Muscle gelsolin: isolation from heart tissue and characterization as an integral myofibrillar protein

J.F. Rouayrenc, A. Fattoum*, J. Gabrion, E. Audemard and R. Kassab

Centre de Recherche de Biochimie Macromoléculaire, CNRS 34033 Montpellier Cédex, France

Received 8 December 1983

A 92-kDa polypeptide present in rabbit and dog cardiac muscle was purified to homogeneity and some of its properties were investigated using biochemical and cytochemical approaches. The protein was found to be similar, if not identical to macrophage gelsolin; it cross-reacts immunologically with anti-rabbit macrophage gelsolin antibody, has a Ca^{2+}-sensitive shortening effect on the actin filaments as judged by the high shear viscometry and sedimentation experiments, and has a similar amino acid composition. In addition, immunoblot and SDS polyacrylamide gel analysis of cardiac muscle extracts obtained at high and low ionic strength showed that this protein is tightly bound to myofibrils, both in the absence and presence of Ca^{2+}, in ventricular as well as in atrial muscle cells. Indirect immunofluorescence microscopy revealed a striated gelsolin staining pattern analogous to that previously observed for the skeletal muscle gelsolin, suggesting that in the muscle cell this protein is sharing the same localisation as actin. Because of its severing and nucleating properties the gelsolin may play a major role in the organization, assembly and turnover of the thin filaments within the muscle cells.

Cardiac muscle *Gelsolin* *Myofibril* *Actin filament* *Immunofluorescence*

1. INTRODUCTION

Actin is a major and essential constituent of the contractile machinery of both muscle and non-muscle cells. It is constantly being reorganized in response to a wide variety of factors controlling the filament number, length and their interaction with each other and with other cellular structures. In addition to the ionic conditions, actin filament assembly in vivo may be affected by numerous actin-binding proteins believed to be involved in maintaining cell architecture and generating movement. Although the mechanism of these interactions is not yet well known, such proteins include a category whose activities are regulated by changes in Ca^{2+} concentration [1–5].

One of these proteins is gelsolin, a Ca^{2+}-dependent regulatory protein of actin gel-sol transformation, first discovered and purified from rabbit lung macrophages [6,7]. Gelsolin acts reversibly by shortening the average length of actin filaments at steady state in the presence of micromolar concentrations of calcium. It also influences both the nucleation and elongation steps of actin assembly by blocking the barbed end [8–11]. Recently, using the gelsolin antiserum, it has been demonstrated, by an indirect immunohistochemical technique, that gelsolin is present in substantial amounts not only in non-muscle cells and tissues, but also in smooth, skeletal and cardiac muscles [12]. This last finding is unexpected and raises the question of great interest about how gelsolin may be involved in the function of the muscle where the actin molecules are assembled into thin filaments which are stable structures arranged in a highly organized manner especially in the sarcomeric muscles. Since Ca^{2+}-sensitive actin assembly mechanism has been investigated only in non-muscle cells, we have been interested in studying the occurrence of such reactions in striated muscle cells. We report here the purification, preliminary biochemical character-

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.

ization and cytochemical localisation of rabbit cardiac muscle gelsolin.

