STUDIES ON ACTIN-RELATED GELATION OF EHRLICH TUMOR CELL EXTRACT.

——— A Ca^{2+}-sensitive Gelation Factor "Actinogelin"———

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Abbreviations: sodium dodecyl sulfate, SDS; Ehrlich tumor cell, ETC; phenylmethylsulfonylfluoride, PMSF; dimethyl suberimidate dihydrochloride, DMS; dimethyl sulfoxide, DMSO; Hemagglutinating virus of Japan, HVJ.

SUMMARY

Cell-free extract of Ehrlich ascites tumor cells undergoes temperature-dependent gelation. This process is similar to "actin-related gelation" which has been observed in extracts of many other non-muscle cells. The gelation in Ehrlich tumor cell extract is reversibly inhibited by free Ca^{2+} at concentrations higher than 10^{-6} M. It is also inhibited by cytochalasin D at concentrations similar to those required for inhibition of microfilament-dependent cellular activities. Biochemical studies of the gel formed have shown that it contains two distinct actin-binding proteins causing gelation of skeletal muscle actin. One is a high molecular weight protein which seems to be identical with filamin, and another is a novel factor producing Ca^{2+}-sensitive gelation. The latter has been tentatively named "actinogelin". Both proteins have been purified to homogeneity. Purified filamin and actinogelin both induce gelation of skeletal muscle actin, but only the gelation induced by actinogelin is sensitive to Ca^{2+}. Both gelation processes are inhibited by cytochalasin D, but the concentration of the inhibitor required is ten times higher than that required for inhibition of microfilament-dependent cellular events. Upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, purified actinogelin migrates as a single protein band and its molecular weight has been determined to be 112,000 to 115,000. On the other hand, a molecular weight of 251,000 to 260,000 has been estimated for native, undenatured actinogelin by sedimentation equilibrium experiments. After chemical cross-

linking, native actinogelin migrates as a dimer having a molecular weight of 230,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One molecule of actinogelin binds to 10-12 actin when mixed with rabbit skeletal muscle F-actin. Addition of filamin to a mixture of actinogelin and F-actin leads to the loss of Ca^{2+} sensitivity of gelation.

INTRODUCTION

Microfilaments, one of the major intracellular filamentous systems, occur widely in eukaryotic cells and consist of non-muscle actin and several accessory proteins. Evidence is now available to indicate that in non-muscle cells microfilaments are not only responsible for cell locomotion and motility (1) but also functional in providing a mechanical basis for diverse cellular activities, including cell division (2), phagocytosis (3,4), secretory processes (5), virus-induced cell fusion (6), cell surface modulations (7), membrane movements (8,9) and maintenance of cell shape (10). Fluorescence and electron microscopic observations have further indicated the redistribution of actin or actin bundles takes place during such cellular processes as virus-induced transformation (11,12), fertilization (13), cell division (14), cell adhesion (15) and others (16). In contrast to the actin filaments in muscle cells, therefore, microfilaments in non-muscle cells seem to change their configurations and aggregation state in response to external circumstances and various cellular events. For a better understanding of the physiology and mechanisms of various microfilament-dependent cellular processes, it is necessary to gather further information at the molecular level concerning the organization of microfilaments and chemical nature of the accesssory proteins in the filaments. For this reason, a number of studies have been carried out attempting to purify and characterize the component proteins of microfilaments, to elucidate the nature of interactions of the accessory proteins with actin, and to reconstitute the micro-

filament-dependent cellular functions from the purified components.

Among the accessory proteins so far identified as components of microfilaments of various cells are those which are also found in skeletal muscle; these include α-actinin (17), tropomyosin (18) and myosin (19). Recent work has, however, shown that microfilaments from several types of cell contain accessory proteins which have not yet been detected in skeletal muscle. For instance, Acanthamoeba cells contain gelactin I, II, III and IV (20), sea urchin eggs 220,000- and 54,000-dalton proteins (21), calf spleen and human platelet profilin (22), macrophage (23) and chicken gizzard (24) filamin ("actin-binding protein"), and brush border microvilli vilin (25). Very recently, however it has been reported by the immunofluorescence technique that anti-filamin antibodies can react with Z band of skeletal muscle myofibril (26).

Actin-related gelation of cell-free extracts is one of the best method for the isolation of these accessory proteins of microfilaments and therefore, has been studied with a variety of cells, such as sea urchin eggs (27), Acanthamoeba (20), macrophages(28), Hela cells (29), Xenopus oocytes (30) and Ehrlich tumor cells (31,3). In all these systems examined, actin has invariably been identified as the major structural component of the gel formed. However, the accessory proteins required for gelation seem to differ depending on the cell type , suggesting the diversity of gelation phenomena. Interestingly, it has recently been shown that actin-related

gelation is sensitive to micromolar concentrations of cytochalasin B and D (29,31-36), which also inhibit microfilament-dependent cellular functions at similar concentrations. Furthermore, it has also been reported that in a number of cell types micromolar concentrations of Ca^{2+} inhibit actin-related gelation in a reversible fashion. Intracellular Ca^{2+} at micromolar concentrations is known to affect cellular activities as motility (37), chemotaxis (38), secretion (39,40) and fertilization (41), and possible involvement of Ca^{2+} has been implicated in HVJ-induced cell fusion (31,42), chemically induced cell fusion (43) and fusion of myoblasts (44). These observations suggest that Ca^{2+} is a regulatory factor for both actin-related gelation and several microfilament-dependent cellular activities. If this is so, then it is likely that Ca^{2+} exerts its regulatory functions on these Ca^{2+}-sensitive cellular activities by affecting the organization of certain types of microfilaments and this effect is similar to that on actin-related gelation of cell-free extracts. In other words, there is the possibility that the properties of the gel formed in the extracts mimic those of the intracellular contractile and cytoskeletal systems. Therefore, it seems that actin-related gelation provides an excellent experimental system for studies of Ca^{2+}-sensitive accessory proteins and regulation of their interactions with actin filaments. The purpose of this paper is to report isolation and characterization of two actin gelation factors from cell-

free extract of Ehrlich tumor cells (ETC). One of them is a high molecular weight protein resembling filamin and produces a Ca^{2+}-insensitive gel, whereas the other is a novel actin-binding protein, termed "actinogelin", and produces a Ca^{2+}-sensitive gel when mixed with skeletal muscle actin.

MATERIALS and METHOD

Preparation of ETC extracts. A 8-azaguanine-resistant strain of ETC (45) was propagated in the peritoneal cavities of ddN mice and harvested two weeks after transplantation of the cancer cells. After the cells were washed three to four times with 0.15 M NaCl containing 15 mM sodium citrate by centrifugation at 80 x g for 7 min, the packed cell volume was determined by centrifugation at 650 x g for 10 min. The resultant pellet was suspended in 1.5 volumes of an extraction medium containing 0.34 M sucrose, 1 mM ATP, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl-fuloride PMSF), 0.1 μg/ml pepstatin, and 20 mM imidazole-HCl (pH 7.0 at 25°C) and then disrupted by means of a cell disruption bomb (Purr) at 1,200-1,500 p.s.i.. The disrupted cells were centrifuged at 30,000 rpm for 1.5 hr in a Hitachi 55P centrifuge, using a RP-30 rotor at 2-4°C, and the resultant clear supernate was used as ETC extract. This procedure is summarized in Fig. 1.

Estimation of gelation of ETC extract and reconstituted systems.

The gelation of ETC extract was usually performed by warming to 25°C for 1 hr after the addition of 1/20 vol. of 20 mM $MgCl_2$ to the extract. Filamin-dependent gelation was routinely conducted using 2 mg/ml of actin at 25°C for 1 hr in the standard reaction medium containing 10 mM imidazole-HCl (pH 7.2), 1 mM $MgCl_2$ and 50 mM KCl. Actinogelin-dependent gelation was routinely conducted using 2 mg/ml actin at 25°C for 1 hr in the standard reaction medium containing 10 mM imidazole-HCl (pH 7.2), 0.5 mM EGTA or 0.05 mM $CaCl_2$, 1 mM $MgCl_2$

and 50 mM KCl. The condition which causes G-actin into F-actin was used for the reconstituted experiments of actinogelin- and filamin-dependent gelation. In the condition, ionic length (50 mM KCl), the concentration of Mg^{2+} (0.5 mM) and concentration of actin (1.7-2.0 mg/ml) are sufficient to induce polymerization of G-actin to F-actin (46).

The following methods were used to estimate the degree of gelation. First, the gelation was observed with naked eyes in the test tube (8 x 90 mm) containing 0.2 ml of reaction mixture at 1 hr after warming the mixture to 25°C, and its extent was qualitatively expressed as follows; +++, firm gel that tightly held its own shape and did not spill out of an inverted tube; ++, relatively solid gel that incompletely maintained its own shape and dropped as a solid mass out of an inverted tube; +, weak gel that was liable to break and crumbled down out of an inverted tube, though it could resist tilting of the tube to some degree; — , no gelation or gel easily broke down with slight disturbance. More quantitatively, the gel formed was compressed by centrifugation and then the volume or total protein of the resultant pellet was measured. In the case of gelation of ETC extracts and the experiment of Fig. 17, the gel was centrifuged at 2,000 x g for 30 min at 20°C and the resultant supernate was removed with Pasteur pipet. After layering of a medium containing 0.1 M KCl and 10 mM imidazole-HCl (pH 7.2) over the compressed gel without disturbance, the gel was centrifuged again at 2,000 x g for 30 min and the total protein in the resultant pellet was determined by Lowry

method (47). Actinogelin- and filamin-dependent gelations were measured in the same way as for the gelation of the extract except that centrifugation at 49,000 x g for 10 min was used instead of at 2,000 x g for 30 min. In some experiments using purified actinogelin, the extent of gelation was expressed in terms of compressed gel volume as follows. The gel was compressed by centrifugation at 16,500 rpm for 10 min in a Sorvall SM-24 rotor and the volume of resultant supernates were measured by a microsyringe. The volume of compressed gel was calculated by subtraction of the volume of the supernate from total volume of the initial reaction mixture.

Assay for the activities of actinogelin and filamin.

Actinogelin-dependent gelation was performed using 1.75 mg/ml of actin in the condition described above. One unit was defined as the amount of actinogelin which precipitated 1 mg of protein from the reaction mixture by centrifugation at 49,000 x g for 10 min. It was calculated from the linear phase of a calibration curve obtained by plotting the amount of precipitate from the reaction mixture by centrifugation at 49,000 x g for 10 min against the amount of actinogelin added. Total activity was calculated from difference in protein precipitation between 0.5 mM EGTA-containing samples and Ca^{2+}-supplemented (0.05 mM) samples. Specific activity was expressed as units/mg protein. Usually the actinogelin content of various fractions was monitored by SDS-polyacrylamide gel electrophoresis. The filamin content of various fractions was also monitored by SDS-polyacrylamide gel electrophoresis

and its activity was measurd according to the method for actinogelin.

Purification of filamin.

Step I: Solubilization from gelated materials.

After addition of 1 mM $MgCl_2$, ETC extract obtained as described above was warmed to 25°C for one hour and then the resultant gel was sedimented at 40,000 x g for 10 min in a Sorvall SS-34 rotor. The pellet was washed twice with 0.1 M KCl in 20 mM imidazole-HCl (pH 7.0) and resuspended in 1/5 of the original volume of a medium consisting of 0.6 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 0.1 μg/ml pepstatin and 10 mM imidazole-HCl (pH 7.0) by pipetting. This suspension was allowed to stand in a cold room (4°C) with stirring overnight to release filamin from the insoluble materials. The turbid insoluble materials containing F-actin were removed by centrifugation at 100,000 x g for 3 hrs at 4°C in a Hitachi 55P centrifuge equipped with a RP-40 rotor. The supernate was collected and subjected to the following ammonium sulfate fractionation.

Step II: Ammonium sulfate fractionation.

Saturated ammonium sulfate solution was adjusted to pH 7.0 with ammonium hydroxide and added to the supernate to give 15 % saturation. After 30 min at 4°C, the suspension was spun at 10,000 rpm for 10 min in a Sorvall SS-34 rotor and the resultant supernate was brought to 35 % saturation by adding saturated ammonium sulfate solution. After 30 min at 4°C, the mixture was centrifuged at 5,000 rpm for 10 min in the Sorvall

SS-34 rotor. The pellet was suspended in 1/5 of the volume of starting ETC extract in o.6 M KI solution containing 20 mM $Na_2S_2O_3$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 0.1 μg/ml pepstatin and 10 mM imidazole-HCl, pH 7.0. The solution was again subjected to ammonium sulfate fractionation and the pellet formed between 15 % and 35 % saturation was dissolved in 1 to 2 ml of the same 0.6 M KI solution.

Step III: Sepharose 4B chromatography.

After 30 min at 4°C, the above solution was immediately applied to a column of Sepharose 4B (1.6 x 83 cm) equilibrated with a low ionic strength buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$ and 0.5 mM 2-mercaptethanol, pH 8.0). On elution of the column with the same buffer at a flow rate of about 6 ml/hr, filamin was eluted as a major peak (Fig. 3). Peak fractions were checked for its purity by sodium dodecyl sulfate (SDS)-poltacrylamide gel electrophoresis (Fig. 3) and the fractions which were found to be homogeneous were pooled and used as a purified filamin. If necessary, the fractions were further purified by DEAE cellulose (Whatman DE32). Upon stepwise elution, filamin was eluted between 0.1 and 0.15 M KCl in 20 mM Tris-HCl (pH 7.6). The purification procedures employed are summarized in Fig. 2.

Purification of actinogelin. Fig. 4 shows the procedure schematically.

Step I: Ammonium sulfate fractionation.

Cell free ETC extract was adjusted to 0.6 M with respect to KCl concentration by adding 3 M KCl and the mixture was

stirring for 2-3 hrs at 4°C. The mixture was fractionated with solid ammonium sulfate and the pellet formed between 15% and 35% saturation was collected. The pellet was dispersed with a buffer containing 10 mM imidazole-HCl (pH 7.0), 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM PMSF and 0.1 μg/ml pepstatin and then dialyzed overnight against 3 liters of the same buffer. The solution was made 0.6 M with respect to KCl concentration by adding 3 M KCl. After stirring for 2 hrs at 4°C, it was spun at 100,000 x G for 3 hrs in a HitachiRP-40 rotor and the clear supernate was fractionated again by ammonium sulfate in the same way (between 15% and 35% saturation). The resultant pellet was dispersed with a buffer containing 20 mM imidazole-HCl (pH 7.2), 0.1 mM PMSF and 0.1 μg/ml pepstatin and then dialyzed overnight against 3 liters of the same buffer.

Step II: DEAE cellulose ion-exchange chromatography.

The insoluble materials were removed by centrifugation at 40,000 x g for 10 min in a Sorvall SS-34 rotor and the clear supernate was applied to a column of DEAE cellulose (Whatman DE32) (3 x 15 cm) equilibrated with 20 mM imidazole-HCl (pH 7.2). As shown in Fig. 5, the column was washed with 100 ml of 20 mM imidazole-HCl (pH 7.2) and subsequently with 400 ml of 0.2 M KCl in 20 mM imidazole-HCl (pH 7.2). Actinogelin was then eluted with 400 ml of 0.4 M KCl in 20 mM imidazole-HCl (pH 7.2) (Fig. 5).

Step III: Hydroxylapatite chromatography.

The actinogelin-containing fractions eluted with 0.4 M KCl were combined and dialyzed against 10 mM potassium phosphate (pH 6.8). The dialyzate was applied to a column of hydroxyl-

apatite column (2 x 6.5 cm) prepared according to the method of Tiselius et al.(48). After washing the column with 70 ml of 50 mM potassiumphosphate (pH 6.8), elution was carried out with a linear gradient prepared from 60 ml of 50 mM potassium phosphate (pH 6.8) and 60 ml of 0.25 M potassiumphosphate (pH 6.8) at a flow rate of 10-12 ml/hr. Actinogelin was eluted as a major peak between 0.1 and 0.2 M potassium phophate (Fig. 6). Those fractions which contained SDS-gel electrophoretically detectable actinogelin were pooled (Fig. 6).

Step IV: Sepharose 6B chromatography.

The pooled fractions were concentrated to 1-1.5 ml with a Sartorius collodion membrane. and applied to a column of Se Sepharose 6B (1.2 x 95 cm) equilibrated with 20 mM Tris-HCl (pH 7.6) containing 50 mM NaCl, 0.1 μg/ml pepstatin and 1 mM NaN_3. Elution was carried out by the same buffer and 1.2 ml fractions were collected. Actinogelin was eluted from the column as a major peak with tailing (Fig. 7). The main fractions whose homogeneity was confirmed by SDS-polyacylamide gel electrophoresis were pooled and used as purified actinogelin.

Estimation of actin content. Semiquantative determination of actin content in a sample was carried out based on the inhibitory effect of actin on DNase I activity. Since this inhibition has been shown to be due to the formation of an inactive equimolar complex between DNase I and actin (49,50), the activity of DNase I after incubation with a given amount of sample can be taken as a measure of the amount of actin contained in the sample. In practice, 10 μl or 20 μl of 80 μg/ml DNase I was

incubated with a 10-40 μl of a sample for 30 min at 25°C, and then the activity of DNase I of the mixture was measured by the method of Lindberg (51). The amount of inactivated DNase I was calculated from the total amount of DNase I added the reaction mixture multiplied by percent inhibition. This was used as a relative actin content (Fig. 6). However, it should be mentioned that inhibition of DNase I by F-actin is a relatively slower process than inhibition by G-actin or by the equimolar complex between profilin and G-actin (profilactin) as reported by Hitchcock et al.(50). Careful interpretation of the results obtained is necessary. Especially, the inhibitory activity observed in the void fraction of Sepharose gel filtration which was probably attributed to highly polymerized actin might not represent the net amount of actin in the fraction. Such limitations of the method should be taken into consideration in interpretation of the experimental results.

Chemical cross-linking. Purified actinogelin at 0.4 mg/ml in 50 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1 μg/ml pepstatin and 1 mM NaN_3 were mixed with an equal volume of 6 mg/ml of dimethylsuberimidate dihydrochloride (pH 7.8) in 50 mM Trisine- NaOH. After the mixture was incubated for 3 hrs at 37°C, 1/2 volume of a medium containing 3% SDS, 30% sucrose, 30 mM Tris-HCl (pH 8.0), 3 mM EDTA and 3% 2-mercaptoethanol was added to stop the cross-linking. The sample was boiled for 3 min and then analyzed by electrophoresis on 4% polyacrylamide gels.

Electrophoresis. SDS-gel electrophoresis was performed according to Fairbanks et al. (52) using 5.6% polyacrylamide gels. The protein samples were heated at 100°C for 3 min in 1% SDS,

10% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1% 2-mercaptethanol. Protein was stained for about 10 hrs with 0.04% Coomassie brilliant blue in 25% isopropyl alcohol and 10% acetic acid. The gel were destained by diffusion in 10% acetic acid.

