fraction, 0% inhibition. Assay conditions: 500 µg/ml actin, 2 mM $MgCl_2$, 10 mM imidazole pH 7, 40% test sample by volume. Concentrated samples equivalent to 250 µl of the original fractions were analyzed by gel electrophoresis in sodium dodecylsulfate.

suggesting that the factor is either a protein or is associated with and requires a protein for its activity. The fractions containing the inhibitor activity are very dilute (Figure 1), but after concentration are found to contain several polypeptides (Figure 4A), including a major component with the same electrophoretic mobility as actin. The peak of viscosity inhibiting activity from the G-150 column can be separated from the bulk of the actin by further chromatography on DEAE-cellulose (Figure 4). The composition of the peak of inhibitor activity from the DEAE column is similar to both the column sample (Figure 4A) and the later column fractions lacking inhibitor activity (Figure 4C). All (Figure 4) contain some actin plus other polypeptides with molecular weights of 85,000, 38,000 and 30,000. However, as illustrated by the gels in Figure 4, the DEAE peak (4B) has a specific activity more than 20 times higher than the gel filtration peak (4A) and

the inactive DEAE fractions (4C). Further purification of the inhibitor activity has not been successful due to the small amount of inhibitor material in muscle actin preparations.

DISCUSSION

These experiments demonstrate that conventional preparations of skeletal muscle actin (1) contain a small amount of a factor which binds to actin filaments and which in 2 mM $MgCl_2$ strongly inhibits the low shear viscosity of actin filament solutions while only minimally inhibiting the rate and extent of polymerization. We suspect that the active material is a large protein from its Stokes' radius of 4.7 nm and its heat and trypsin sensitivity. However, the identification of the inhibitor molecule will require further purification of the active material. This may be difficult using conventional actin as starting material because the factor represents considerably less than 1% of the protein.

We have considered the possibility that the inhibitor is an altered form of actin, and while this remains a possibility, the inhibitor activity is separated from the bulk of the actin during both gel filtration (Figure 1) and ion exchange (Figure 4) chromatography. In particular, the inhibitor activity is clearly distinct from the void volume fractions of the gel filtration column (Table 2) which contain a higher concentration of aggregated actin (Figure 1). However, whether this factor is an altered form of actin or some other molecule, it is necessary to acknowledge the existence of this activity in muscle actin preparations and the effect it may have on the analysis of actin function.

Although the exact mechanism of action of the inhibitor factor(s) could not be established in these initial experiments, the effect resembles the action of cytochalasin on actin filaments. Both the inhibitor activity and cytochalasins bind to actin filaments, but not actin monomers (10,11,12). Both inhibit the low shear viscosity of filaments of gel filtered actin without substantially reducing the extent of polymerization (3,10). Both

reduce the rate of actin polymerization, more in some buffers such as 20 mM KCl, than others like 2 mM $MgCl_2$ (3,10,11,12,13).

Other proteins can reduce the extent of actin polymerization or the length of actin filaments and thereby inhibit network formation. For example β-actinin (14), plasmodium actinin (15,16), gelsolin (17) and profilin (18) all seem to act principally by altering the extent of polymerization or the length of the filaments. This distinguishes them from the factor studied here which can reduce the low shear viscosity more than >90% with only minimal (<10%) inhibition of high shear viscosity, a measure of filament length.

The discovery of this viscosity inhibiting factor in muscle actin led us to search for similar molecules elsewhere. In a subsequent paper (19) we describe the purification of such a protein from Acanthamoeba. Like the muscle inhibitor, the Acanthamoeba protein has a Stokes' radius of about 4.4 nm. Moreover, at very low concentrations it inhibits the low shear viscosity of actin filaments and reduces the rate of actin polymerization. Just like cytochalasin (3), the Acanthamoeba protein inhibits polymerization by blocking monomer addition at the "barbed" end of the actin filaments. Consequently we call the Acanthamoeba protein a "capping protein." The strong similarities suggest that the muscle inhibitor may have a related mechanism.

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