2. MATERIALS AND METHODS

Muscle gelsolin was isolated as follows: 100 g of rabbit heart previously perfused and washed several times in ice-cold 0.08 M NaCl solution was either frozen until use or homogenized in 5 vols of 0.3 M KCl, 0.2 mM ATP, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM imidazole-HCl (pH 7.2), 5 mM EGTA, 0.1 mM NaN_3. Antiproteases such as phenylmethylsulfonylfluoride (0.1 mM), trypsin inhibitor (10 μg/ml) and α-macroglobulin (10 μg/ml) were added before blending for twice 30 s at 4°C. After standing for 30 min, the mixture was centrifuged at 100 000 × g for 60 min. The supernatant was then filtered carefully through two layers of gauze and fractionated with solid ammonium sulfate. The pellet resulting from 20% saturation was discarded and the supernatant made up to 50% saturation. The harvested ammonium sulfate pellet was dissolved in a buffer consisting of 20 mM Tris-HCl (pH 7.8), 5 mM EGTA, 1 mM dithiothreitol, 0.1 mM NaN_3, 0.1 mM PMSF and 10% glycerol and dialyzed overnight against the same buffer. Insoluble materials were removed at 100 000 × g for 60 min and the clear supernatant was applied to a (2.5 × 20 cm) column of DEAE-Sepharose CL-6B (Pharmacia) equilibrated in the same buffer and eluted with a linear 0-0.6 M KCl gradient. Gelsolin-containing fractions were pooled, dialyzed against 0.8 M KCl, 1 mM DTT, 1 mM EGTA, 10 mM Tris-HCl (pH 7.5), concentrated to 5 ml by ultrafiltration through an Amicon PM 30 and applied to a 1.5 × 150 cm column of Sephadex G-150 equilibrated and eluted with the same buffer. The eluate was analyzed by 5-15% polyacrylamide gel electrophoresis in the presence of 0.1% SDS (SDS-PAGE) using the discontinuous buffer system in [13] and was checked for its ability to decrease the viscosity of F-actin solutions. Gelsolin-containing fractions were combined, concentrated, dialyzed against 50 mM potassium phosphate (pH 7.5) and applied to a column of hydroxyapatite (1.5 × 12 cm). The column was first washed with 100 ml of 100 mM potassium phosphate, and then eluted with a linear gradient of 0.1-1 M phosphate buffer, pH 7.5 (2 × 100 ml). Fractions containing the 92-kDa protein and emerging at approximately 0.15 M phosphate were pooled and identified as pure gelsolin by SDS-PAGE. These fractions were either stored at −30°C in 40% glycerol (v/v) or dropped in liquid nitrogen to form frozen protein balls and stored at −70°C until use.

Macrophage gelsolin was prepared from rabbits as in [7].

Serum gelsolin was purified from healthy human serum as indicated for muscle gelsolin except that in the starting step the collected serum was directly cut by crystallized ammonium sulfate (30-50% saturation).

Actin was isolated from acetone powder of rabbit skeletal muscle as in [14].

Protein concentrations were obtained using $E_{cm}^{1\%}$ at 290 nm of 6.3 for G-actin and of 6.6 for F-actin [15]. Gelsolin concentration was assayed as in [16] and [17], using crystalline bovine serum albumin as standard.

Amino acid analysis was performed on a Beckman model 120 C autoanalyzer using a single column. For this purpose, samples of purified cardiac muscle gelsolin were dialyzed extensively against distilled water, lyophilized and hydrolyzed with 6 M HCl at 105°C for 48 h in an evacuated, sealed tube.

Myofibril preparation was essentially as in [18].

Viscosity was measured at 25°C by a Cannon-Manning semimicroviscosimeter (extra low charge, size 100) with a charge volume of 0.25 ml. A thermostatted rolling ball viscometer was also used to detect active fractions after each purification step [19].

Immunological experiments: antibodies to rabbit cardiac muscle gelsolin were obtained by immunisation of goat with rabbit alveolar macrophage gelsolin which was further purified by SDS-PAGE. Immunoglobulins IgG from immune serum precipitated by ammonium sulfate (50% saturation). In some cases, analytical quantities of specific antibodies to gelsolin were purified using nitrocellulose paper as in [20]. Thin frozen sections (0.1-0.2 μm) of rabbit atrial and ventricular cardiac muscle were obtained as in [21]; small pieces of tissue were fixed in 2% formaldehyde, infused in 1.2 M sucrose and frozen in liquid nitrogen.

Thin sections were then obtained with a Reichert Ultrocut microtome with a FE_4 cryoattachment and treated for immunofluorescence staining; they

were first incubated for 30 min in purified antibodies against gelsolin at 60 μg/ml in phosphate buffered saline (PBS) with 3% bovine serum albumin and 3% calf serum. After thorough washing in the buffer, specimens were stained 30 min in rhodamine-conjugated rabbit antigoat IgG (50 μg/ml in PBS) prepared as in [22]. Sections were examined with a Zeiss epifluorescence microscope equipped with interferential filters and with Planapochromat objectives 40 × and 63 ×. Photographs were taken on Kodak Tri X films. Controls were made by omitting the first specific antibody.