Ultracentrifugal analysis. Sedimentation equilibrium experiments were performed at 6,400 rpm with Spinco Model E ultracentrifuge . at 20°C in the medium containing 50 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1 μg/ml pepstatin and 1 mM NaN_3. The scanning of sedimentation pattern was carried out using an ultraviolet sccanner at 280 nm or an interference optical system. The photographic reads of the results obtained using an interference optical system were measured on an Olimpus microcomparator. The calculations used for molecular weight the same as those described elesewhere by Cherninka (53).

Other analytical methods. The activity of calmodulin was measured by the activity which mediates Ca^{2+}-dependent activation of cyclic nucleotide phosphodiesterase according to the method of Kakiuchi and Yamazaki (54). Protein was determined by the method of Lowry et al. The absorption spectrum of actinogelin was measured in a Cary model 219 spectrophotometor. Densitometric scan was carried out with a Joyce-Loebl Chromoscan 200 scanning spectrophotometer.

Purification of rabbit skeletal muscle actin. Rabbit skeletal muscle actin was prepared from acetone powder according to the method of Spudich and Watt (55). Purified actin (G-actin) was stocked in cold room (4°C) in 2 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 1 mM NaN_3 and 0.2 mM ATP. This stocked solution was used within two weeks.

Chemicals RNA polymerase B was purchased from Seikagaku Chemical Co. and bovine serum albumin, RNA polymerase from E. coli (Core enzyme) and soybean trypsin inhibitor were obtained Boehringer Mannheim GmbH as marker proteins for SDS-polyacrylamide gel electrophoresis. Acrylamide was obtained from Seikagaku Chemical Co. and N,N'-methylene bis acrylamide, sodium dodecyl sulfate (SDS) and ammonium persulfate were purchased from Wako Pure Chemical Industries, Ltd., and 2-mercaptoethanol was obtained from Sigma as reagents for SDS-polyacrylamide gel. ATP was obtained from Kohjin Co. Ltd. Cytochalasin D was a gift from Shionogi Co. Phenylmethylsulfonylfluoride (PMSF) and bovine pancreatic DNase I (DN-EP) were purchased from Sigma. Dimethyl suberimidate dihydrochloride (DMS) was purchased from Wako Pure Chemical Industries, Ltd. Ammonium sulfate was obtained from Nakarai Chemicals Ltd. All other reagents were obtained commercially and were of analytical grade.

RESULTS

Gelation of cell-free extract of ETC. The cell-free extract prepared from ETC as described in "Materials and Methods" (see Fig. 1) was found to form a solid gel on warming to room temperature (Fig. 8). Gel formation was observable within 20 min after raising the temperature and could be reversed by lowering the temperature to 4°C. The revesibility was, however, lost gradually when the gel was allowed to stand for more than one hour at room temperature. Upon further incubation, the gel began to shrink and after about 10 hours it became a relatively dense and opaque coagulate which seemed to be similar to the "contraction" or "syneresis" reported for cell-free extracts from other types of cells (20,28,56). The gelation was inhibited by omitting ATP from the cell extraction medium or by lowering the ionic strength of the extract by Sephadex G-25 gel filtration. The gels formed under these conditions were mechanically labile and readily undergo fragmentation and subsequently converted to the sol state by brief shaking.

Inhibition of gelation by cytochalasin D. As in the cases of extracts of other cells (20,27,28,29,33-66), the gelation of ETC extract seemed to be a process in which microfilaments are involved. To confirm this possibility, the effect of cytochalasin D on the gelation was examined, since this compound has been shown to affect microfilaments and thus inhibit

microfilament-dependent functions (4,6,57,58). As shown in Fig. 9, cytochalasin D actually blocked the gelation of ETC extract at concentrations similar to thos required for inhibition of various microfilament-dependent processes. At a concentration of 0.4 μg/ml cytochalasin D inhibited the gelation almost completely when the gelation was estimated by visual observations. When the gelation was assayed by determining the amount of protein recovered in the pelleted gel, on the other hand, it was found that 0.1 μg/ml of cytochalasin D was sufficient for complete inhibition of the gelation. The reason for the discrepancy between the two assay methods is at present unknown. In anyway, the fact that the gelation was sensitive to cytochalasin D indicated that the process was dependent on microfilaments and the gelation system retained at least one in vivo property of microfilaments.

Polypeptide components of gel. To obtain information concerning the components of the gel, the gelated extract was centrifuged at 10,000 rpm for 10 min to separate the gel from soluble materials, and the polypeptide compositions of the precipitated gel, supernate and the whole extract were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 10, the gel pellet (P-1) was found to contain so numerous polypeptides that it was not possible to distinguish the gel components from contaminating polypeptides. After

washing the gel two times with 20 mM imidazole-HCl buffer (pH 7.2) containing 0.1 M KCl by agitating with Pasteur pipet, four major polypeptide bands were detected together with some contaminating proteins. The four major bands were termed band 1, 2, 3 and 4 from the slowest moving to fastest moving bands. Among them, band 4 was most abundant and was found to co-migrate with rabbit skeletal muscle actin in the electrophoresis. Band 2 was second prominent; it had a molecular weight of 310,000 and, therefore, seemed to be identical with or similar to filamin (24) or "actin-binding protein" (23) previously reported to be present in other cells. Accordingly, "filamin" was used hereafter to represent this protein.* Band 1, which showed the highest molecular weight, was also concentrated in the gel pellet. This band was suggested to be an aggregated form of band, the filamin-like protein, because purified filamin, after trichloroacetic acid precipitation, occassionally gave a band having a similar molecular weight on

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* The 310,000 dalton protein is strikingly similar to group of proteins such as filamin from smooth muscle (24), "actin-binding protein" from alveolar macrophages (23) and "high molecular weight protein" from Hela cells (29) not only in its high molecular weight but also in its ability to induce gelation of actin. In this paper, therefore, we call the protein filamin.

SDS-polyacrylamide gel electrophoresis (data not shown), although its final identification must await further investigations. Band 3 appeared to be a minor component of the cell-free extract and could not be detected on SDS-polyacrylamide gel electrophoresis of the extract and even the pelleted gel (P-1), probably because of the presence of large amounts of proteins having similar molecular weights. Band 3 became detectable only after washing of P-1 and could be finally shown to be a true component of the gel, as will be described below. Before washing, the gel pellet ws contaminated by many adsorbed and trapped proteins, and the washing treatment may have caused disintegration of the gel structure. Therefore, it was not possible to quantitate the ratio of gel component based on the results of SDS-polyacrylamide gel electrophoresis.

Involvement of actin in gelation. The above gel electrophoretic experiments indicated that the gel contained a protein having a same molecular weight as actin as the major component, a finding strongly suggesting the participation of actin in the gelation. To confirm further this point, the effect of DNase I on the gelation was examined. Lazarides and Lindberg (49) and Hitchcock et al.(50) have shown that DNase I specfically binds to G-actin to form a stable 1:1 complex and thus inhibits the polymerization of G-actin to F-actin.

It has also been shown that interaction of DNase I with F-actin leads to the formation of the DNase I - actin complex and subsequent depolymerization of F-actin (49,50). These findings indicate that DNase I can be used as a specific inhibitor of F-actin-related functions. As shown in Fig. 11, DNase I was found to be a potent inhibitor of the gelation of ETC extract. Under the experimental conditions employed, addition of 0.1 mg of DNase I caused almost complete inhibition. Incubation of DNase I with 1.5 times weight of purified skeletal muscle actin prior to the addition to the gelation mixture resulted in complete abolishment of the inhibition. Furthermore, the preformed gel was dissolved by layering a DNase I solution over the gel. It was thus certain that actin was an indispensable participant in the gelation phenomenon.

Polymerization state of actin in ETC extract. It is expected that only the actin in the polymerized state can participate in the gelation. To test this possibility, the polymerization of actin in the extract was examined by Sepharose 4 B gel filtration; the amount of actin eluted from the column was assayed by measuring the inhibitory action of each eluate on DNase I activity. As shown in Fig. 12, the results thus obtained suggested that actin was present in two distinct polymerization states. Thus, a small amount of actin was eluted at the void volume and seemed to be in a highly polymerized state, whereas a much larger amount of actin was retarded and, therefore, appeared to be in a smaller molecular weight state. However, care must be taken in inter-

pretation of this result, because it has been shown that F-actin cannot inhibit DNaseI as effectively as G-actin under certain conditions (48,50) and, therefore, the actin contents obtained in void fraction may not be true values. In any way, the smaller sized population did not seem to represent G-actin itself, because its Stokes radius calculated from the gel filtration results was higher than that of haemoglobin, and therefore, that of G-actin. Although further work is needed, it was assumed that G-actin is complexed with some unknown protein(s). This smaller sized population, though occupying a large portion of actin in ETC extract, was unable to be polymerized into F-actin at least under the conditions employed. Preliminary experiments showed further that this population of actin could not form a gel even when purified filamin was added. Thus, it was clear that the gelation ability of ETC extract was limited by the content of the polymerized actin. In confirmation of this, it was found that the supernate obtained from the gelated ETC extract by centrifugation at 10,000 x g for 10 min could undergo gel formation at room temperature when muscle actin was added and the extent of gelation increased as the amount of added actin was increased (Fig. 13). It was thus evident that the gelation capacity of ETC extract was limited by the content of filamentous actin.

Control of gelation by physiological concentrations of Ca^{2+}. As reported previously (31), the gel-sol transformation of ETC extract was controlled by micromolar concentrations of Ca^{2+}. For studies of this control, ETC extract was subjected to gel filtration through a Sephadex G-25 column equilibrated

with 40 mM Tris-maleate buffer (pH 7.0), 0.34 M sucrose, 1 mM ATP, 1 mM dithiothreitol and 1 mM EGTA to remove endogeneous Ca^{2+} and to make the EGTA concentration 1 mM. $CaCl_2$ was then added to the treated extract to concentrations of 0 to 0.8 mM. The concentration of free Ca^{2+} was calculated from the Ca^{2+}/EGTA ratio at pH 7.0 or 6.7 for each preparation (59). As shown in Fig. 14 and Table I, gel formation did not occur when the free Ca^{2+} concentration was higher than about 1 μM. This Ca^{2+}-induced inhibition could be reversed by addition of EGTA to a final concentration of 10 mM. Extensive gel formation became observable within 10 min after the addition of EGTA. As shown in Fig. 14, the amount of protein recovered in the pelleted gel was also restored to the nearly normal level after the addition of EGTA. Fig.14 also shows that the protein in the pelleted gel increased when the free Ca^{2+} concentration was decreased from 3.1 μM to 0.15 μM. These findings ruled out the possibility that the inhibition of gelation by Ca^{2+} was due to irreversible destruction of the protein components by a Ca^{2+}-activated protease(s). When excess Ca^{2+} was layered over the preformed gel, the gel was partially converted to the sol state within a few minutes from the surface. Upon subsequent shaking, the destruction of gel structure proceeded further, though complete dissolution of the gel was not attained (Fig. 8).

Purification of filamin. For further characterization of the gelation mechanism of ETC extract, it seemed highly desirable to purify and identify the structural and regulatory

components of the gel other than actin. Since a high molecular weight protein called filamin was found to be a major component of the gel as described above, an attempt was first made to purify this protein from ETC extract. It was thus possible to obtain a purified filamin preparation by the method described in "Materials and Method". Filamin thus purified was found to be homogeneous upon SDS-polyacrylamide gel electrophoresis. It induced gelation of skeletal muscle actin under the conditions which induced polymerization of actin; 0.12 mg/ml protein of pure filamin was sufficient for complete gelation of 2 mg/ml protein of actin (Fig. 15). The properties of the gel thus formed were, however, different from those formed from crude ETC extract in that the former failed to show sensitivities to Ca^{2+} and cytochalasin D and temperature-dependent reversibility, all of which could be clearly observed in the latter system. Fig. 15 shows that the formation of Ca^{2+}-insensitive gel was dependent on the amount of purified filamin added in the presence of a fixed amount of skeletal muscle actin.

The insensitivity of filamin-induced gelation of muscle actin to Ca^{2+} suggested that ETC extract contained an unknown factor conferring Ca^{2+} sensitivity to the gelation system. It is to be noted that the sensitivity of filamin-dependent gelation to cytochalasin D was also decreased to one tenth of that observable with the gelation of ETC extract. As shown in Fig. 16, the filamin-dependent gelation was completely inhibited by 10 μg/ml of cytochalasin D, but a concentration of the drug as low as 1 μg/ml was effective to prevent the gelation of the extract completely. Although the reason for

this difference is still unclear, it was likely that the inhibition of filamin-dependent gelation was due to direct action of cytochalasin D on actin filament, because Spudich and Lin (60) have reported that cytochalasin B decreased the viscosity of actin solutions. On the other hand, cytochalasin D at lower concentrations (10^{-6} M) has been shown to inhibit the activities of many types of non-muscle cells, which seems to be dependent on microfilaments (4,6,57,58). This suggests the possibilty that cytochalasin D inhibits these activities as well as the gelation of ETC extract by interacting with an unknown actin-linked accessory protein(s) which has a high affinity for the drug. To elucidate these problems, we began to search for protein factors conferring Ca^{2+} and cytochalasin D sensitivities to the filamin-dependent gelation of muscle actin.

Isolation of a novel Ca^{2+}-sensitive gelation factor.

Our initial attempts to find out the postulated Ca^{2+} sensitivity conferring factor were unsuccessful. Despite a number of efforts, no factors conferring Ca^{2+} sensitivity to filamin-dependent gelation of muscle actin could be detected in any fractions of ETC extract. We therefore decided to find out a factor capable of transforming actin filaments into gel in a Ca^{2+}-sensitive fashion, and we could detect this activity in the supernate obtained after centrifugation of the gelated ETC extract as shown in Fig. 13. The protein factor having this activity was then purified by the method detailed in "Materials and Methods". The purified protein, which we

named "actinogelin", gave a single protein band on SDS-polyacrylamide gel electrophoresis and caused the gelation of F-actin in a Ca^{2+}-sensitive fashion even in the absence of filamin. Its electrophoretic mobility in SDS-polyacrylamide gels was identical with that of band 3 observed in the washed gel pellet. Like band 3, no band corresponding to actinogelin could be clearly identified on SDS-polyacrylamide gel electrophoresis of the whole ETC extract, because of the presence of a highly stainable protein having similar molecular weight. During its purification, actinogelin could be separated from both filamin and myosin in the DE-32 column chromatography step. The elution profile obtained in this step is shown in Fig. 5. SDS-polyacrylamide gel electrophoresis and the experiment using purified filamin indicated that filamin was eluted 0.1 - and 0.2 M KCl, but this fraction did not induce the gelation of muscle actin (Fig. 17), probably because large portion of filamin in this fraction had been incorporated into the aggregate formed during second dialysis. The actinogelin activity was eluted by 0.4 M KCl (Fig. 17). Separation of actinogelin from other proteins was most effectively achieved in the subsequent hydroxylapatite column chromatography step; the elution profile obtained in this step is shown in Fig. 6. In the pooled actinogelin fractions obtained in this step the major band in SDS-polyacrylamide electrophoresis now corresponded to actinogelin. The pooled fractions were then concentrated to 1 to 1.5 ml by means of a Sartorius collodion membrane and subjected to gel filtration on a Sepharose 6B

column. Fig. 7 shows the elution pattern thus obtained. SDS-gel electrophoretic examinations showed that actinogelin was present as ahomogeneous protein in the fractions indicated in the figure. Starting from about 6 g protein of crude ETC extracts, 2.5 mg of protein of pure actinogelin were recovered in this fraction. If fractions containing almost pure(more than 90%) actinogelin were collected together with the pure fractions, usually 5 to 6 mg of the protein was obtained at Sepharose 6B step (Table II). The fractions containing some contaminants (3.7 mg protein in case of Table II) were either used as almost pure actinogelin or further purified by DE32 chromatography as described in "Materials and Methods".
It is to be noted that a significant loss of actinogelin was caused during the first low ionoic strength extraction step and during the concentration by means of a Sartorius collodion membrane. Fig. 18 shows the actin-gelation activities of the fractions obtained from several purification steps. The final preparation at a concentration of 9.6 μg/ml could cause the gelation of 400 μg/ml of muscle actin. Table II shows the specific gelation activities of the fractions obtained after several purification steps, which were tentatively calculated from Fig. 18. The specific activity of purified actinogelin was even higher than that of purified filamin.

Molecular properties of purified actinogelin. The apparent molecular weight of actinogelin was estimated by SDS-polyacrylamide gel electrophoresis by using 5.6 % gels. Actinogelin was found to have a slightly lower mobility than the X subunit of RNA polymerase B which possesses a molecular weight of 100,000. In two

experiments using different sets of molecular weight markers, the apparent molecular weight of actinogelin was determined to be 115,000 (Flg. 19a) and 112,000 (Fig. 19b). A sedimentation equilibrium study, which was conducted at 20°C at an actinogelin concentration of 0.74 mg/ml by using an ultraviolet scanner, showed that native (undenatured) actinogelin had a molecular weight of 260,000. In another experiment, in which an interference optical system was used and the actinogelin was 2.5 mg/ml, a molecular weight of 251,000 was obtained for native actinogelin. These observations suggested that native actinogelin existed as a dimer. Both sedimentation equilibrium experiments also indicated that actinogelin contained highly aggregated population.

To confirm the dimeric nature of native actinogelin, it was subjected to the chemical cross-linking treatment described by Davies and Stark (61). As shown in Fig. 20, after this treatment native actinogelin gave a new band having an estimated molecular weight of 230,000 on SDS-polyacrylamide gel electrophoresis; the molecular weight was estimated by using cross-linked hemocyanin as an internal standard. Under the experimental conditions employed, however, only one third of the total actinogelin used was converted to the new band. The reason for such incomplete cross linking is unknown, but several proteins have been shown to undergo incomplete cross linking among their subunits (61). In any way, it was certain that native actinogelin was composed of two subunits. When actinogelin was analyzed by SDS-polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol, the reducing agent, it exhibited the same electrophoretic mobility as in the presence of reducing agent (data not shown), indicating that actinogelin

had no intersubunit disulfide bonds.

Actinogelin was coloress and showed no optical absorptions in the visible region. As shown in Fig. 21, its ultraviolet absorption spectrum showed a maximum at 280 nm together with a shoulder at 290 nm, which was due to tryptophan residues. Small shoulders in the 260-270 nm region were probably due to phenylalanin residues. Based on the protein determination by the Lowry method, an $E_{280}^{1\%}$ value of 8.7 was estimated for actinogelin.

