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# Conformational changes in subdomain-2 of G-actin upon polymerization into F-actin and upon binding myosin subfragment-1

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The susceptibility of subdomain-2 of actin to different proteases has been examined, for G-actin, F-actin, G-actin-$S_1(A_2)$ and F-actin-$S_1(A_2)$ complexes on a comparative basis. The sites of subtilisin, α-chymotrypsin and trypsin attack, exposed on G-actin, are protected in F-actin, F-actin-$S_1(A_2)$ as well as in the G-actin-$S_1(A_2)$ complex. In contrast, a new cleavage site ($Arg^{39}$–$His^{40}$) for ArgC protease, which is protected in G-actin, is exposed in G-actin-$S_1(A_2)$ as well as in F-actin and F-actin-$S_1(A_2)$. These results are consistent with the previously proposed structural analogy between the ternary $(G\text{-actin})_2S_1$ and the F-actin-$S_1$ complexes, and provide information on the mechanism of $S_1$-induced polymerization of G-actin.

Actin; Myosin; Actomysin interaction; Conformational change; Limited proteolysis

## 1. INTRODUCTION

It is well known that myosin subfragment-1 ($S_1$) induces the polymerization of G-actin into decorated F-actin-$S_1$ filaments, even in low ionic strength buffers in which actin alone remains monomeric [1,2]. We have shown [3] that the first intermediate in the assembly pathway was a ternary $G_2S$ complex, in which $S_1$ interacts tightly with 2 G-actin molecules, in low ionic strength buffer and in the absence of ATP. The $G_2S$ complex then self-assembles into short $(G_2S)_n$ oligomers [4]. EDC-crosslinking experiments [5] have shown that the electrostatic close contacts between actin and myosin were very similar, if not strictly identical, in $G_2S$ and F-acto-$S_1$ complexes. These data suggested that the orientation of the two actin molecules interacting with $S_1$, in the $G_2S$ complex, might be structurally similar to the F-acto-$S_1$ complex, in which $S_2$ interacts mainly with the N-terminal and C-terminal regions in subdomain-1 of one actin subunit, and more weakly with the protruding helix 79–95 of a second actin subunit, adjacent to the first one at the barbed end, along the long pitch helix of the filament [6,7]. In this longitudinal actin dimer-$S_1$ complex, subdomain-2 of the second actin molecule appears 'clamped' by the myosin head. Conformation changes of actin subdomain-2 therefore are expected to occur upon formation of F-actin, or $G_2S$ or F-acto-$S_1$ complexes. In the present work, the nature of such structural changes has been investigated by limited proteolysis, since this region of actin is known to be susceptible to proteolytic attack [8]. The results show that the $Arg^{39}$–$His^{40}$ bond, which is protected in the G-actin state, is exposed to ArgC cleavage both in G-actin-$S_1$, F-actin and F-actin-$S_1$ complexes; on the other hand, loop 45–52 and segment 61–69 which are susceptible to subtilisin, α-chymotrypsin and trypsin on G-actin, are both protected from proteolytic attack in the G-actin-$S_1$, F-actin and F-actin-$S_1$ complexes. Our results complement and extend the recent work of Chen et al. [9], and are in support of the previously proposed structure of the $G_2S$ complex [3–5], and of its being a precursor in $S_1$-induced polymerization of G-actin.

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## 2. MATERIALS AND METHODS

G-actin and myosin were purified from rabbit skeletal muscle as described [3–5]. G-actin (~2 mg/ml) was stored on ice in buffer G (5 mM Tris-HCl pH 7.8, 0.1 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ATP, 0.01% $NaN_3$) and used within 2 weeks. The $S_1(A_1)$ and $S_1(A_2)$ isoforms of chymotryptic myosin subfragment-1 were resolved by cation exchange chromatography on SP-trisacryl (IBF) [5]. Trypsin (TPCK-treated) and α-chymotrypsin were purchased from Worthington. Arg-C protease was from Boehringer and subtilisin carlsberg (P5380) from Sigma.