3. RESULTS AND DISCUSSION

3.1. *Isolation of cardiac gelsolin and its association with myofibrils*

The present investigation demonstrates that rabbit cardiac muscle contains significant amounts of a 92-kDa actin-severing protein which was isolated in a pure state by ammonium sulfate fractionation and chromatography on DEAE-Sepharose, Sephadex G-150 and hydroxyapatite. The developed procedure for purification of gelsolin has the advantage of being a simple, generated method for isolation of gelsolin-like proteins from muscle and non-muscle cells. Fig. 1 illustrates each of these purification steps. The final fraction (fig. 1, lane D) shows homogeneity of over 95% as estimated from a densitometric scan. The yield of the protein was about 5 mg from 100 g of heart muscle.

The cardiac muscle protein was proved to be similar to alveolar macrophage gelsolin [7] and human serum gelsolin [23] in its molecular and biochemical properties. Its amino acid composition, depicted in table 1, shows a great similarity with that of some other severing proteins. Using the immune replica technique, we found that the anti-serum to macrophage gelsolin specifically recognized the protein band with a relative mass of 92 kDa present in the high-speed supernatant of extracts from the atria (fig.2B) as well from the ventricles (fig.2C). The replica of purified cardiac gelsolin (fig.2D) comigrated with the band corresponding to gelsolin in macrophage extract (fig.2A) slightly ahead of the replica of pure human serum gelsolin (fig.2E). The weakness of the intensity of the immunoreaction in clarified

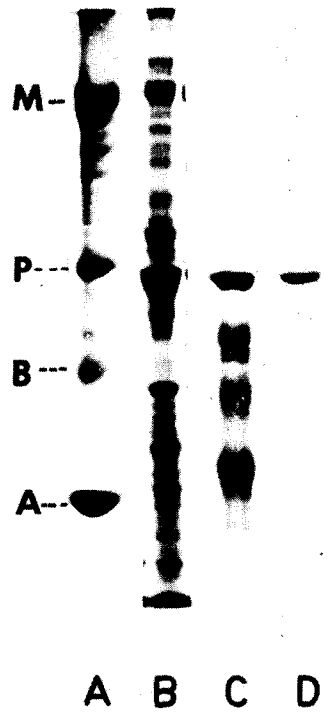

Fig. 1. SDS-PAGE (7.5%) of fractions obtained through the preparation of cardiac muscle gelsolin. Lane (A) represents M_r markers (from top to bottom): M, myosin heavy chain, 200 kDa; P, phosphorylase *b*, 94 kDa; B, serum albumin, 68 kDa; A, actin, 43 kDa. Lane (B) contains pooled fractions with actin-modulating protein eluted from the DEAE-Sepharose column. Lane (C) contained pooled fractions with actin modulating protein eluted from the Sephadex G-150 column. Purified cardiac gelsolin eluted from the hydroxyapatite column is shown in lane (D).

cardiac muscle extracts prepared at a moderate ionic strength (60 mM KCl) (fig.2B,C) led us to further investigate by SDS-PAGE and immune replica, the possible association of the protein with myofibrils. The analysis of supernatants and pellets issued from cardiac muscle extracted at different salt concentrations (0.1–1 M KCl) revealed that the cardiac gelsolin was not a readily soluble fraction component. Its solubility from myofibrils was a function of salt concentration but independent of Ca^{2+}/EGTA ratios since up to 90% of the intensity of the immunoreaction was found in cardiac myofibrils (fig.3A,B) and since the same pattern was found in the presence of Ca^{2+} or EGTA. Fig.3C,D and fig.3F,G represent the supernatants

Table 1
Amino acid composition of cardiac muscle gelsolin and comparison with some actin-severing proteins

Amino acid	Human serum[a] gelsolin (mol%)	Rabbit macrophage[b] gelsolin (mol%)	Cardiac muscle gelsolin (mol%)
Asx	10.1	10.5	9.5
Thr	5.2	5.2	5.6
Ser	7.7	6.4	6.8
Glx	12.5	12.8	11.8
Pro	5.2	6.8	6.8
Gly	7.5	8.9	8.1
Val	6.9	7.2	6.9
Met	1.7	1.9	1.6
Ileu	3.3	3.5	4.1
Leu	7.2	9.2	8.1
Tyr	2.9	3.1	2.7
Phe	4.5	4.3	4.1
Lys	5.8	5.8	6.0
His	4.1	9.2	3.3
Arg	5.5	5.5	5.2