Ca^{2+}-sensitivity of actinogelin-dependent gelation of muscle actin. As described above, actinogelin purified to homogeneity could induce the gelation of skeltal muscle actin in a Ca^{2+}-sensitive fashion. Fig. 27 shows that optimal gelation was observed in the KCl concentration range between 20 mM and 100 mM. KCl concentrations above 100 mM and below 20 mM resulted in deceased extents of gelation. Fig. 28 shows that optimal gelation requires above 1.5 mg/ml actin was enough to produce actinogelin-dependent gel. By contrast, the volume of pellet of actinogelin-actin mixture in the presence of Ca^{2+} was the same value as that of F-actin only. Actinogelin-dependent gelation did not require ATP and Mg^{2+}. If both reagents were removed from the reaction medium, gelation occured normally (data not shown). Fig. 24 shows the effect of free Ca^{2+}concentration (controlled by using Ca^{2+}-EGTA buffer) on this reconstituted gelation system. The inhibition of gelation in the reconstituted system was observed at free Ca^{2+}concentrations which were required for inhibition of the gelation of crude ETC extract.

In both systems half maximal inhibition was observed at a free Ca^{2+} concentration of about 10^{-6}M. The inhibition could be reversed by reducing the Ca^{2+} concentration by adding EGTA (Table III). When 10 µl of 10 mM $CaCl_2$ was placed on the gel formed from 400 µg of actin and 20 µg of actinogelin (fraction of hydroxylapatite step), the gel began to undergo solation from the upper surface and complete solation was effected within one hour. Thus, in this case the solation was extensive than in the corresponding experiment with crude extract. At any rate, it was evident that actinogelin plays an essential role in the Ca^{2+}-sensitive gel formation of crude extract.

In the above experiments, gelation was started by mixing G-actin with actinogelin. It was,therefore, unclear whether actinogelin inhibited the polymerization of G-actin in the presence of Ca^{2+} or the gel formation per se from F-actin and actinogelin was inhibited by Ca^{2+}. Table III shows the results of experiments in which actinogelin was added after completion of actin polymerization. It can be seen that in the presence of Ca^{2+} the addition of actinogelin to preformed F-actin could not induce gelation, whereas normal gelation of preformed F-actin was observed upon addition of actinogelin when free Ca^{2+} was depleted by EGTA. It was, therefore, suggested that the observed inhibition of actinogelin-dependent gelation by $Ca^{2+}$was not due to the prevention of the polymerization of G-actin. Table III also shows that the inhibited gelation of preformed F-actin in the presence of Ca^{2+} could be readily reversed

by addition of excess EGTA. More convincing evidence against the possibility that Ca^{2+} inhibited the polymerization of F-actin came from centrifugal experiments shown in Fig. 25, which compares the sedimentation profile of F-actin with those of an actin-actinogelin mixture at various g x min values. As can be seen, in the presence of enough Ca^{2+} to inhibit the gel formation the actin-actinogelin mixture behaved similarly to F-actin alone, although there was a tendency that the former was slightly more sedimentable than the latter. In the absence of Ca^{2+}, on the other hand, actinogelin markedly increased the sedimentation of actin at lower centrifugal force, though under these conditions (e.g. 12,000 x g for 10 min) the pellet formed was still loose leading to significant errors in estimation of the extent of gelation. Centrifugation at 50,000 x g for 10 min was, therefore, usually used for estimation of gelation. In any way, these observations indicated clearly that free Ca^{2+} did not affect the polymerization of G-actin to F-actin. Instead, it was likely that free Ca^{2+} somehow inhibited the assembly of F-actin and actinogelin into the three-dimensionally arranged gel structure.

Other characteristics of actinogelin-dependent gelation.

Actinogelin-dependent gelation of muscle actin was also found to be sensitive cytochalasin D, but the drug concentration required for complete inhibition of the reconstituted system was about 10 times higher than that needed for inhibition of the gelation of crude ETC extract (Fig. 26). The cytochalasin D concentration required for inhibition of actinogelin-dependent system was, however, similar to that for the filamin-dependent gelation of muscle actin (cf. Fig. 12), suggesting

that simlar mechanisms were operating in the cytochalasin inhibitions of both reconstituted systems.

A titration experiment shown in Fig. 27 indicated that in the gelation reaction 20 μg of actin could precipitate a maximum of 10 μg of actinogelin in the form of sedimentable gel; further addition of actinogelin caused no increase in the amount of protein recoverable in the pellet. Assuming molecular weights of 230,000 and 43,000 for actinogelin dimer and G-actin, respectively, it was calculated that each actinogelin dimeric unit could maximally bind 10 to 12 G-actin units. If actin was in the form of F-actin filament in the gel, it could be stated that there was one actinogelin-binding site in each F-actin segment containing 10 to 12 G-actin units. Almost all the actinogelin added could form sedimentable gel with actin below the saturation point. The presence of a stoichometric relationship in the binding of actinogelin and actin suggested that this binding was specific.

A specific Ca^{2+}-binding protein, called calmodulin (62), calcium-dependent regulator (63) or calcium modulator protein (64), has been found in various mammalian tissues and shown to mediate Ca^{2+}-dependent activation of such enzymes as cyclic nucleotide phosphodiesterase (54,64), brain adenylate cyclase (63), erythrocyte membrane Ca^{2+}/Mg^{2+}ATPase (65,66), a membrane-bound protein kinase (67) and a cytosolic protein kinase which efficiently catalyzes the phosphorylation of myosin light chain (68,69). It has further been reported that this protein can replace troponin C partially in the regulation of myosin ATPase in vitro (70,71). It was therefore, of interest to examine

if the Ca^{2+}-sensitivity of actinogelin-dependent gelation of F-actin was due to a calmodulin-like activity of actinogelin. However, practically no Ca^{2+}-dependent activation of cyclic nucleotide phosphodiesterase was elicited by addition of pure actinogelin. Furthermore, gel filtration of actinogelin through a Sephacryl S-200 in the presence of high salt concentration and EGTA did not abolish the capacity of actinogelin to induce Ca^{2+}-sensitive gelation of actin, though this treatment has been reported to separate calmodulin-like leiotonin C from leiotonin A, both of which are claimed to be responsible for Ca^{2+}-regulation of smooth muscle (72). These findings led to the conclusion that calmodulin-like activity was not involved in the Ca^{2+}-sensitive gelation of actin by actinogelin.

In the presence of Ca^{2+} addition of filamin to a mixture of F-actin and actinogelin induced gel formation, though the extent of gelation was somewhat less extensive than the addition of filamin to F-actin alone (Fig. 28). In the absence of Ca^{2+}, where actinogelin could induce the gelation of F-actin, further addition of filamin enhanced the extent of gelation when sub-maximal amounts of actinogelin were present in the mixture. Moreover, addition of a large amount of filamin to a mixture of F-actin and actinogelin resulted in a loss of Ca^{2+}-sensitivity of the gel formation.

DISCUSSION

Extracts of a number of cells have been shown to undergo revesible gelation upon warming to room temperature and involvement of actin in the process has been fairly well established (27-36). The gelation is temperature-dependent and sensitive to cytochalasins and Ca^{2+} (30-32,36). During the course of studies of the gelation phenomena, several actin-binding proteins have been isolated from cell-free extracts and shown to induce the gelation of skeletal muscle actin. The proteins so far isolated include 220,000- and 58,000-dalton proteins from sea urchin eggs (21), gelactins from Acanthamoeba (20) and filamin ("actin-binding protein") from macrophages (23). However, none of the gelation systems reconstituted from these purified proteins and actin exhibited Ca^{2+}-sensitivity, leading to the postulation that Ca^{2+}-sensitivity of crude extracts must be due to a third factor(s) which confers the sensitivity to the gelation of actin with accessory proteins. In view of the possible physiological importance of Ca^{2+} sensitivity (31,36), many laboratories have attempted to detect such a factor(s) without any success. The problem of Ca^{2+}-sensitivity of gelation of cell-free extracts has, therefore, still remained to be elucidated.

In this study we have shown that an extract of ETC can also undergo gelation in similar ways to those reported for extracts from other cells. We have also been able to purify a protein closely resembling filamin, which can induce the gelation of muscle actin. However, this gelation was again insensitive to Ca^{2+}, and all attempts to detect a factor confer-

ring Ca^{2+}-sensitivity to the filamin-dependent gelation of actin were unsuccessful. In view of this failure, we decided to take another approach and began a search for a factor which induces the gelation of actin in a Ca^{2+}-sensitive fashion. It was thus possible for us to isolate a protein, termed actinogelin, which could induce the gelation of actin only in the absence of free Ca^{2+}, as described above. It is thus clear that ETC extracts contain two actin-gelation factors, i.e. filamin and actinogelin; the gelation of actin induced by the former is insensitive to Ca^{2+}, whereas the latter possesses both actin gelation activity and Ca^{2+}-sensitivity in the same molecule.

The fact that actinogelin induces the Ca^{2+}-sensitive gelation of actin cannot unequivocally explain the Ca^{2+}-sensitivity of gelation of ETC extract, because filamin, another gelation factor in the same extract, neutralizes the Ca^{2+}-sensitivity of actinogelin-dependent gelation (cf. Fig. 28). For evaluation of the gelation of crude extract, therefore, quantitation of the two gelation factors in ETC extract is necessary. The content of filamin in the extract was roughly estimated to be about 0.1% from the intensity of filamin band in an SDS-polyacrylamide gel electrophoretogram of the extract. On the other hand, the content of actinogelin was estimated to be 0.3 % (probably more) based on its recovery in the final purification step starting from the second ammonium sulfate fractionation step. From the data shown in Figs.18 and 28, it can be concluded that the estimated actinogelin content

in ETC extract (containig approx. 20 mg protein/ml) is sufficient to induce Ca^{2+}-sensitive gelation even in the coexistence of the estimated amount of filamin. Furthermore, it has recently been found that the gelation of an ascites tumor cell extract was enhanced after brief trypsin digestion (73); the gel formed after digestion was almost free from filamin-like protein, whereas it was concentrated in the gel formed before trypsin treatment. Although these experiments were conducted in the presence of EGTA (its Ca^{2+}-sensitivity was not tested), these findings support the view that a gelation factor other than filamin is present in the extract. It should be noted, however, that other actin-interacting proteins such as myosin and tropomyosin in the extract may affect the gelation. It is also probable that the extract contains a hitherto unknown gelation factor(s). Nevertheless, the participation of actinogelin in the gelation of ETC extract is a very likely possibility in view of the followings. (1) Actinogelin is concentrated in the gel from the extract. (2) The gelation of crude extract and that of the reconstituted system consisting of actinogelin and actin are inhibited by the same concentrations of Ca^{2+}. (3) No fractions in ETC extract other than those containing actinogelin induce Ca^{2+}-sensitive gelation or confer Ca^{2+}-sensitivity to the filamin-dependent system. (4) The estimated content of actinogelin in the extract is sufficient to account for the gelation of extract. The failure of previous workers to detect Ca^{2+}-sensitivity factors seems to be due to the fact that their reconstitution experiments were carried out in the

absence of added EGTA, which removes endogeneous free Ca^{2+}.

At a Cold Spring Harbour laboratory conference this year, Condeelis has reported that extracts of Dictyostelium discoidium ontain two gelation factors having molecular weights of 250,000 and 120,000 and partially purified 120,000-dalton protein induces Ca^{2+}-sensitive gelation of actin. These findings are strikingly similar to our results and it seems that 250,000- and 120,000-dalton proteins correspond to filamin and actinogelin, respectively. However, while 120,000-dalton protein loses its activity when separared from three contaminating proteins of 90,000, 80,000 and 42,000 daltons by gel filtration, actinogelin retains its activity even after Sepharose 6B gel filtration. At a Gordon Research Conference this year, Lin and Stossel reported that a protein named "gel-solin", isolated from pulmonary macrophage, can confer Ca^{2+}-sensitivity to the gel formed from filamin and actin and that this protein is composed of two identical subunits of 90,000 daltons.* Since purified actinogelin occasionally contains small amounts of contaminants which is similar to gel-soin in size, it might be possible that one of the contaminants is gel-solin. However, this possibility can be excluded because purified actinogelin, unlike gel-solin, does not inhibit the filamin-dependent gelation in the presence sufficient concentrations of Ca^{2+}. Furthermore, our estimation indicates that 10 μg of purified actinogelin contains only 0.3 μg of the contaminants at most. Even if this amount of the contaminants is all gel-solin, then one molecule of gel-solin

* We thank Prof. S. Kakiuchi for this information.

is present per 25 molecules of actinogelin and 5,000 molecules of actin in the reconstituted system containing 10 μg of actinogelin and 400 ug of actin. A calculation indicates that under these conditions only one molecule of gel-solin is available for a length of F-actin filament of 12.5 μm. Since an average length of each actin filament is 1.7 μm (74), only one out of more than seven F-actin filaments can be attacked by the putative gel-solin. It is, therefore, hardly conceivable that the contaminants in purified actinogelin can convert the gel into the sol state in the presence of Ca^{2+}. Calmodulin is a ubiquitous regulator of a number of Ca^{2+}-dependent cellular processes. However purified actinogelin is devoid of calmodulin activity, excluding the possibility of participation of calmodulin in the Ca^{2+}-sensitivity of actinogelin-dependent gelation. A much less likely possibility might be that a calmodulin-like protein of a small size is tightly bound to actinogelin and in this state does not mediate Ca^{2+}-dependent activation of cyclic nucleotide phosphodiesteras.

Sedimentation equilibrium experiments and SDS-polyacrylamide gel electrophoresis indicate that native actinogelin is a dimeric molecule consisting of two subunits of identical size. The molecular weight of the subunit has been determined to be 112,000 to 115,000 and that of native actinogelin to be 251,000 to 260,000. Chemical cross-linking studies have also supported the dimeric nature of actinogelin; a molecular weight of 230,000 has been obtained by this method for the native molecule. Hydrodynamic data further suggest that actinogelin has a tendency to undergo aggregation. In fact, aggregate formation can

be readily observed upon freeze-thawing of actinogelin and concentration by means of a Sartorius collodion membrane. Electron microscopic observations of actinogelin (data not shown) are also consistent with these findings.

Actinogelin binding to F-actin becomes saturated when dimeric actinogelin molecule binds to 10-12 monomer units of F-actin. This may be compared with the molar ratios of binding of other actin-binding proteins, i.e. 1:8-12 for filamin (75), 1:7 for tropomyosin (76), and 1:9-11 for α-actinin (77). Among these actin-binding proteins, α-actinin is similar to actinogelin in that both are dimeric proteins of similar sizes (subunit molecular weight of α-actinin is 90,000 (78)) and bind to F-actin at similar stoichiometry. However, gelation of actin induced by α-actinin is not sensitive to Ca^{2+} (K. Maruyama, personal communication).

In ETC extract there is an appreciable amount of unpolymerized actin whose molecular weight, estimated by Sepharose 6B gel filtration, is somewhat higher than that of monomeric actin or hemoglobin (Fig. 12). Gordon et al. (79) have recently demonstrated that Ca^{2+}, at higher concentrations than 10^{-4} M, increases dramatically the critical monomer concentration of non-muscle actin. However, the requirement of such high concentrations of Ca^{2+} for this effect seems to exclude the possibility that this effect is involved in the Ca^{2+}-induced solation of the crude extract reported in this paper.

Cytochalasins inhibit a number of cellular events which are dependent on microfilaments (4,6,57), but it is still

unknown how cytochalasins affect microfilaments. To elucidate this problem, the effects of cytochalasins on purified contractile proteins, such as actin, myosin and their mixture, have been extensively studied (60,80,81). Significant effects of the drugs have, however, been usually observed at concentrations which are 10- to 100-fold higher than those required for inhibitions of cellular functions. On the other hand, the gelation of ETC extract reported in this study as well as those of extracts of macrophages (34) and Hela cells (29) can be avolished by low concentrations of cytochalasins, which are just enough to inhibit microfilament-dependent functions in vivo. This indicates that these gelation systems retain the cytochalasin sensitivity observable in vivo and, therefore, suggests that microfilaments in these systems retain their physiological structural features. It is likely that cytochalasins affect the three-dimensional organization composed of actin filaments and actin accessory proteins rather than attack the actin filaments themselves when the drugs inhibit cellular functions. Hartwig and Stossel (34) have reported that 1 μM cytochalasin B prevents the gelation of macrophage extract and a reconstituted system consisting of macrophage actin and "actin-binding protein" and claimed that the main site of cytochalasin B action is the "actin-binding protein". However, neither the gelation of muscle actin induced by filamin nor that induced by actinogelin is inhibited by such a low concentration of cytochalasin D, though this concentration of the drug is inhibitory to the gelation of ETC extract and micro-

filament-dependent cellular functions *in vivo*. Ten times higher concentrations of cytochalasin D are required for inhibition of the gelation of these reconsituted systems. In these cases, it is likely that the drug affects actin filaments directly, because such high concentrations of cytochalasin D can modify the actin filaments. The different cytochalasin sensitivities between crude ETC extract and reconstituted systems suggest the presence in the extract a factor(s) which confers a high cytochalasin sensitivity to microfilaments.

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EXTRACTION MEDIUM

20 mM	imidazol-HCl(7.0)
1 mM	ATP
1 mM	EGTA
1 mM	DTT
0.34 M	sucrose
0.1 mM	PMSF
0.1 µg/ml	pepstatin

Ehrlich tumor cells suspended in 1.5 vol. of the extraction medium are disrupted by cell disruption bomb at 1,100 to 1,300 psi.

"100,000 x g sup." is obtained from this homogenate by centrifugation at 100,000 x g for 1 h.