Before each experiment actin and $S_1(A_2)$ were dialyzed separately overnight against buffer $G_0$, which is buffer G without ATP. The G-actin-ATP 1:1 complex was then prepared by Dowex-1 treatment of the G-actin solution [3].

All proteolytic digestions of G-actin, $S_1$ and G-actin-$S_1$ complexes were done with 5 μM G-actin and 5 μM $S_1A_2$, at 10°C in buffer $G_0$ (or 5 mM HEPES, pH 8.0, 0.1 mM $CaCl_2$ in the case of ArgC digestion). Under these conditions no polymerization into decorated filaments took place in the time course of the experiments [5], which was further controlled by light scattering. Proteolytic digestion of F-actin and F-actin-$S_1(A_2)$ complexes were done under identical conditions except for the presence of 0.1 M KCl in the buffer. F-actin had been prepolymerized for 90 min at 25°C in the presence of 0.1 M KCl. The digestions were done under the following conditions: trypsin 5 μg/ml, 5–30 min, digestion was stopped by addition of 10 μg/ml trypsin

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soybean inhibitor; α-chymotrypsin 0.1 mg/ml, 3 min, digestion was stopped by 1 mM PMSF; subtilisin 0.1 μg/ml, 5–30 min, reaction was stopped by 1 mM PMSF; ArgC 20 U/ml, 180 min, digestion was stopped by rapid thermodenaturation at 100°C following addition of boiling 5 mM β-mercaptoethanol and 2% SDS.

Samples were denatured and submitted to SDS-PAGE according to Laemmli [10] using a 8% to 18% acrylamide gradient and a Tris-borate electrophoresis buffer [8]. 20 μg of actin and/or 50 μg $S_1A_2$ were loaded in the slots. Molecular weight markers were: myosin (212 kDa), $\alpha_2$ macroglobulin (170 kDa), β-galactosidase (116 kDa), phosphorylase b (94 kDa), transferrin (76 kDa), bovine serum albumin (67 kDa), glutamic dehydrogenase (53 kDa), ovalbumine (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (soybean) (21 kDa), α-lactalbumin (14 kDa).

The location of ArgC cleavage site on G-actin was determined by sequencing the 37 kDa proteolytic fragment, following SDS-PAGE and electrotransfer on a PVDF membrane (Problot) using an Applied Biosystem instrument and 50 mM Tris-100 mM borate pH 8.0 buffer. Sequencing was performed in gas phase by sequential Edman degradation.

## 3. RESULTS

The regions of the actin molecule that are susceptible to proteases are known to be located essentially in subdomain 2 [8]. The cleavage sites of subtilisin ($Met^{47}$–$Gly^{48}$), α-chymotrypsin ($Met^{44}$–$Val^{45}$ and $Leu^{67}$–$Lys^{68}$) and trypsin ($Arg^{62}$–$Gly^{63}$ and $Lys^{68}$–$Tyr^{69}$) have been well characterized [8,11–13]. The susceptibility to these proteases as well as to ArgC proteolysis was examined for G-actin, F-actin, G-actin-$S_1(A_2)$ and F-actin-$S_1(A_2)$ complexes on a comparative basis. Fig. 1 shows that subtilisin attack was more effective on G-actin (lane c) than on F-actin (lane d), and that binding of $S_1(A_2)$ exerted a protective effect, both in G-actin-$S_1$ (lane f) and F-actin-$S_1$ (lane g) complexes. $S_1(A_2)$ was practically not cleaved by subtilisin under the same conditions (lane e). This result is at variance with the recent report by Chen et al. [9] who found that binding of $S_1(A_2)$ to G-actin did not protect G-actin against subtilisin attack; the most likely explanation for this discrepancy is the presence of 0.2 mM ATP in the digestion assay used in [9], which is known to weaken the G-actin-$S_1(A_2)$ interaction [3].