The analysis of cardiac gelsolin is compared to:
[a] Human serum gelsolin prepared as described in the text
[b] Rabbit lung macrophage gelsolin as in [7]

and their immune replica obtained when the rabbit cardiac muscle was extracted in the presence of 0.1 M and 0.6 M KCl, respectively. At 50–100 mM KCl, most of the gelsolin remained tightly associated with the insoluble myofibrillar fraction containing the bulk of the actin, myosin, tropomyosin and troponins (fig.3A,B). This finding was strongly suggestive of the presence of gelsolin in cardiac muscle as an integral part of the myofibril. In this context, it was demonstrated that in muscle cells, glycolytic enzymes, tropomyosin-troponin complex [25] and myosin light chain kinase [26] are tightly associated to filamentous actin at ionic strengths comparable to, or higher than, those found in vivo. As shown in fig.4, cardiac gelsolin was found to reduce greatly the steady-state viscosity of preformed F-actin filaments increasing the critical concentration for actin assembly in presence of μM Ca^{2+} and controlling filament lengths similarly to macrophage gelsolin [8,9], actin depolymerizing factor [23] microvilli villin [28] and physarum fragmin [29]. A mixture of cardiac gelsolin and actin was prepared at a 1:50 molar ratio, and the effect on viscometry occurred either when the gelsolin was added after

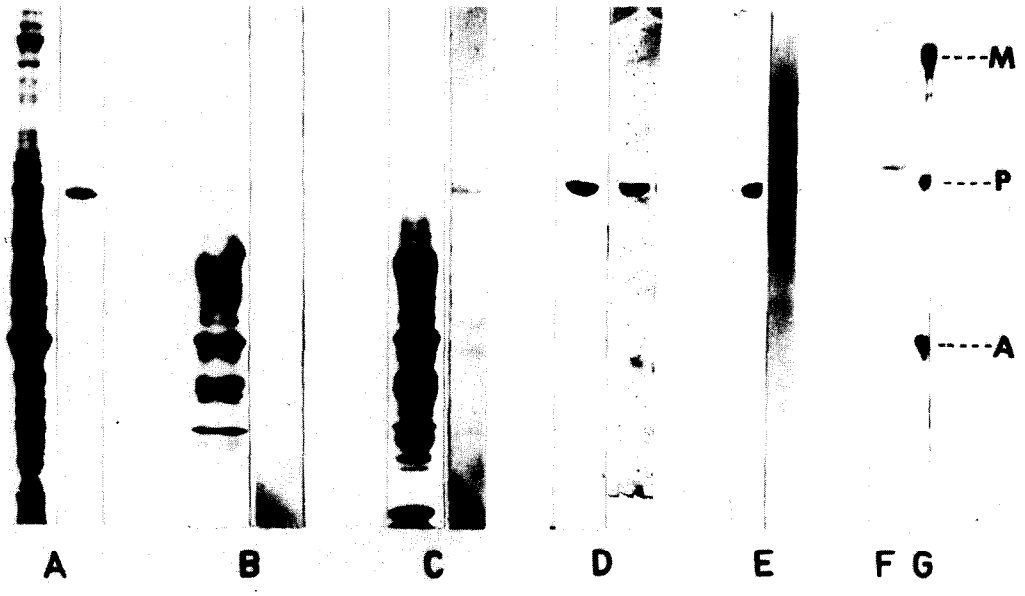

Fig. 2. Immunoblot analysis of macrophage, cardiac and serum gelsolin. The Coomassie blue-stained gel is shown on the left and the corresponding immunohistochemically stained gel replica of each sample on the right. (A) Homogenate of rabbit alveolar macrophages; (B) extract of rabbit atrial cardiac muscle; (C) extract of rabbit ventricular cardiac muscle. Soluble extracts of A, B and C were prepared as in [12]. (D) pure cardiac muscle gelsolin; (E) pure human serum gelsolin; lanes (F) and (G) represent, respectively, α-actinin and a mixture of myosin heavy chain (M), phosphorylase *b* (P) and actin (A) as markers.