Fig. 1 Method of preparation of ETC extract

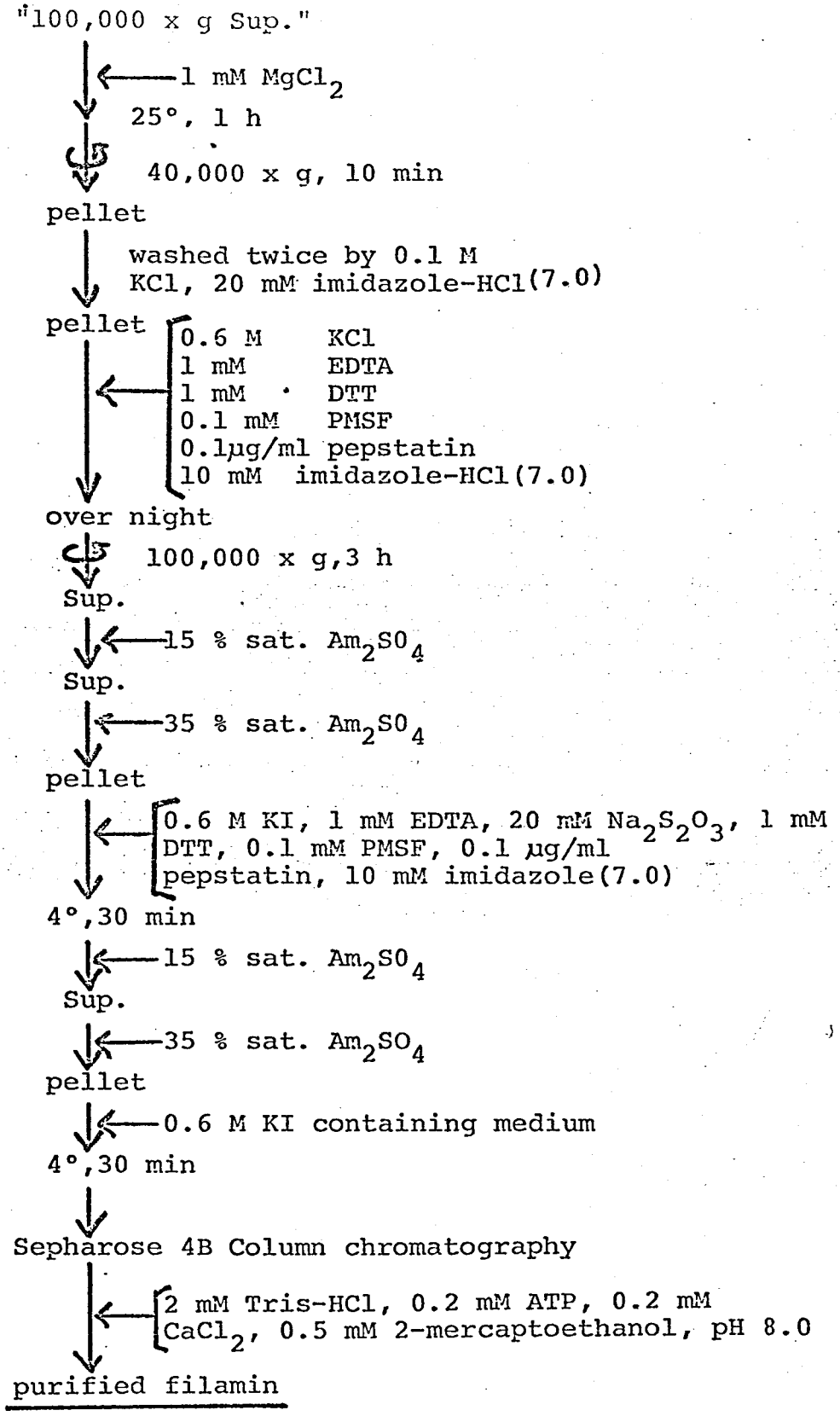
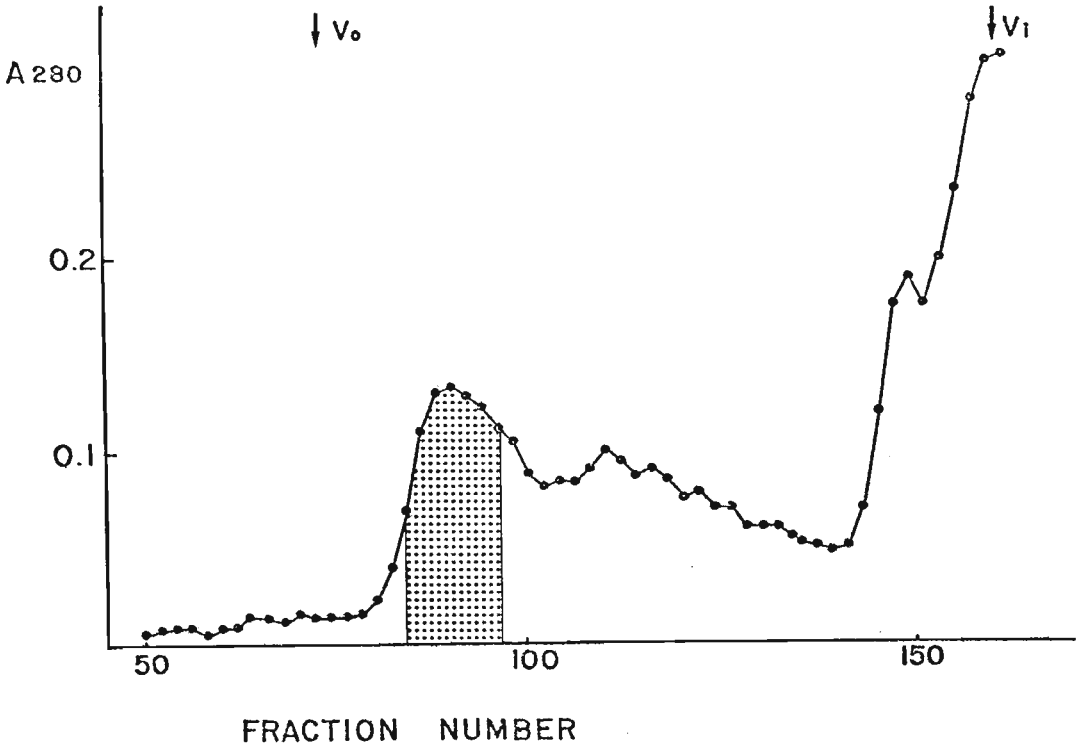


Fig. 2 Method of purification of filamin.



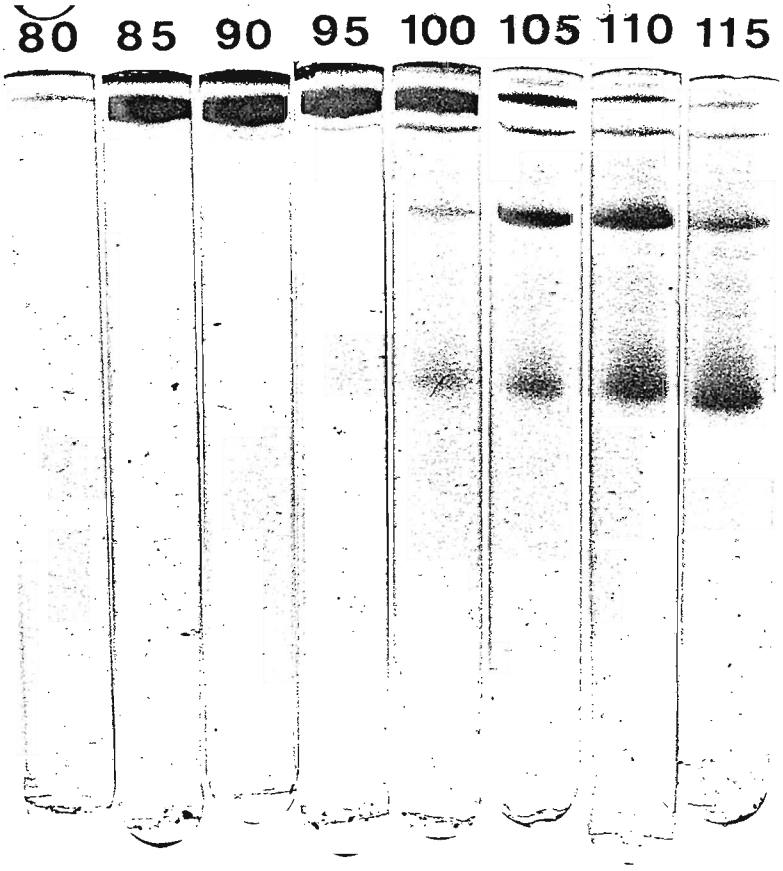


Fig. 3 Elution profile of filamin from a Sepharose 4B column.

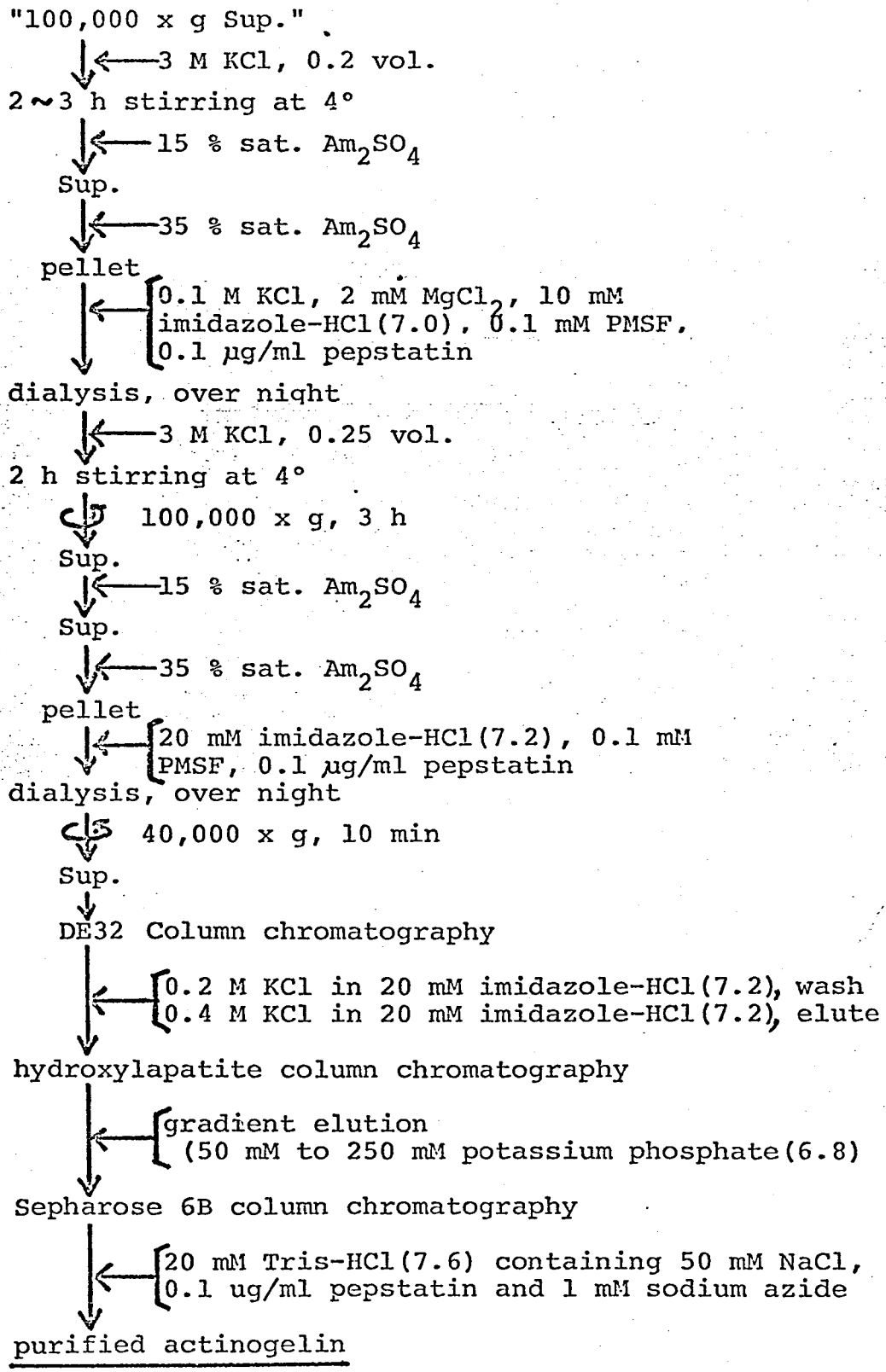


Fig. 4 Method of purification of actinogelin.

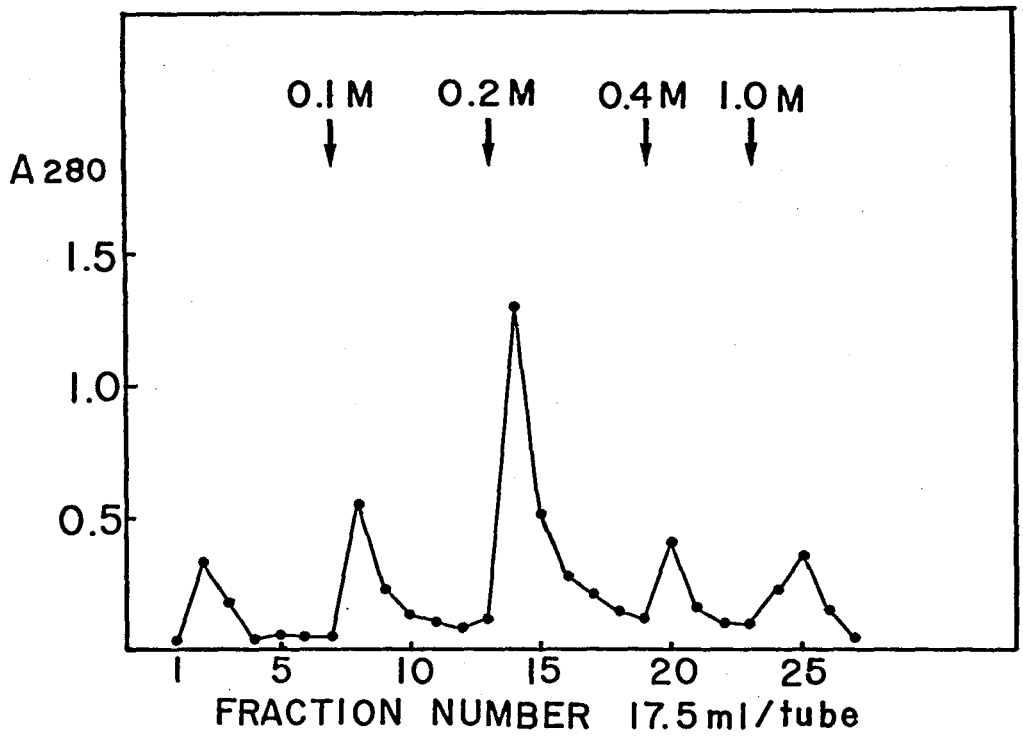
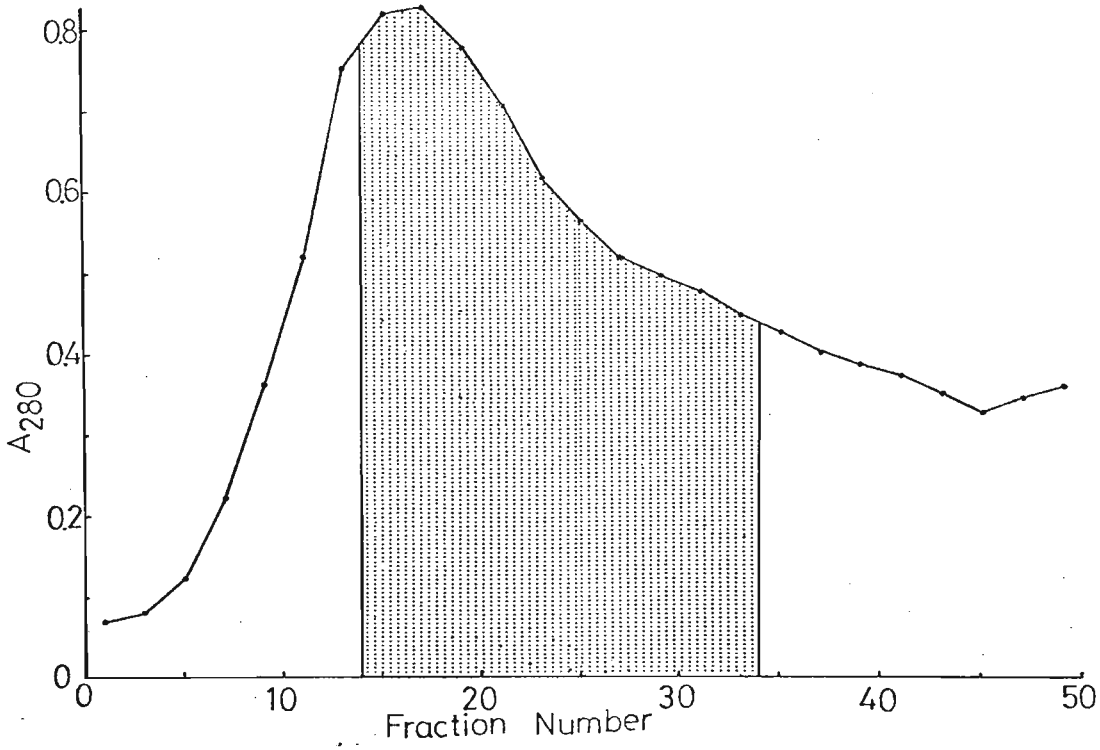


Fig. 5 Elution profile of actinogelin from DEAE-cellulose column.



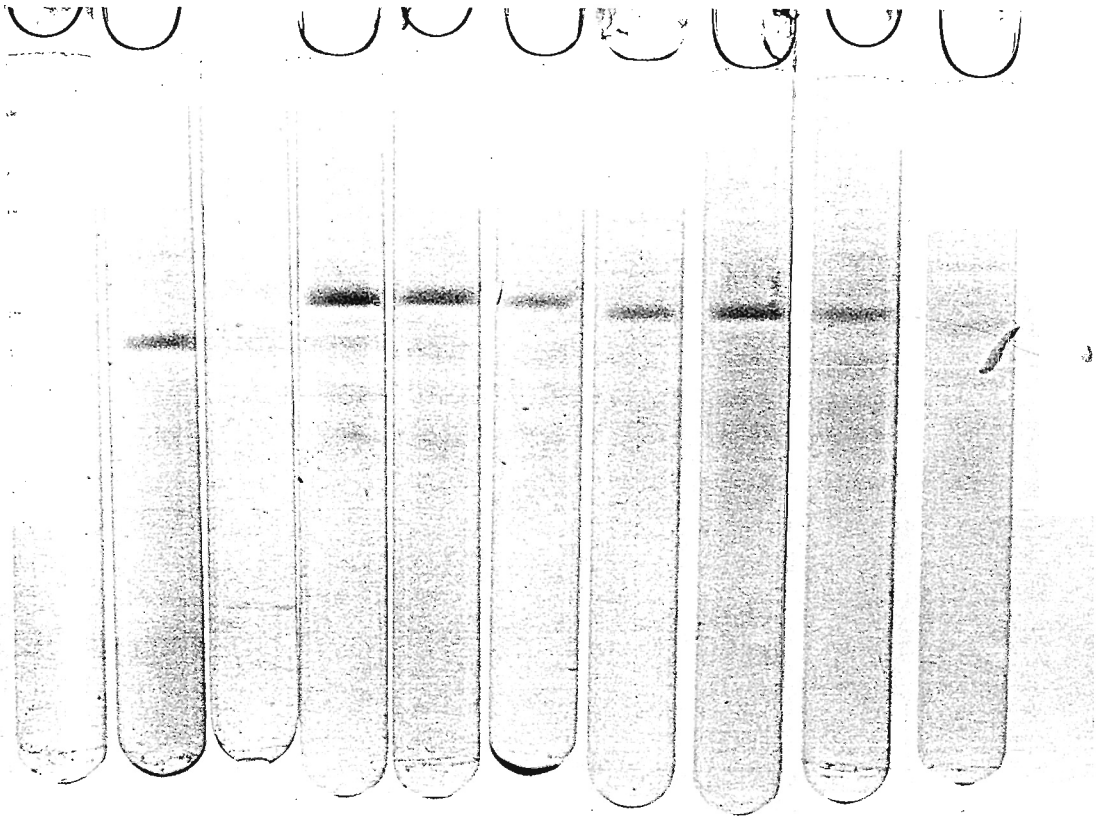


Fig. 6

Elution profile of partially purified actinogelin from a hydroxylapatite column.