The results concerning α-chymotrypsin digestion of actin are displayed in Fig. 2. The data show that both cleavages of G-actin at $Met^{44}$ and $Leu^{67}$, leading to the 35 kDa and 33 kDa fragments (lane c) are greatly inhibited when $S_1(A_2)$ is bound to G-actin (lane f): only the 35 kDa (30% of the total actin) and no 33 kDa polypeptides were observed under conditions where G-actin alone was 90% proteolyzed into 33 kDa. When F-actin was submitted to α-chymotrypsin attack, only a low amount of 35 kDa was produced, and no 33 kDa (lane d). Total protection of the first cleavage was afforded by $S_1(A_2)$ binding to F-actin (lane g). One also should

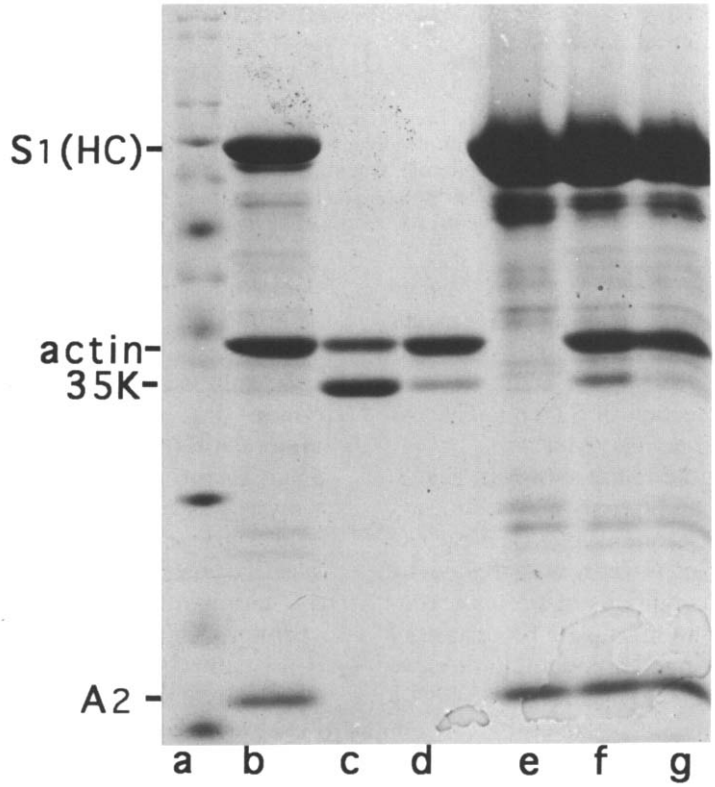


Fig. 1. Subtilisin digestion of G- and F-actin in the presence and absence of $S_1(A_2)$. Actin (5 μM) and/or $S_1(A_2)$ (5 μM) were submitted to digestion by subtilisin (0.1 μg/ml) for 5 min at 10°C in buffer $G_0$ (5 mM Tris pH 7.6, 0.2 mM DTT, 0.1 mM $CaCl_2$, no ATP), as described in section 2. Lane a, molecular weight markers; lane b, undigested actin and $S_1(A_2)$; lane c, ATP-G-actin (1:1 complex) + subtilisin; lane d, F-ADP-actin + subtilisin; lane e, $S_1(A_2)$ + subtilisin; lane f, G-actin-$S_1(A_2)$ + subtilisin; lane g, F-actin-$S_1(A_2)$ + subtilisin.