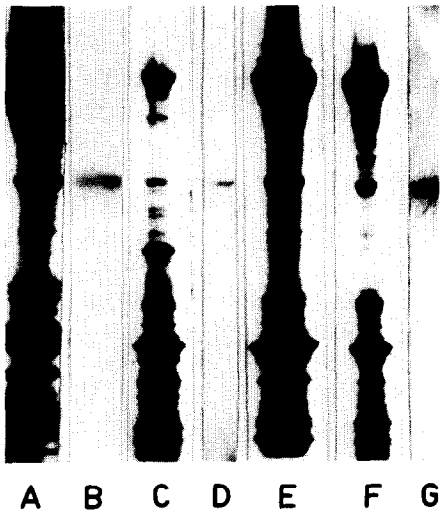

Fig. 3. Ionic strength-dependent solubilization of myofibrillar gelsolin. Cardiac muscle was extracted with 40 mM borate, 0.5 mM $MgCl_2$, 0.5 mM DTT, 5 mM EGTA, 10 mM NaN_3, 10 mM PMSF (pH 7.2) containing 0.1 M or 0.6 M KCl and centrifuged. The resulting pellet (lanes A,E) and supernatants (lanes C,F) were electrophoresed. Cross-reactivity with anti-gelsolin antibody was performed on myofibrils (lane B) and on supernatants (lanes D and G). Note the solubilization of cardiac gelsolin and its recovery in the supernatant.

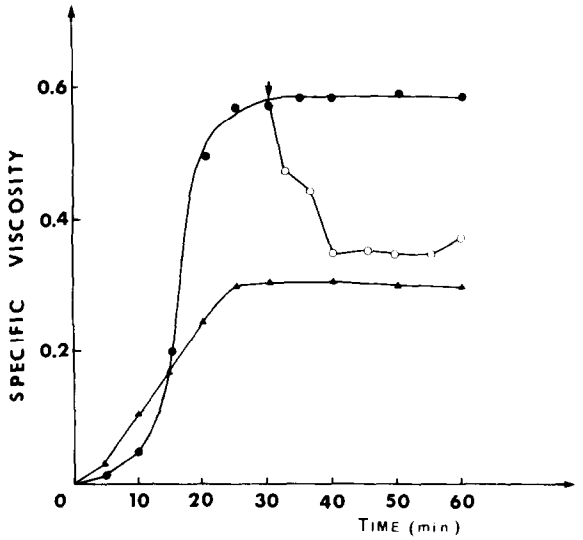

Fig. 4. Time course of the effect of purified cardiac gelsolin on the viscosity of actin in the presence of calcium. Polymerization of G-actin (0.7 mg/ml in 2 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$ and 0.2 mM 2-mercaptoethanol, pH 8.0) was initiated by the addition of KCl to 0.1 M and $MgCl_2$ to 2 mM final concentration (●—●) and monitored by viscometry. After a steady-state viscosity was reached, cardiac gelsolin was added to preformed F-actin at a final molar ratio of 1:50. Note the rapid drop in viscosity (○—○) after addition of gelsolin at the time indicated by the arrow. In a parallel sample (▲—▲), cardiac gelsolin was mixed with G-actin in the same buffer, 15 min before adding salt to induce actin polymerization.

polymerization was achieved or when it was present during the course of the actin assembly. The elimination of the lag phase could be interpreted by the fact that gelsolin binds to monomeric actin and stimulates nucleus formation.

3.2. *Cytochemical localization of gelsolin in cardiac muscle*

To localize gelsolin in rabbit cardiac muscle, thin frozen sections were studied by indirect immunofluorescent labelling, using purified antibodies elicited against alveolar macrophage gelsolin. The analysis of the cellular patterns clearly demonstrates that gelsolin was present in ventricular (fig.5a,b) and atrial (fig.5c) cardiac muscle. As shown for the latissimus dorsi muscle [12], the antibody binds specifically to gelsolin in the myofibrillar bands of myocardium. The immunoreaction was more intense in the ventricular than in the atrial myocardium. Gelsolin was also located in subsarcolemmal sarcoplasm, forming a regular underlining of the cardiomyocytes. In contrast, contact regions (intercalated discs) as well as nucleus and internal sarcoplasm were always negative. Some cardiomycytes appeared brighter especially in the ventricle. The cardiac vessel smooth muscle also exhibited a strongly positive reaction in this immunostaining. This is in agreement with the recent observation [12] suggesting that smooth muscles have a very high gelsolin content. Finally this specific localization of gelsolin was not found in the control, where the specific gelsolin antibody was omitted (not shown).