Fig. 1 Method of preparation of ETC extract.
For details, see "Materials and Methods".

Fig. 2 Method of purification of filamin.

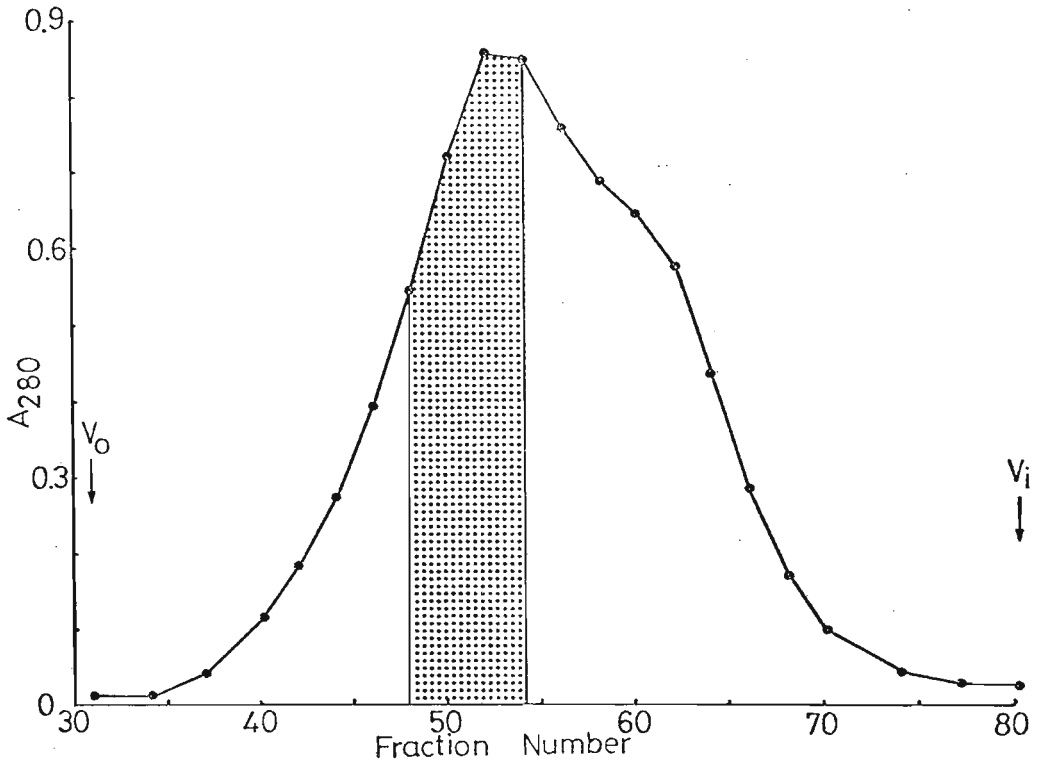
Fig. 3 Elution profile of filamin from Sepharose 4B column.
Fractions were subjected to SDS-polyacrylamide gel electrophoresis as shown in lower part of the figure. And fractions found to be free from contamination were pooled as indicated by a dotted area and used as purified filamin. Fraction volume was 0.82 ml.

Fig. 4 Method of purification of actinogelin.

Fig. 5 Elution profile of actinogelin from DEAE-cellulose column.
The volume of fraction is 17.5 ml. Fractions eluted by each KCl concentration were pooled and gelation activity was measured as shown in "Materials and Methods".

Fig. 6 Elution profile of partially purified actinogelin from a hydroxylapatite column. Fractions were subjected to SDS-polyacrylamide gel electrophoresis. Electrophoretograms shown in lower part of the figure correspond to fractions, 1,7,11,16,19, 22,25,28,31, and 35, respectively from left to right. Thirty μg protein of each fraction was used.

Dotted area indicates the pooled fractions which were used for next purification step. Fraction volume was set to 1.6 ml.



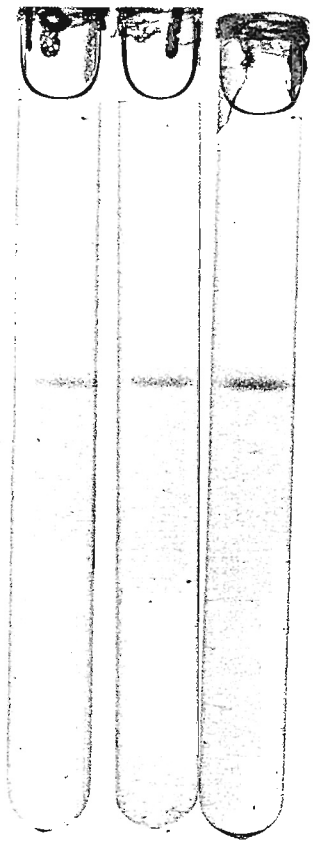


Fig. 7

Elution profile of partially purified actinogelin from a Sepharose 6B column.

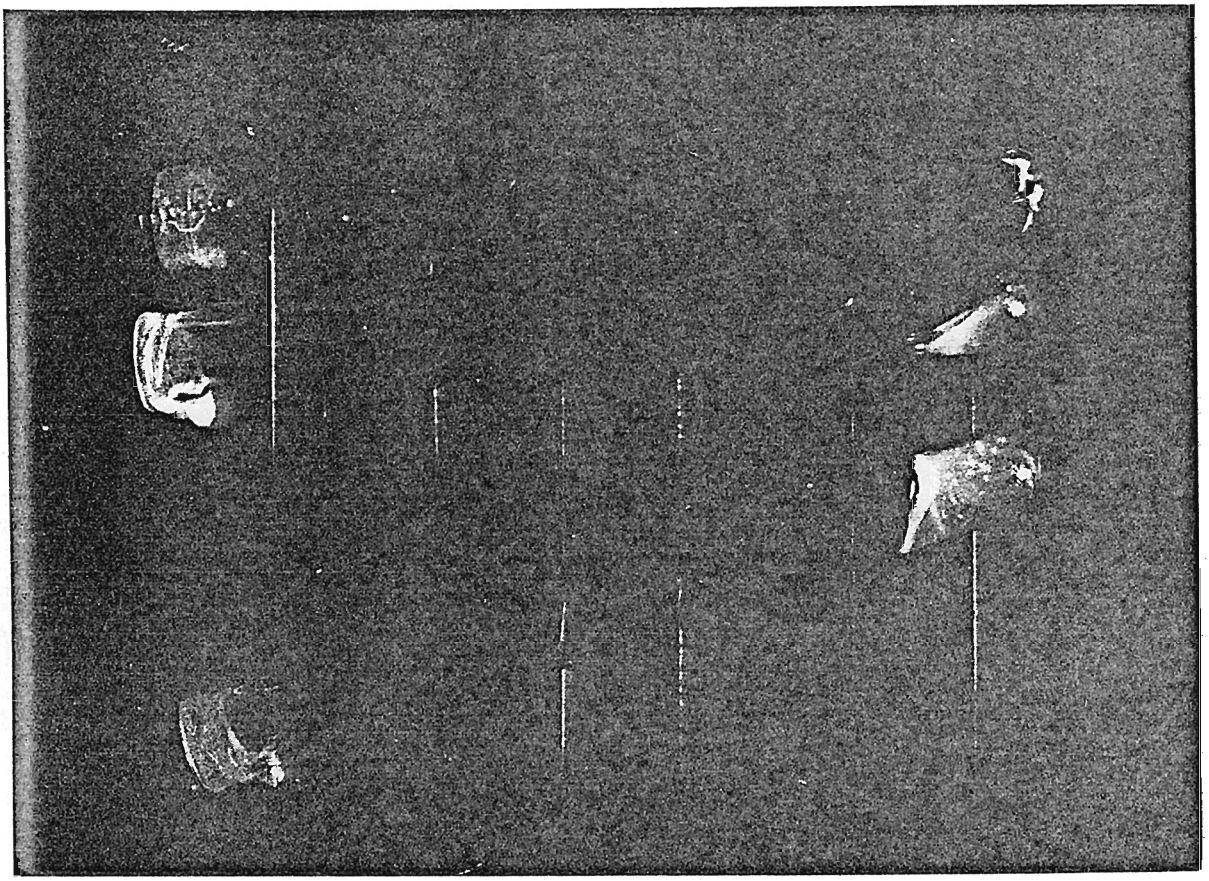


Fig. 8 | Dissolution of the gel and inhibition of gelation by Ca^{2+}.

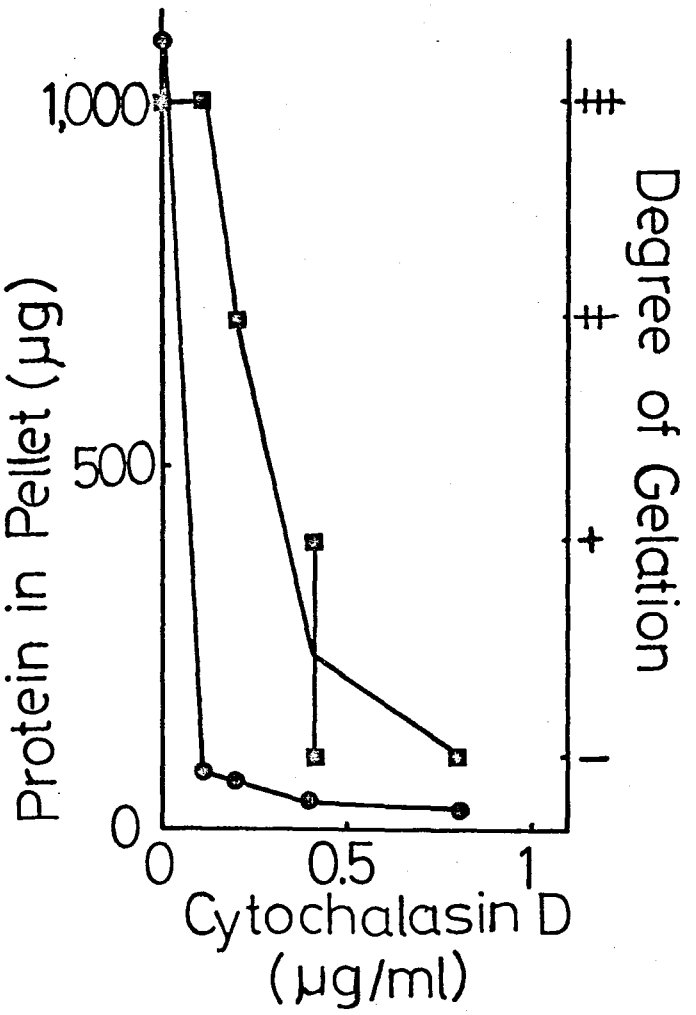


Fig. 9 Inhibition of gelation with cytochalasin D

Fig. 7 Elution profile of partially purified actinogelin from a Sepharose 6B column. Fractions were subjected to SDS-polyacrylamide gel electrophoresis,and resultant electrophoretograms which correspond to fractions, 48,52 and 56 are shown from left to right. Dotted area indicates the pool made from 1.33 ml fractions.

Fig. 8 Dissolution of the gel and inhibition of gelation by Ca^{2+}. Top tube, $Ca^{2+}$was added to a final concentration of 0.8 mM. Middle tube, preformed gel was partially liquified by the addition of Ca^{2+} to a final concentration of 0.8 mM (final free Ca^{2+} concentration was calculated to be more than 3.1 μM). Duration of incubation with $Ca^{2+}$was 30 min without disturbance. Bottom tube, gel formed by incubation of un treated extracts at 20°C for 150 min. Total protein contents were 10 mg in 0.4 ml.

Fig. 9 Inhibition of gelation by cytochalasin D.
Ten μl of various concentrations of cytochalasin D dissolved in dimethylsulfonylfluoride (DMSO) or 10 μl of DMSO were added to ETC extracts (15.4 mg/ml). Gelation was performed and the extent of gelation was estimated as shown in "Materials and Methods"; (■), degree of gelation (visual observation); (◉), protein in pellet obtained by centrifugation at 2,000 x g for 30 min.

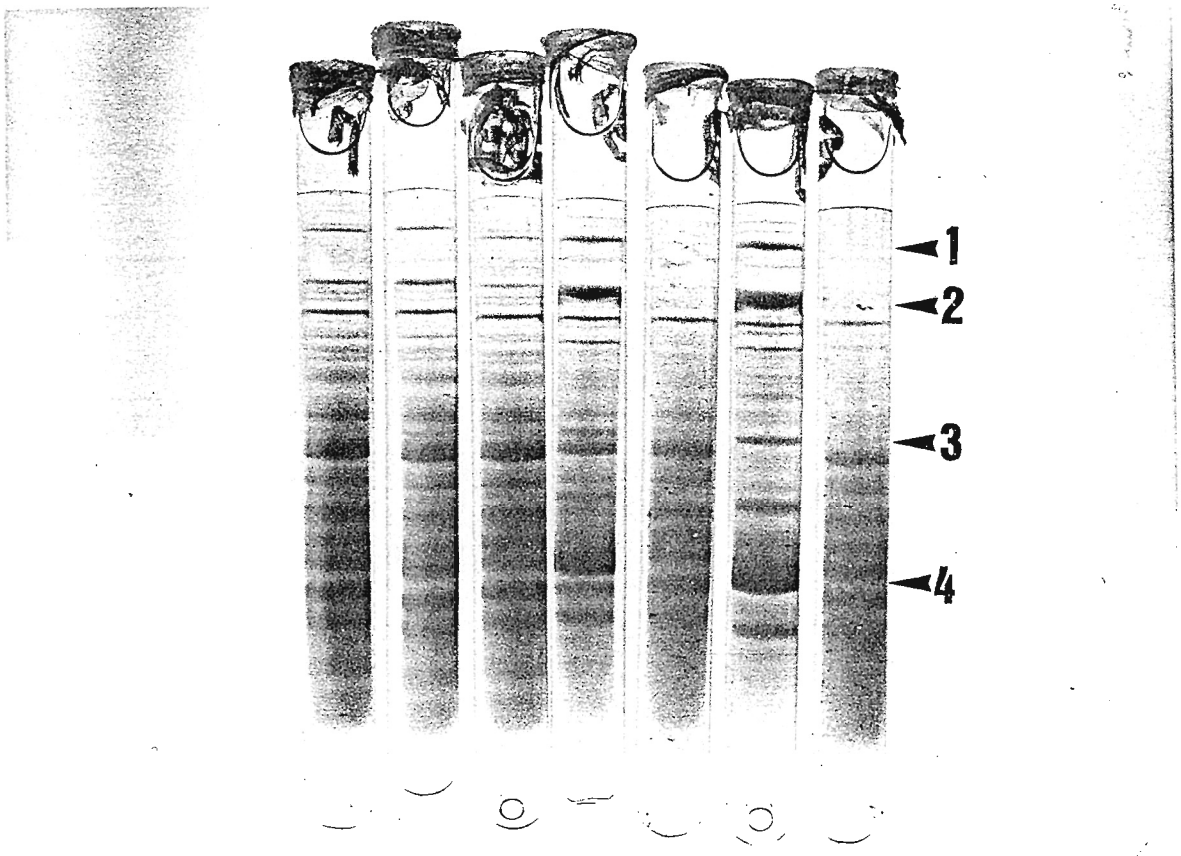


Fig. 10 Analysis of the structural components of gel formed from crude extracts by SDS-polyacrylamide gel electrophoresis

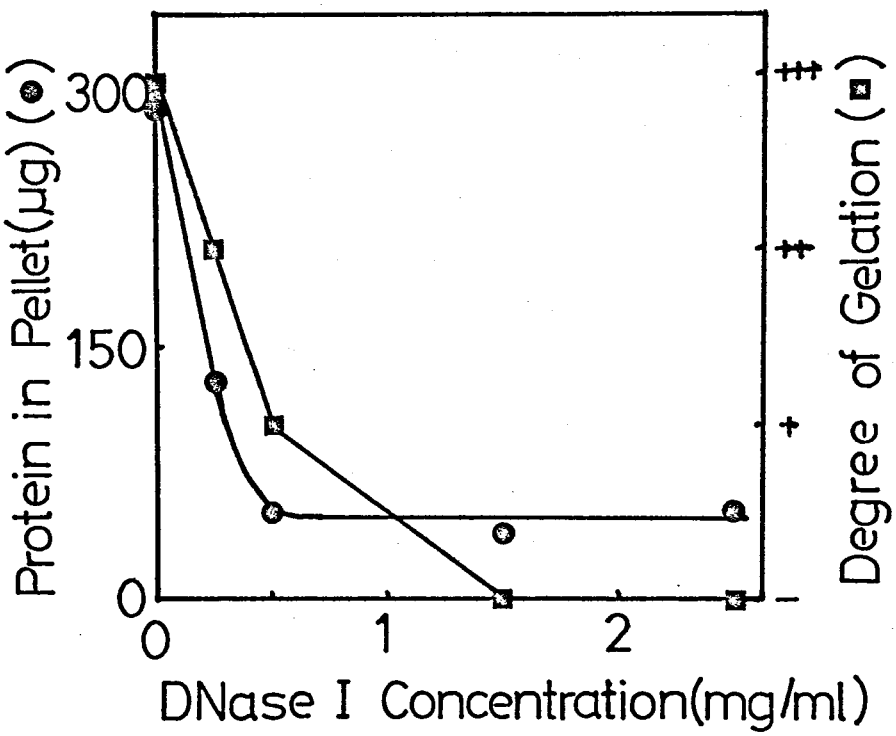


Fig. 11 Inhibition of gelation with DNase I.

Fig. 10 Analysis of the structural components of gel formed from crude extracts by SDS-polyacrylamide gel electrophoresis.
(A), supernate from gelated extract centrifuged at 9,200 x g for 10 min after incubation for 1 hr at 25°C (S-1).
(B), transparant pellet resulting from centrifugation at 9,200 x g for 10 min of gelated extract (gelation was performed by warming the extract to 25°C for 1 hr) (P-1).
(C), supernate obtained by centrifugation at 9,200 x g for 10 min of P-1 after it was resuspended with 20 mM imidazole-HCl(pH 7.2) containing 0.1 M KCl to wash out soluble components from gel materials. (S-2).
(D), resultant pellet from P-1 washed as described for S-2 (P-2).
(E), supernate obtained from P-2 washed again as shown in (C) (S-3)
(F), resultant pellet from P-2 washed again as shown in (C) (P-3).
(G), crude extract of ETC.
Eighty μg of protein was applied to each gel.(5.6% acrylamide).
(A) to (G) were shown from left to right. Band 1,2,3,and 4 correspond to filamin dimer (?), filamin, actinogelin and actin, respectively.