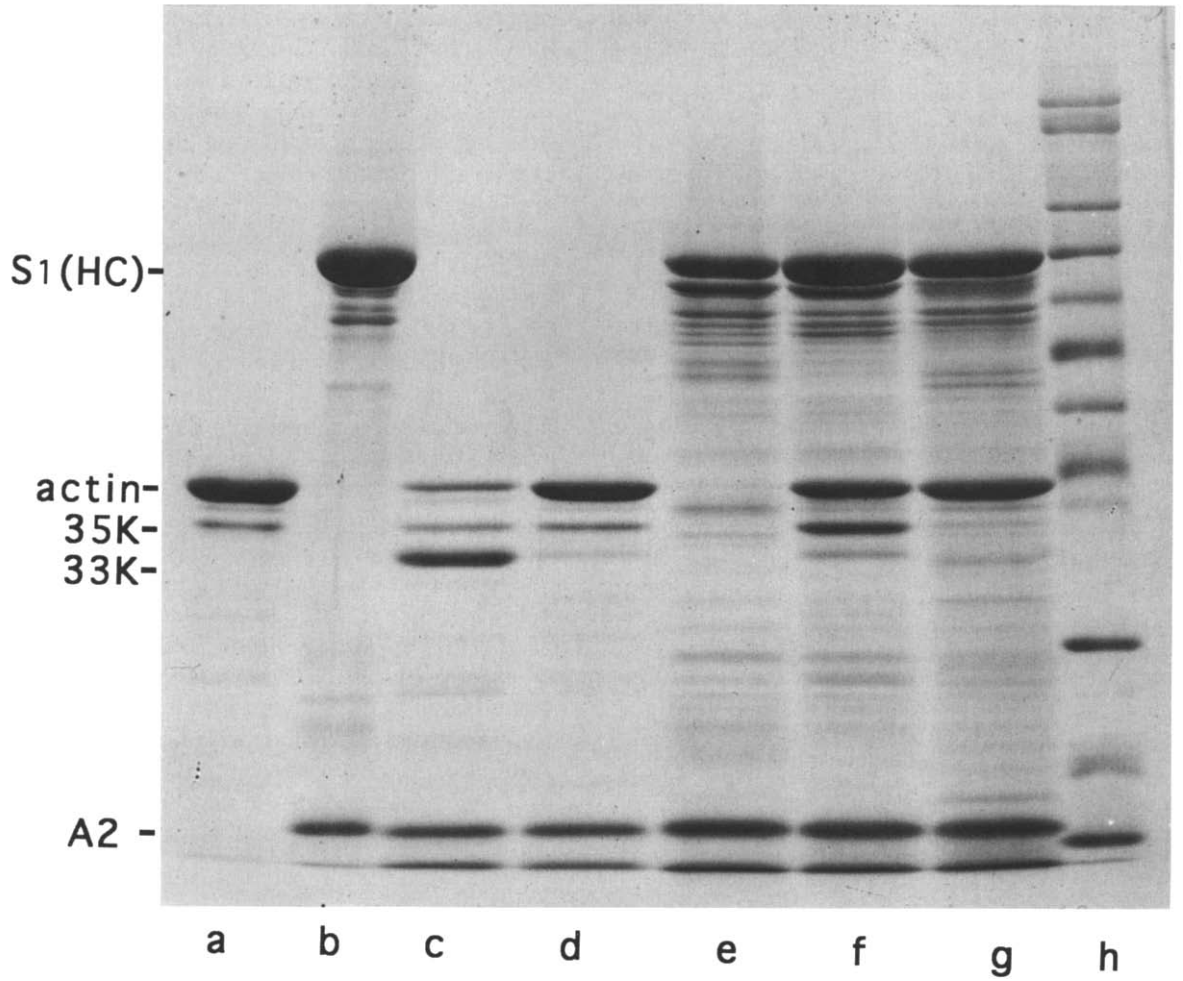


Fig. 2. α-Chymotrypsin digestion of G- and F-actin in the presence and absence of $S_1(A_2)$. Actin and/or $S_1(A_2)$ were submitted to limited α-chymotryptic digestion as described in section 2. Experimental conditions are as under Figure 1. Lane a, undigested ATP-G-actin (1:1 complex); lane b, undigested $S_1(A_2)$; lane c, ATP-G-actin + α-chymotrypsin; lane d, F-actin + α-chymotrypsin; lane e, $S_1A_2$ + α-chymotrypsin; lane f, G-actin-$S_1A_2$ + α-chymotrypsin; lane g, F-actin-$S_1(A_2)$ + α-chymotrypsin; lane h, molecular weight markers.

note that the α-chymotryptic cleavage of $S_1(A_2)$ heavy chain was protected by G-actin, and to a greater extent F-actin binding (compare lanes b, e, f, g). Again our results agree only partially with Chen et al. [9] who observed essentially no protection of $S_1(A_2)$ against α-chymotrypsin cleavage of G-actin at $Met^{44}$.