Intracellularly, the gelsolin was suggested to solvate actin gels in the presence of $Ca^{2+} > 10^{-6}$ M and may thus regulate the consistency of cytoplasm [9], whereas the major role of the serum gelsolin may be in clotting or in clearing actin filaments from the circulation as a consequence of tissue damage or turnover [23,24]. Possible identi-

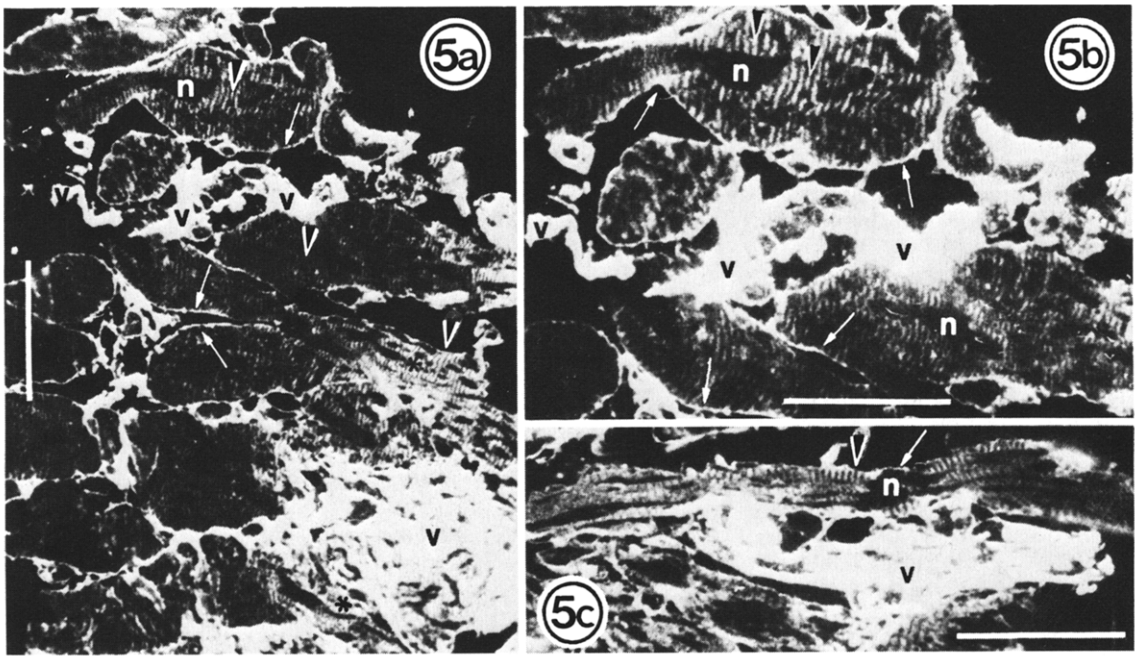

Fig. 5. Indirect immunofluorescence staining of myocardium using anti-gelsolin antibody. Longitudinal sections of rabbit ventricular (5a,b) and atrial (5c) cardiac muscle cells demonstrate an periodically distributed reaction (▼). Moreover, the subsarcolemmal cytoplasm (➞) appears clearly stained whereas the nucleus (n) was always negative. Vessel smooth muscle (v) appears strongly fluorescent. Some heterogeneity of the immuno-reaction is observed in neighbouring muscle cells (*). Bar, 25 μm. (5a) × 720, (5b,c) × 900.

ty between these two intra- and extracellular actin-severing proteins was already suggested [12] and the action of tropomyosin in protecting F-actin from severing by macrophage gelsolin was also recently reported [30]. At present, we have no knowledge of the physiological function of gelsolin in cardiac or skeletal muscle. The data described here show that cardiac gelsolin which is physically and immunologically similar to non-muscle gelsolin seemed to form quite a tight complex with the myofibrils in vivo and in vitro and thus could contribute to the stability of the polymeric structures of the sarcomere. The gelsolin which presumably binds to F-actin in the presence of the regulatory proteins, could be involved essentially in the nucleation step of G-actin in situ and possibly in the turnover of the thin filaments within the myofibrils.