Fig. 11 Inhibition of gelation with DNase I. DNase I was added to ETC extract (16.25 mg/ml) as indicated and then incubated for 1 hr at 25°C. The extent of gelation was expressed as shown in "Materials and Methods". Ten μl of 20 mM $MgCl_2$ was usually added to ETC extracts before they were warmed to 25°C. The final volume of reaction mixture was 0.2 ml.

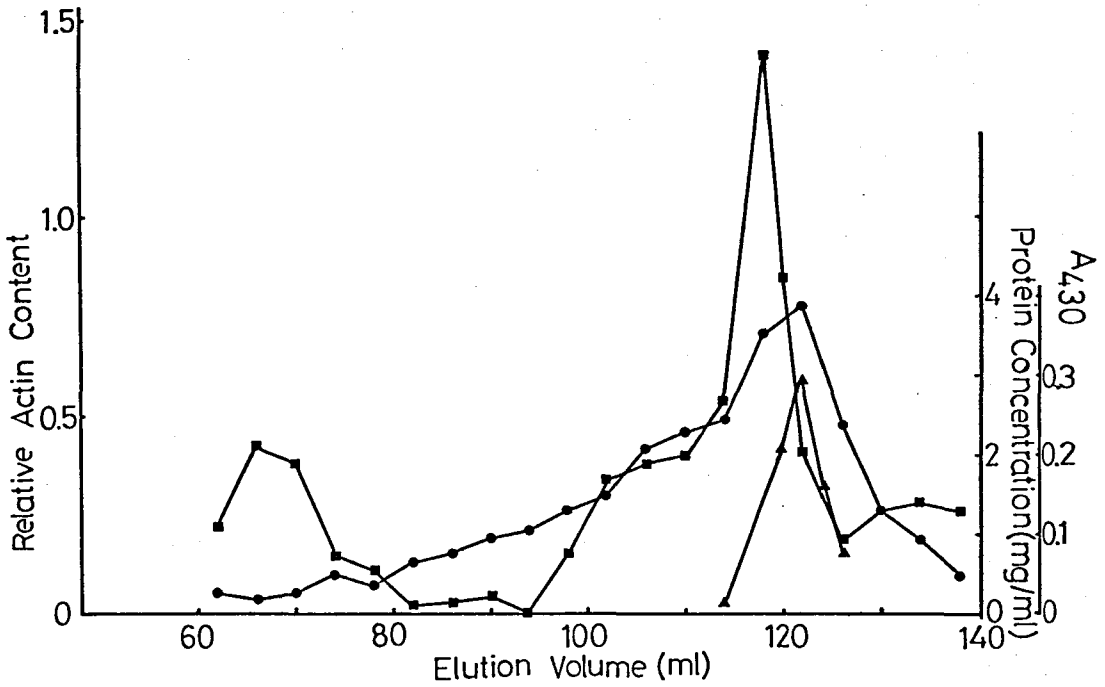


Fig. 12 | Fractionation of actin contained in ETC extract by gel filtration.

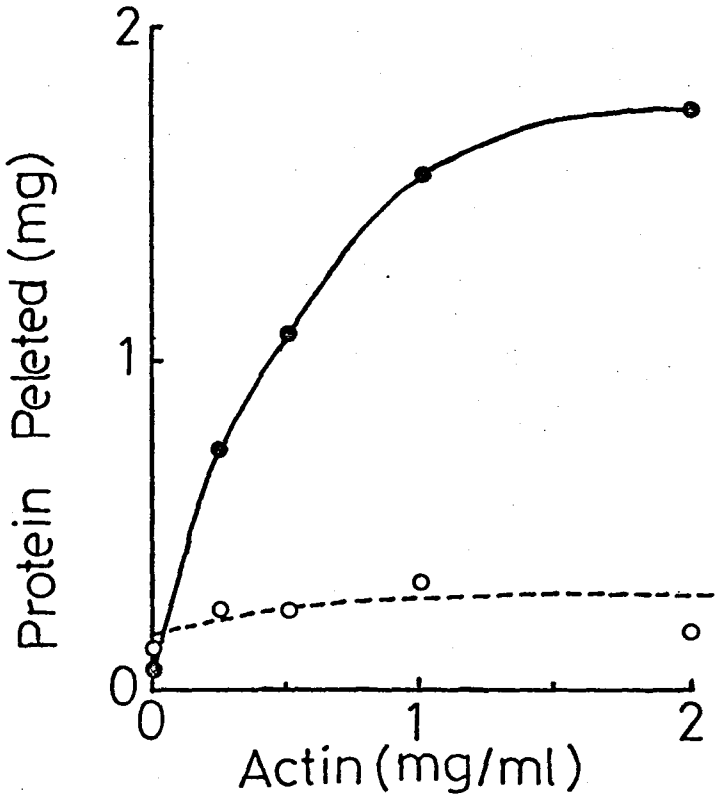


Fig. 13 The ability for the supernate to induce gelation of actin.

Fig. 12 Fractionation of actin contained in ETC extract by gel filtration. The 1.6 x 80 cm column of Sepharose 4B was equilibrated with 50 mM imidazole-HCl (pH 7.0), 0.2 mM EGTA, 1 mM dithiothreitol, 0.2 M sucrose, 0.1 mM PMSF, 0.1 μg/ml pepstatin, 1 mM ATP and 1 mM $MgCl_2$. Two ml of ETC extract was applied to this column and eluted by the same buffer. (●), protein concentrations (mg/ml), (■), relative contents of actin estimated by the method described in "Materials and Methods", (▲), contents of contaminating haemoglobin which was confirmed by its unique spectrum (measurement was carried out at OD_{430nm}).

Fig. 13 The ability for the supernate to induce gelation of actin. The supernate was obtained from gelated extract by centrifugation at 10,000 x g for 10 min. It could not produce a gel by itself in cntrast to the crude extract. Indicated concentrations of muscle actin were added to the supernate (19.1 mg protein /ml) and the mixture was incubated for 1 hr at 25°C in the presence or absence (less than 10^{-7} M) of Ca^{2+}. The gelation was performed as the same conditions described for the crude extract.; (●—●), protein in pellet (mg) in the absence of Ca^{2+}; (○···○), protein in pellet in the presence of Ca^{2+}.

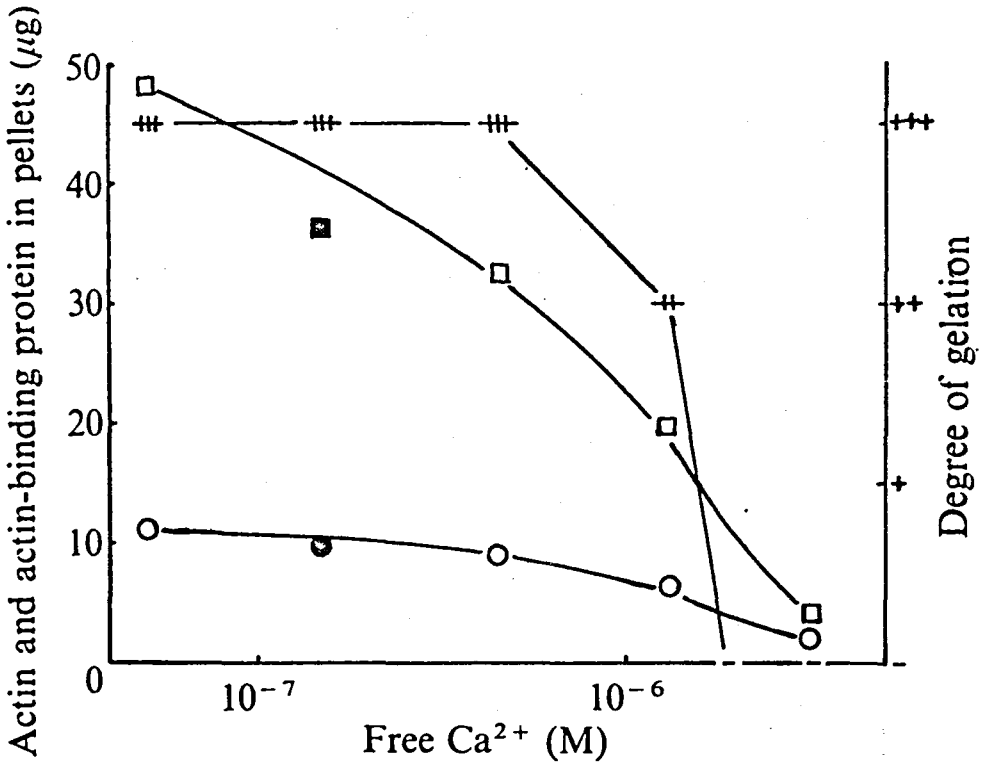


Fig. 14 Inhibition of gelaion with Ca^{2+}.

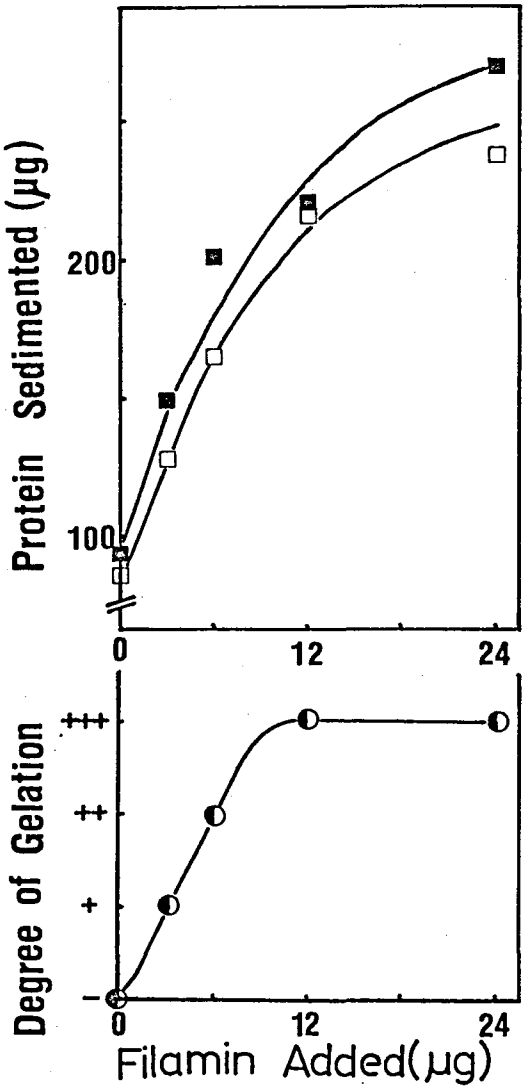


Fig. 15

Effect of Ca^{2+} on filamin-dependent gelation of rabbit skeletal muscle actin.

Fig. 14 Inhibition of gelation by Ca^{2+}. Cell free extracts which were subjected to gel filtration as described in the legend of Table I were used. A $CaCl_2$ solution prepared from $CaCO_3$ was added and free Ca^{2+} concentrations were calculated according to Ogawa(59). Final pH after gelation was 6.7. The eluate containing 8.17 mg protein in 0.4 ml was used in each tube, and pellets were washed two times with 0.1 M KCl. Open symbols, amounts of actin (□) and filamin (○) in the pellets. Calculation was based on the assumption that all protein was stained equally by Coomassie brilliant blue and amounts were expressed as μg protein. Solid symbols, amounts of actin (■) and filamin (●) in a sample brought to a free Ca^{2+} concentration of 3.1 μM, incubated for 30 min at 20°C and then brought back to 0.15 μM by the addition of EGTA. Duration of incubation for gelation at 20°C was 60 min. The extent of gelation (degree of gelation) was estimated as described in "Materials and Methods".

Fig. 15 Effects of Ca^{2+} on filamin-dependent gelation of rabbit skeletal muscle actin. Reaction conditions were similar to that described in "Materials and Methods", except that 2.0 mg/ml of rabbit skeletal muscle actin and either 1.15×10^{-4} M $CaCl_2$ (■) or 5×10^{-4} M EGTA and 6.5×10^{-5} M $CaCl_2$(□) were added as indicated. Filamin purified from Ehrlich tumor cells was added as indicated to a final volume of 0.2 ml. Duration of incubation was 60 min at 20°C. Then, extent of gelation was estimated as described in "Materials and Methods", either in the presence of Ca^{2+} or EGTA (◐).

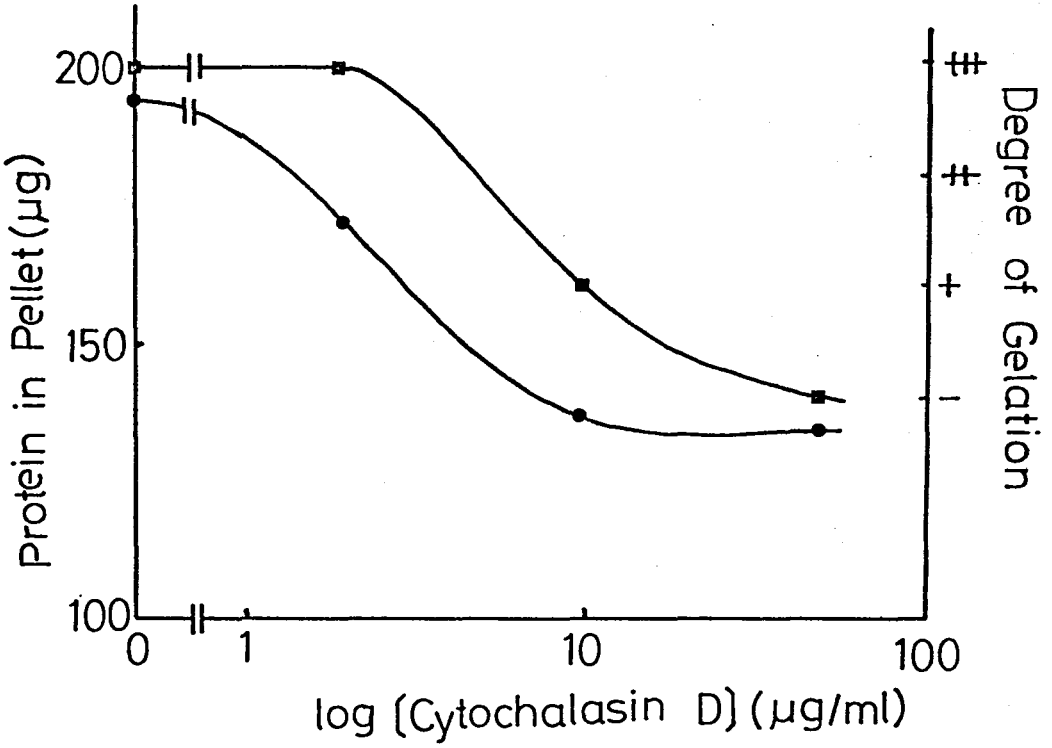


Fig. 16

Effect of cytochalasin D on filamin-dependent gelation of rabbit skeletal muscle actin.

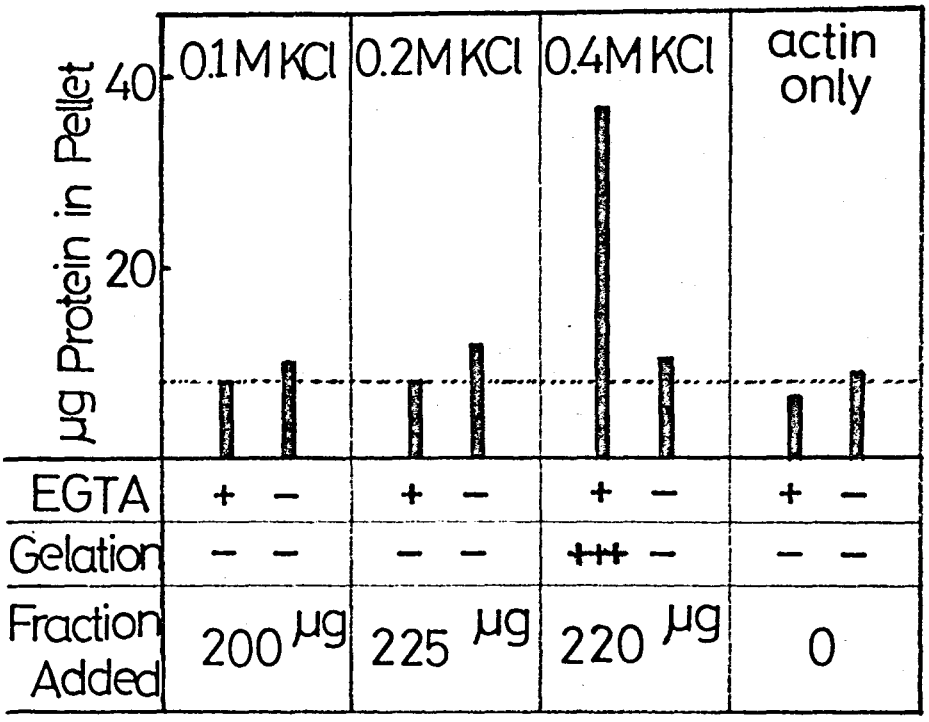


Fig. 17

Gelation activities of three fractions eluted from DEAE-cellulose column.

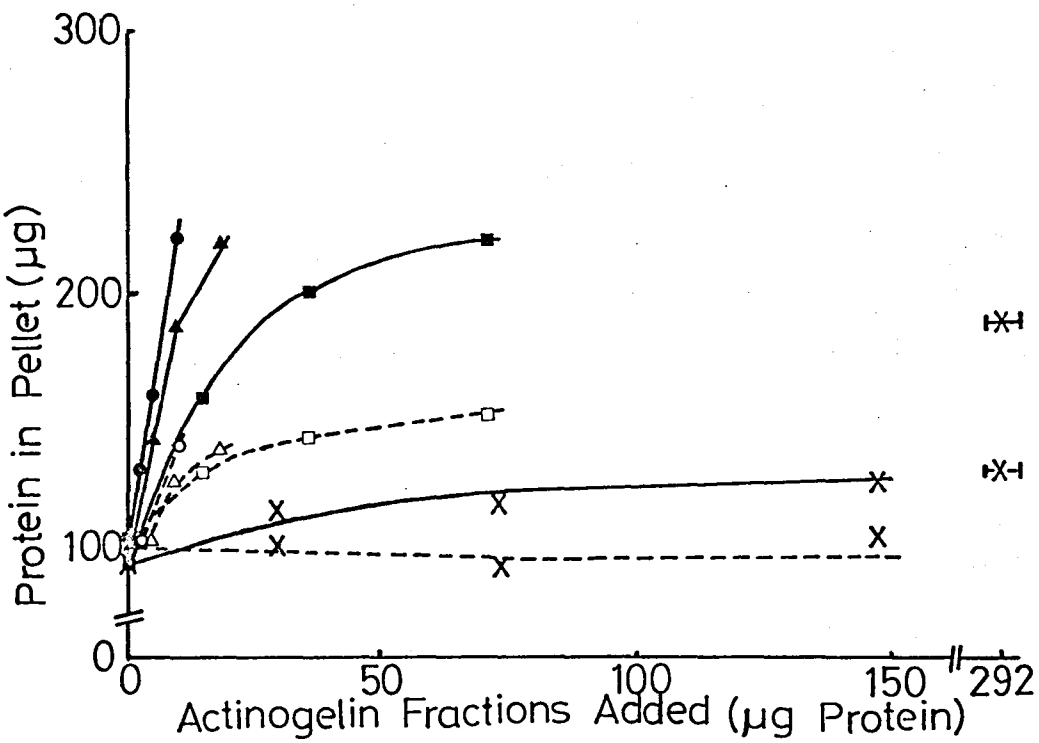


Fig. 18 Gelation activities of samples from each purification steps.