The results of trypsin digestion are shown in Fig. 3. In agreement with Chen et al. [9], only a 34 kDa fragment (corresponding to the second tryptic cleavage site at $Lys^{68}$) was obtained when G-actin was treated by trypsin (lane d), under all conditions of protease concentration and digestion times. Complete protection of the trypsin cleavage was observed in F-actin (lane e), as well as in G-actin-$S_1(A_2)$ (lane g) and F-actin-$S_1(A_2)$ (lane h) complexes. Note that, as observed by Chen et al. the protective effect of $S_1$ was exerted despite its extensive cleavage into 75 kDa, 50 kDa and 20 kDa fragments during the digestion.

The results of ArgC digestion shown in Fig. 4, demonstrate that while G-actin was not cleaved by ArgC (lane c), a fragment of 37 kDa was obtained when F-actin was digested by ArgC (lane d). The cleavage was also observed in G-actin-$S_1(A_2)$ complex (lane g), and somewhat more accentuated in F-actin-$S_1(A_2)$ complex (lane e). In agreement with a previous report [14], binding of actin (both G-actin and F-actin) to $S_1$ protected against ArgC cleavage of $S_1$ at $Lys^{640}$. When ArgC proteolysis was carried out using G-actin fluorescently labeled on $Cys^{374}$ by DACM [14], the 37 kDa fragment was fluorescent (data not shown), showing that it was a C-terminal fragment. Sequencing of the 37 kDa polypeptide yielded HQGVM only as N-terminal sequence, consistent with the location of ArgC cleavage at the $Arg^{39}$–$His^{40}$ bond. Therefore this bond is more exposed in the actin-$S_1$ complexes and in the F-actin state than in the G-actin state. It is noteworthy that in the presence of EDTA, which leads to actin denaturation, via the sequential dissociations of tightly bound Ca and ATP [16], a similar proteolytic cleavage of the $Arg^{39}$–$His^{40}$ bond was obtained by thrombin [8].