In this context, it will be of great interest to know if muscle gelsolin has such an important role during the physiological processes of moulting, and/or during pathological phenomena of damaged tissue or cellular injury such as myocardial infarctus and coronary occlusion. Studies are currently underway to examine the precise localization of the gelsolin within the myofibril itself and the possible role of this protein in myofibril assembly in striated muscle.

ACKNOWLEDGEMENTS

We are grateful to Dr D. Louvard (Institut Pasteur, Paris) for a generous gift of rhodamine-conjugated rabbit antigoat IgG and to the Centre de Transfusion Sanguine de Montpellier for supplying human serum. This work was supported by grants from the Centre National de la Recherche Scientifique, the Direction Générale de la Recherche et de la Technologie (convention no. 5-11384) and the Institut National de la Santé et de la Recherche Médicale (CRE Convention no. 5-11850). This paper is part of the Thèse de Doctorat d'Etat et Sciences of J.F.R. (Université des Sciences et Techniques du Languedoc, Montpellier).

REFERENCES

[1] Schliwa, M. (1981) Cell 25, 587-590.
[2] Craig, S.W. and Pollard, T.D. (1982) Trends Biochem. Sci. 7, 88-92.
[3] Weeds, A. (1982) Nature 296, 811-816.
[4] Korn, E.D. (1982) Physiol. Rev. 62, 672-737.
[5] Geiger, B. (1983) Biochim. Biophys. Acta 737, 305-341.
[6] Yin, H.L. and Stossel, T.P. (1979) Nature 281, 583-586.
[7] Yin, H.L. and Stossel, T.P. (1980) J. Biol. Chem. 255, 9490-9493.
[8] Yin, H.L., Hartwig, J.H., Maruyama, K. and Stossel, T.P. (1981) J. Biol. Chem. 256, 9693-9696.
[9] Yin, H.L. and Stossel, T.P., Cheung, W.Y (eds) Calcium and Cell Function, vol 2, pp. 325-337, Academic Press.
[10] Kurth, M.C., Wang, L-L., Dingus, J. and Bryan, J. (1983) J. Biol. Chem. 258, 10895-10903.
[11] Harris, H.E. and Weeds, A.G. (1983) Biochemistry 22, 2728-2741.
[12] Yin, H.L., Albrecht, J.H. and Fattoum, A. (1981) J. Cell. Biol. 91, 901-906.
[13] Laemmli, U.K. (1970) Nature 227, 680-685.
[14] Pardee, J.D. and Spudich, J.A. (1982) Methods Enzymol. 85, 164-181.
[15] Houk, W.T. and Ue, K. (1974) Anal. Biochem. 62, 66-74.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
[18] Perry, S.V. (1952) Biochem. J. 51, 495-499.
[19] Pollard, T.D. (1982) Methods Cell. Biol. 24A, 301-311.
[20] Coudrier, E., Reggio, H. and Louvard, D. (1983) EMBO J. 2, 469-475.
[21] Tokuyasu, K.T. (1980) Histochem. J. 12, 381-403.
[22] Brandtzaeg, P. (1973) Scand. J. Immunol. 2, 273-290.
[23] Thorstensson, R., Utter, G. and Norberg, R. (1982) Eur. J. Biochem. 126, 11-16.
[24] Harris, D.A. and Schwartz, J.H. (1981) Proc. Natl. Acad. Sci. USA 78, 6798-6802.
[25] Westrin, H. and Backman, L. (1983) Eur. J. Biochem. 136, 407-411.
[26] Cavadore, J-Cl., Molla, A., Harricane, M.C., Gabrion, J., Benyamin, Y. and Demaille, J.G. (1982) Proc. Natl. Acad. Sci. USA 79, 3475-3479.
[27] Yin, H.L., Zaner, K.S. and Stossel, T.P. (1980) J. Biol. Chem. 255, 9494-9500.
[28] Bretscher, A. and Weber, K. (1980) Cell 20, 839-847.
[29] Hasegawa, T., Takahashi, S., Hayashi, H. and Hatano, S. (1980) Biochemistry 19, 2677-2683.
[30] Fattoum, A., Hartwig, J. and Stossel, P.T. (1983) Biochemistry 22, 1187-1193.