Fig. 16 Effects of cytochalasin D on filamin-dependent gelation of muscle actin. Either 10 μl of cytochalasin D dissolved in DMSO or 10 μl of DMSO are added to the reaction mixture as indicated. Total volume of reaction mixture is 0.2 ml. Other conditions are the same as in Fig. 15: (■), extent of gelation (visual observation), (●), total protein in pellet.

Fig. 17 Gelation activity of three fractions eluted from DEAE-cellulose column. To the reaction mixture, indicated amount of actinogelin fractions, 90 μl of actin-solution (6.4 mg/ml) and either 10 μl of 20 mM EGTA (pH 7.0) dissolved in 20 mM imidazole-HCl or 10 μl of imidazole-HCl were added as shown. Final volume of the reaction mixture is 0.2 ml. Gelations are perfomed at 25°C for 1 hr.

Fig. 18 Gelation activities of samples from each purification steps. Reaction mixture is similar to that described in "Materials and Methods", except that 0.1 ml of actin solution (3.52 mg/ml) and 0 to 20 μl of samples dialyzed against 20 mM imidazole-HCl (pH 7.2) were added. The total volume of the reaction mixture was made up to 0.2 ml with 20 mM imidazole-HCl (pH 7.2). Gelation was performed at 25°C for 1 hr in the presence of 0.5 mM EGTA (solid line) or of 0.05 mM Ca^{2+} (dashed line). (●),(○), Sepharose 6B fraction; (▲), (△), hydroxylapatite fraction; (■), (□), DE32 fraction; (X), 2nd ammonium sulfate fraction.

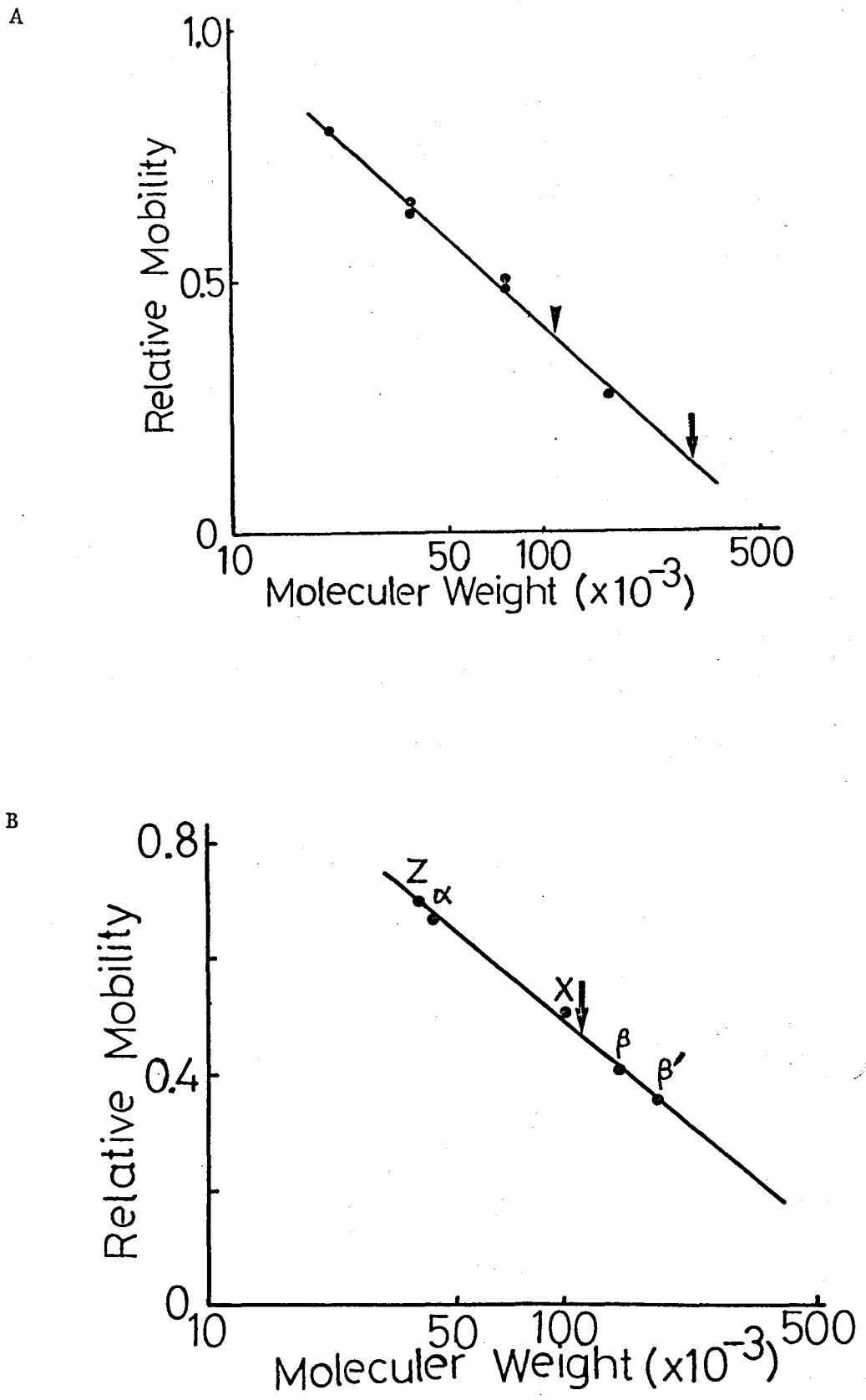
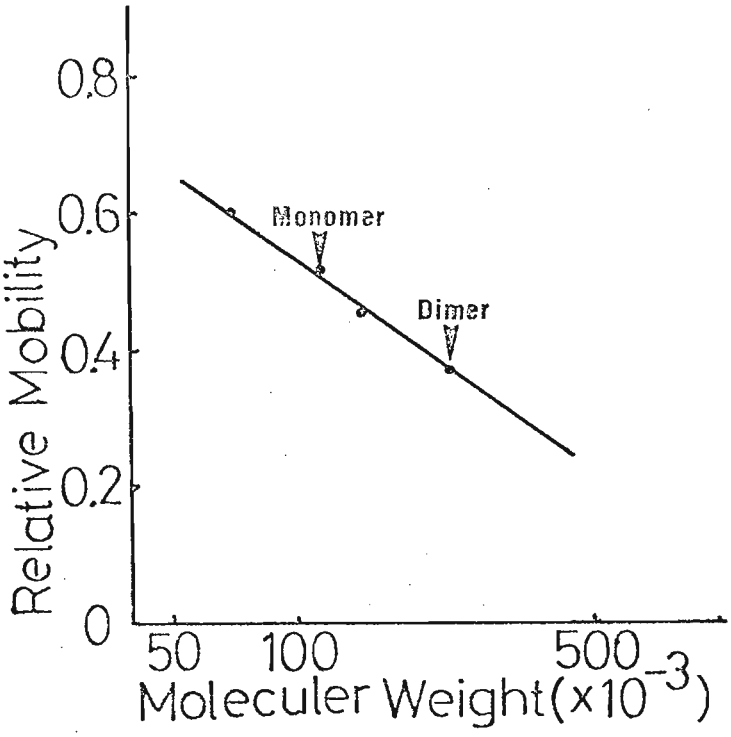


Fig. 19 Calibration of subunit molecular weight of actinogelin.

a



b

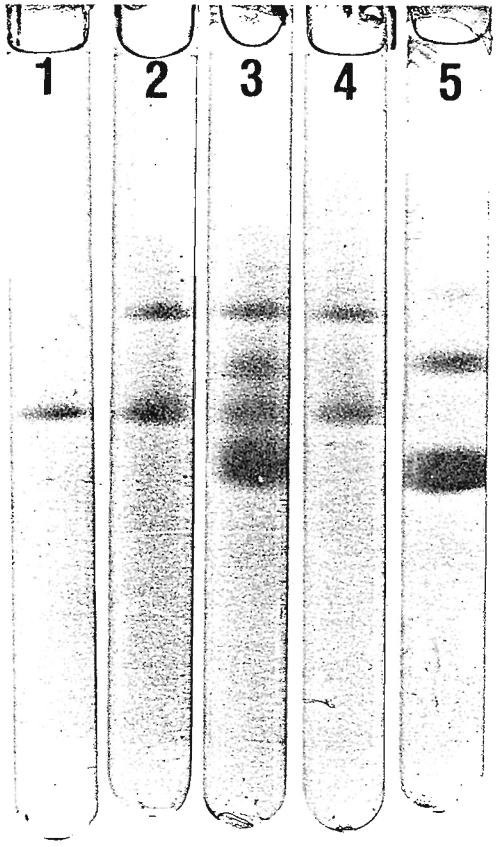


Fig. 20. Chemical cross-linking of actinogelin.

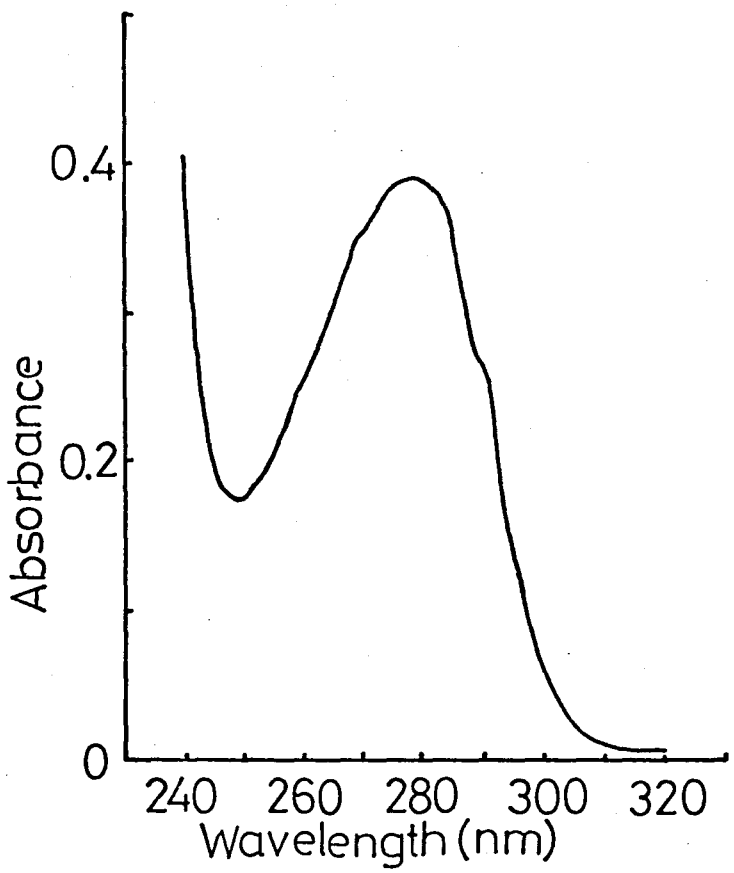


Fig. 21. Ultraviolet absorption spectrum.

Fig. 19 Calibration of subunit molecular weight of actinogelin. Electrophoresis was performed as described in "Materials and Methods". Standard marker proteins used were as follows. A: β and β' chains of RNA polymerase (E. coli), 160,000; bovine serum albumin, 68,000; α chain of RNA polymerase (E. coli), 39,000; soybean trypsin inhibitor, 21,500. An arrowhead corresponds to actinogelin and an arrow corresponds to filamin. B: Chains of RNA polymerase B; β chain, 180,000; β' chain, 140,000; X chain, 100,000; α chain, 42,000; Z chain, 39,000. An arrow corresponds to actinogelin.

Fig. 20 Chemical cross-linking of actinogelin.
(a): Cross-linked haemocyanin purchased from Sigma was used for marker protein. Monomer is 70,000 dalton and dimer is 140.000 dalton. Arrowheads indicate cross-linked actinogelin, corresponding to monomer and presumed dimer. (b); column 2 and 4, 16 μg of cross-linked actinogelin was applied; column 3, 16 μg of actinogelin and 20 μg of cross-linked hemocyanin were applied together on the column; column 5, 20 μg of cross-linked was applied. The results indicate cross-linked actinogelin has molecular weight of 230,000 and suggests it is a dimer of 115,000 dalton protein. 4% Acrylamide gel was used.
(column 1, 10 μg of untreated actinogelin was used.)

Fig. 21 Ultraviolet absorption spectrum. Purified actinogelin was dissolved in 20 mM Tris-HCl (pH 7.6) containing 50 mM NaCl, 0.1 μg/ml pepstatin and 1 mM NaN_3 at a concentration of 0.45 mg/ml. The spectrum shown was obtained by the use of autobaseline against the medium at 25°C.

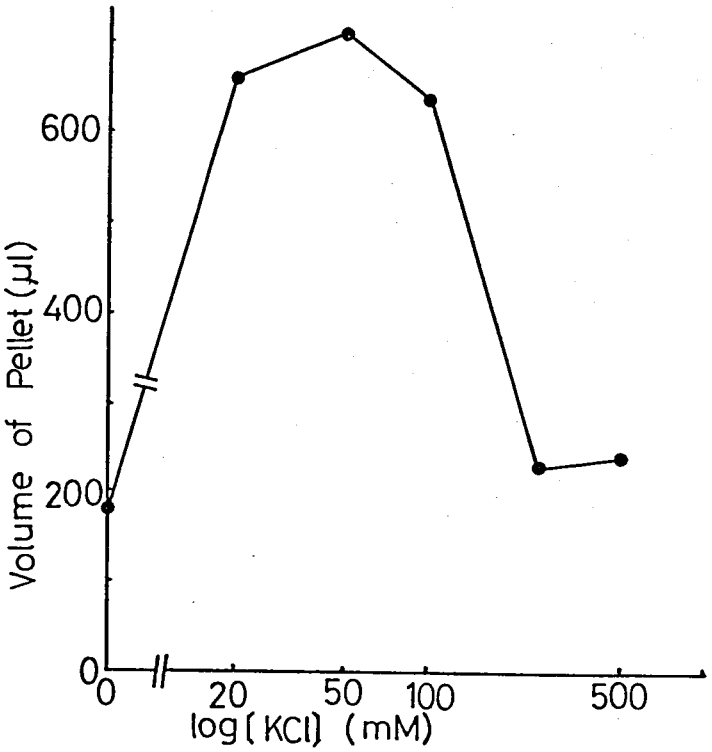


Fig. 22 Effect of KCl on actinogelin-dependent gelation of skeletal muscle actin

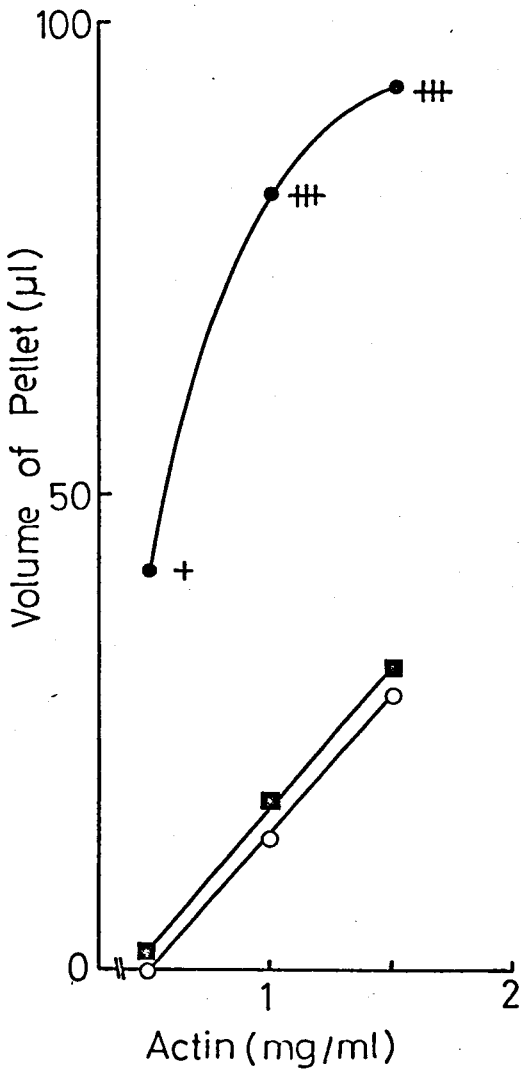


Fig. 23

Effect of concentrations of skeletal muscle actin on the extent of actinogelin-dependent gelation.

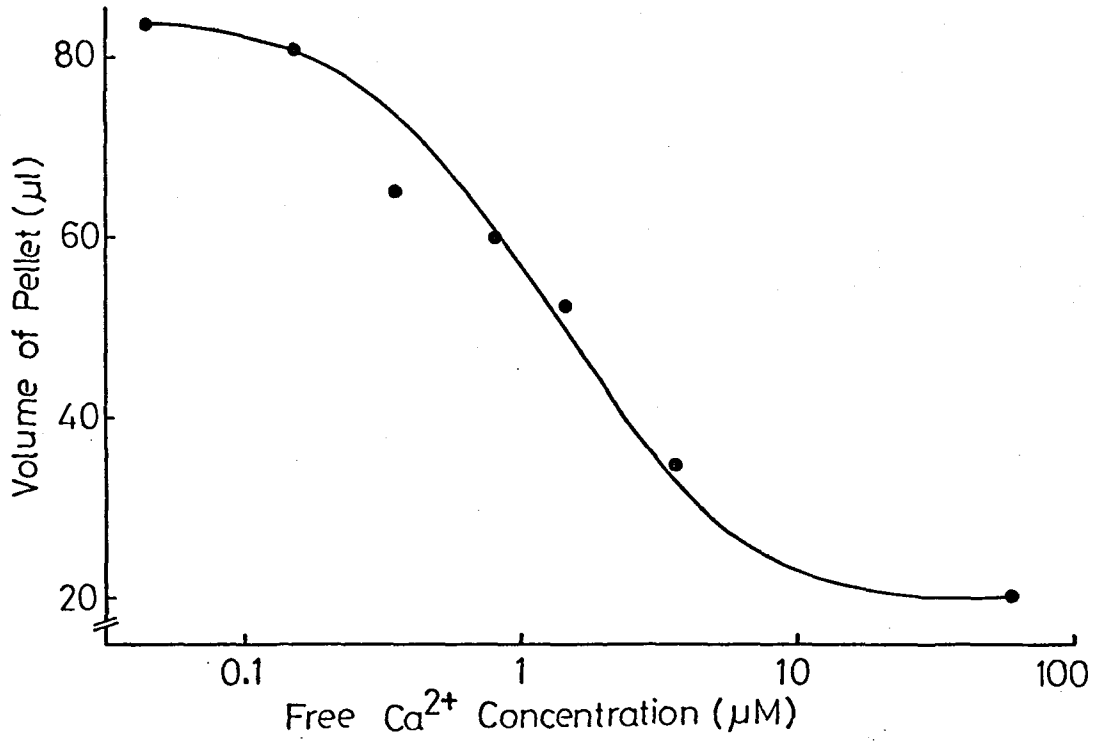


Fig. 24

Effect of free Ca^{2+} concentrations on extent of gelation of a reconstituted system consisted of skeletal muscle actin and purified actinogelin.