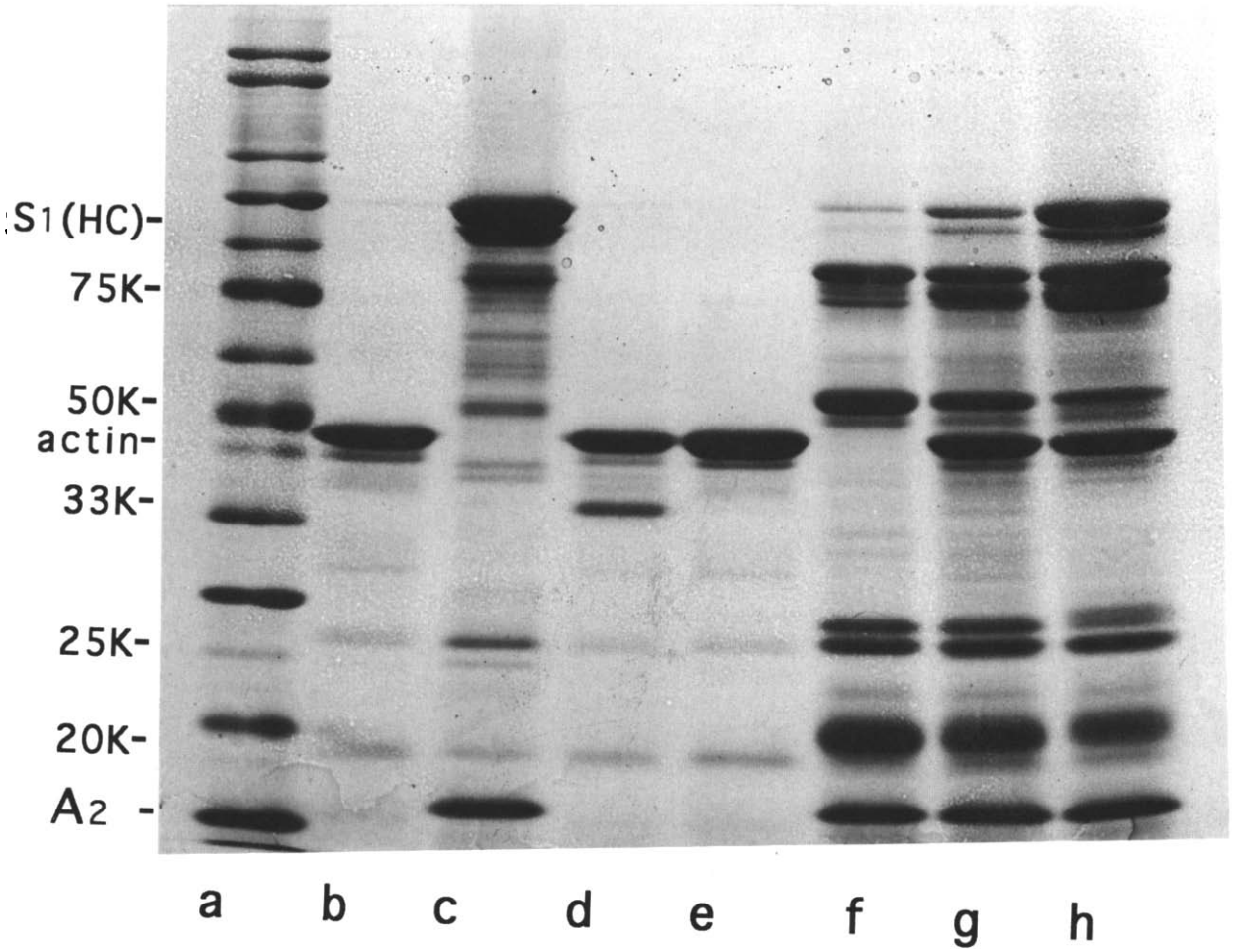


Fig. 3. Tryptic digestion of G- and F-actin in the presence and absence of $S_1(A_2)$. Actin and/or $S_1(A_2)$ were submitted to limited trypsin digestion for 8 min as described in section 2. Experimental conditions are as in Fig. 1. Lane a, molecular weight markers; lane b, undigested actin; lane c, undigested $S_1(A_2)$; lane d, ATP-G-actin (1:1 complex) + trypsin; lane e, F-actin + trypsin; lane f, $S_1(A_2)$ + trypsin; lane g, G-actin-$S_1(A_2)$ + trypsin; lane h, F-actin-$S_1(A_2)$ + trypsin.

## 4. DISCUSSION

Proteolytic digestion by subtilisin, α-chymotrypsin, trypsin and ArgC has been used to examine the conformation changes of regions of subdomain-2 of actin upon filament assembly, and upon interaction of G-actin and F-actin with the myosin head. Two main observations have been made: upon binding of $S_1$ to G-actin, the segments 44–50 and 60–69 are protected from proteolytic attack, while the 39–40 bond is exposed to ArgC proteolysis. Similar changes in subdomain 2 occur upon polymerization of G-actin in filaments. Therefore these regions of subdomain-2 of actin adopt, in the $G_2S$ complex, a conformation very similar to the conformation in the F-actin state. While binding of $S_1$ to G-actin greatly affects the conformation of subdomain-2, binding of $S_1$ to F-actin does not greatly affect its susceptibility to proteases.

These results do not prove, but are supportive of the structure proposed [3–5] for the $G_2S$ complex, within which the two G-actin molecules interacting with the myosin head are connected to each other via longitudinal bonds similar to the actin-actin bonds along the long pitch helix of the F-actin filament. In this interaction, subdomain-2 of the pointed end of one actin molecule interacts with subdomain-1 at the barbed end of the other molecule. This interaction would be responsible for the change in subdomain-2 leading to the exposure of the $Arg^{39}$–$His^{40}$ bond and protection of segments 45–52 and 60–69. These conclusions agree with the structural differences in subdomain-2 between F- and G-actin that could be recently estimated from a Monte Carlo refinement of the positions of the actin domains [17].

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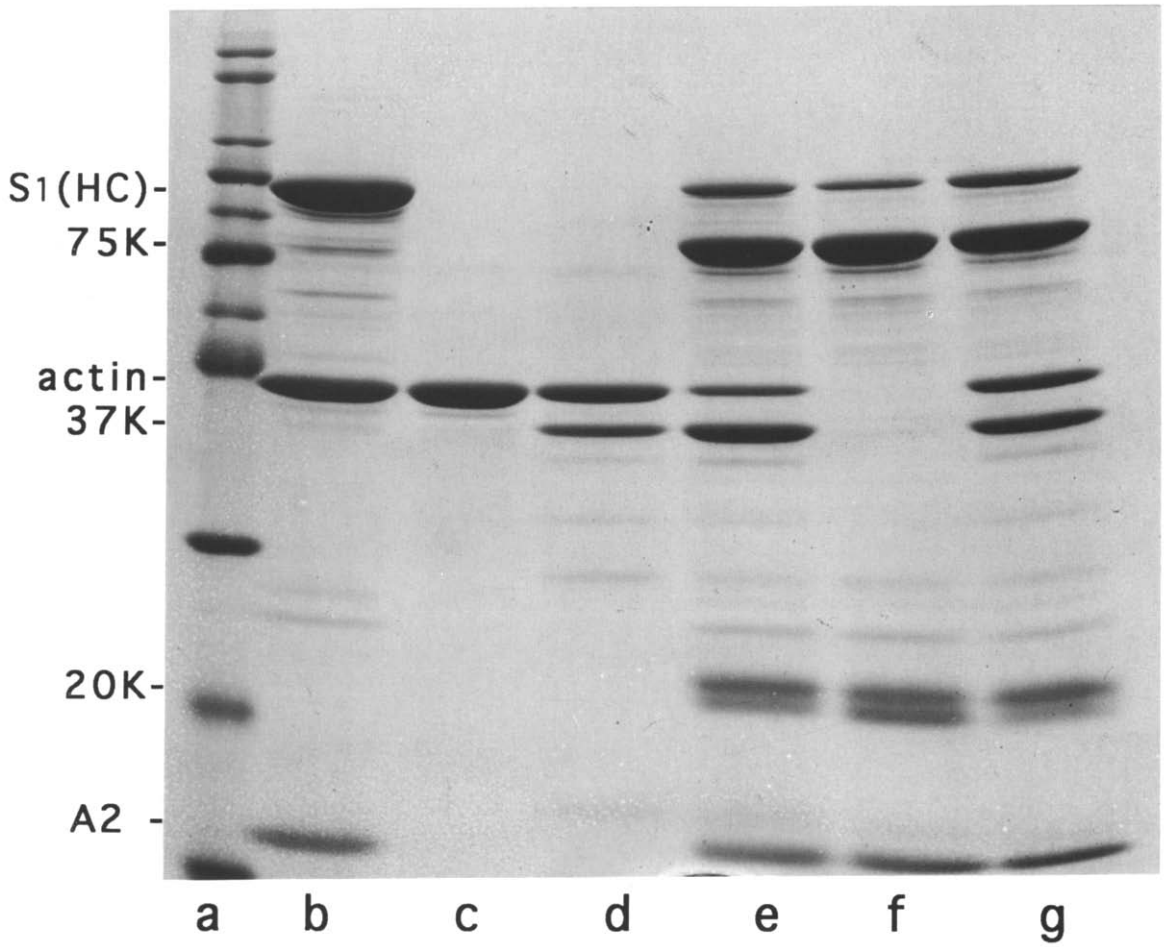


Fig. 4. ArgC digestion of G- and F-actin in the presence and absence of $S_1(A_2)$. Actin (3 $\mu$M) and/or $S_1(A_2)$ (3 $\mu$M) were submitted to ArgC digestion as described in section 2. Lane a, molecular weight markers; lane b, undigested actin and $S_1(A_2)$; lane c, ATP-G-actin (1:1 complex) + ArgC; lane d, F-actin + ArgC; lane e, F-actin-$S_1(A_2)$ + ArgC; lane f, $S_1(A_2)$ + ArgC; lane g, G-actin-$S_1(A_2)$ + ArgC.

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