Fig. 22 Effect of KCl concentrations on actinogelin-dependent gelation of skeletal muscle actin. Reaction mixture contained 4 mM imidazole-HCl (pH 7.2), 0.5 mM $MgCl_2$, 0.5 mM EGTA, 0.07 mg/ml purified actinogelin, 2 mg/ml actin and indicated concentrations of KCl in total volume of 0.2 ml. Reaction was performed at 25°C for 1 hr.

Fig. 23 Dependence on actin concentrations of the extent of actinogelin-dependent gelation. The reaction mixture consisting of 20 mM imidazole-HCl (pH 7.2), 50 mM KCl, 1 mM $MgCl_2$, either 0.5 mM EGTA (●) or 0.05 mM $CaCl_2$(○), 0.07 mg/ml purified actinogelin and indicated concentrations of actin. Control experiments (without addition of actinogelin) (■) was performed in the presence of 0.5 mM EGTA.

Fig. 24 Effects of free Ca^{2+} concentrations on extract of gelation of a reconstituted system consisting of skeletal muscle actin and purified actinogelin. Reaction mixture contained 4 40 mM imidazole-HCl (pH 7.20), 50 mM KCl, 0.5 mM EGTA, 0.25 mM $MgCl_2$, 0.07 mg/ml actinogelin and 2 mg/ml actin-solution consisting of the same medium as described in "Materials and Methods". Various concentrations of Ca^{2+} was added to the reaction-mixture to manipulate free Ca^{2+} concentrations by EGTA-Ca^{2+} buffer system. Total volume of reaction mixture was 0.2 ml. The extent of gelation was evaluated by the volume of resultant pellet by centrifugation as described in "Materials and Methods". For stability constant of EGTA-Ca^{2+} complex at pH 7.2, 3.16×10^6 M^{-1} was used according to Ogawa (59)

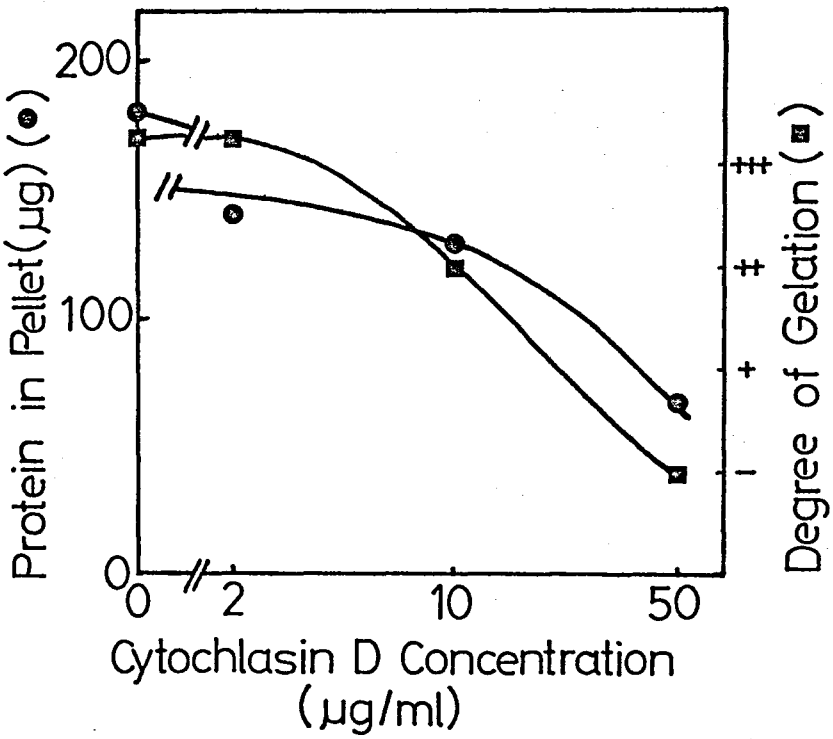


Fig. 25

Effect of cytochalasin D on actinogelin-dependent gelation of rabbit skeletal muscle actin.

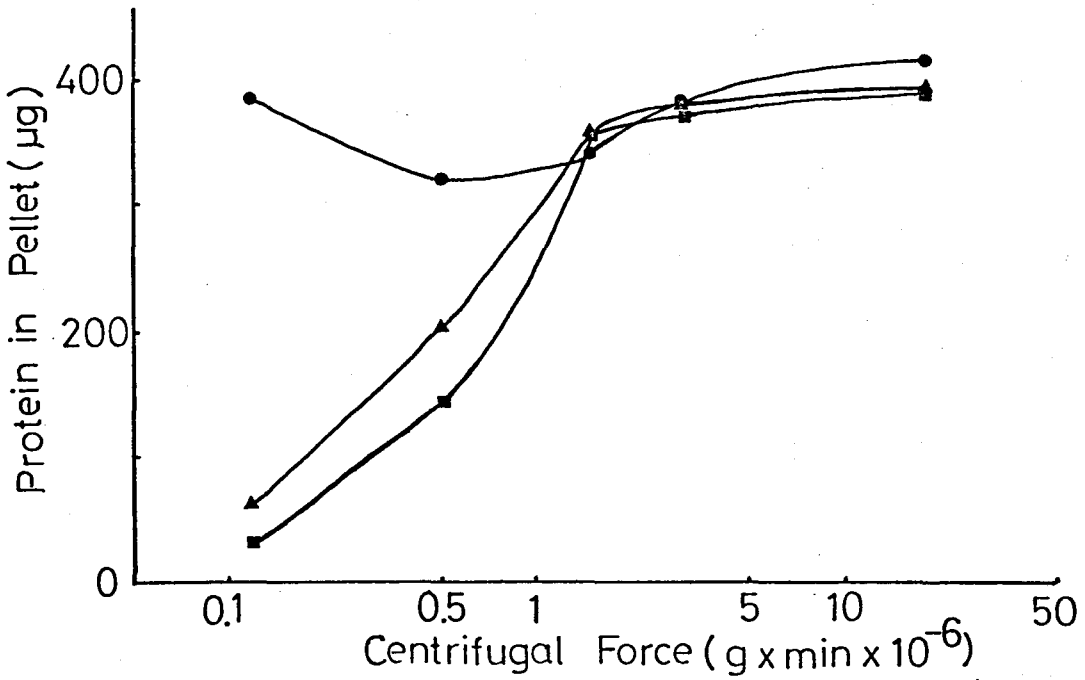


Fig. 26 | Sedimentation profile of F-actin and actin-actinogelin mixture in the presence and absence of Ca^{2+} at various g x min values.

Fig. 25 Effects of cytochalasin D on actinogelin-dependent gelation of rabbit skeletal muscle actin. Reaction mixture contains 1 mM $MgCl_2$, 50 mM KCl, 10 mM imidazole-HCl (pH 7.2), 0.5 mM EGTA, 0.75 mg/ml crude actinogelin (DE32 fraction) and 2.36 mg/ml actin solution consisting of the same medium as described in "Materials and Methods". Cytochalasin D dissolved in 10 µl of DMSO or 10 µl of DMSO were added to the reaction mixture to give final concentrations indicated. Total volume of reaction mixture is 0.2 ml. The extent of gelation was evaluated by the total protein of resultant pellet by centrifugation as described in "Materials and Methods".

Fig. 26 Sedimentation profile of F-actin and actin-actinogelin mixture in the presence or absence of Ca^{2+} at various g x min values. - Reaction mixture consisting of 21 µg-purified actinogelin, 370 µg-actin, 50 mM KCl, 1 mM $MgCl_2$, 10 mM imidazole-HCl (pH 7.2) and either 0.5 mM EGTA or 0.05 mM $CaCl_2$ as indicated in total volume of 0.2 ml. After incubation at 25°C for 1 hr, they were sedimented by centrifugation at various g x min values as shown in the figure. Total proteins of resultant pellets and supernates were determined as described in "Materials and Methods", except that washing of the pellet with 10 mM imidazole-HCl (pH 7.2) containing 0.1 M KCl was omitted; (●), total protein sedimented in the presence of EGTA; (▲), that of the mixture in the presence of Ca^{2+}; (■), that of control tubes without addition of actinogelin, i.e. F-actin alone in the presence of EGTA.

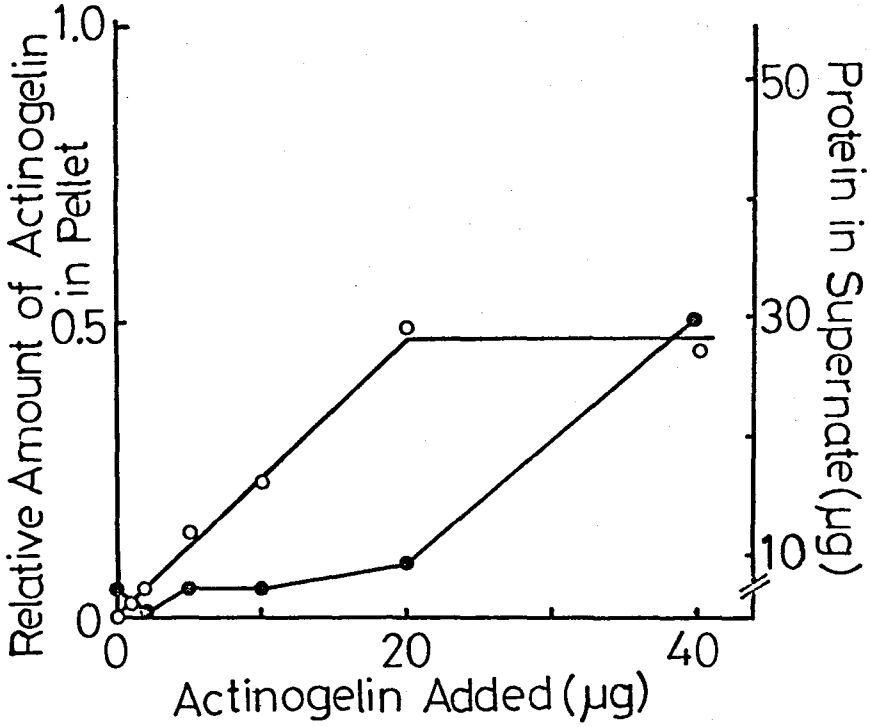


Fig. 27(A) Stoichiometric binding of actinogelin to actin.

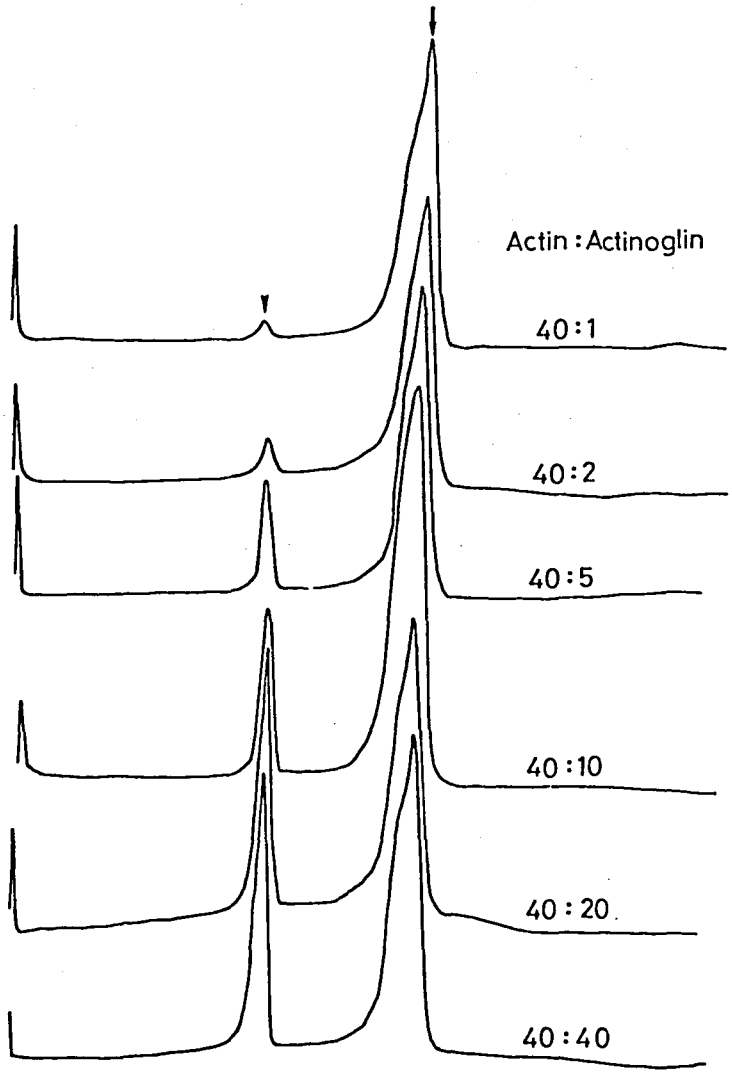


Fig. 27(B) Stoichiometric binding of actinogelin to actin.

Fig. 27 Stoichiometric binding of actinogelin to actin. Actinogelin solution containing 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 0.1 μg/ml pepstatin and 1 mM NaN_3, and G-actin solution containing 2 mM Tris-HCl (pH 8.0), 0.5 mM 2-mercapto-ethanol, 0.2 mM $CaCl_2$ and 0.2 mM ATP were centrifuged at 1,000 x g for 3 hrs to remove insoluble materials, if any. Resultant supernates were used for this experiment. Pretreated actin (40 μg protein) were mixed with indicated amounts of pretreated actinogelin in the medium containing 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA and 20 mM imidazole-HCl (pH 7.2) in total volume of 80 μl. After incubation at 30°C for 1 hr, the mixture were centrifuged at 100,000 x g for 3 hrs and resultant pellets were analyzed by SDS-polyacrylamide gel electrophoresis. The ratio of these two proteins in pellet fraction was calculated from measurement of corresponding peak area in the densitometric scan of electrophoretograms stained by Coomassie brilliant blue. A; (○), relative amount of actinogelin to actin in pellet, (●), protein in supernate (μg). B; from top to bottom, weight ratio of actinogelin to actin added in the reaction mixture was as follows; 1:40, 2:40, 5:40, 10:40, 20:40, 40:40. Electrophoretograms of pellet fraction were shown.

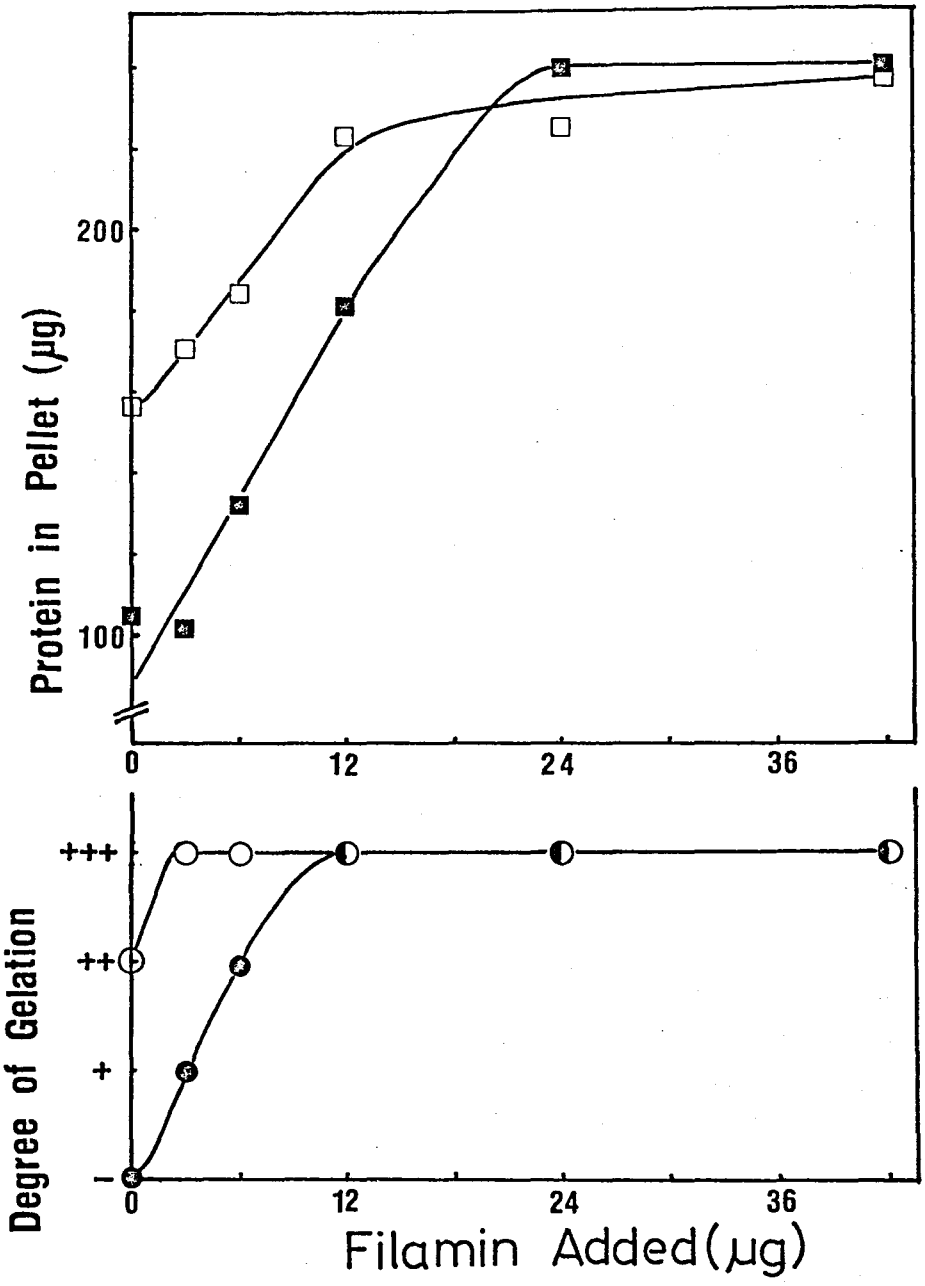


Fig. 28 Ca^{2+}-sensitivity of gelation reconstituted from skeletal muscle actin, actinogelin and filamin.

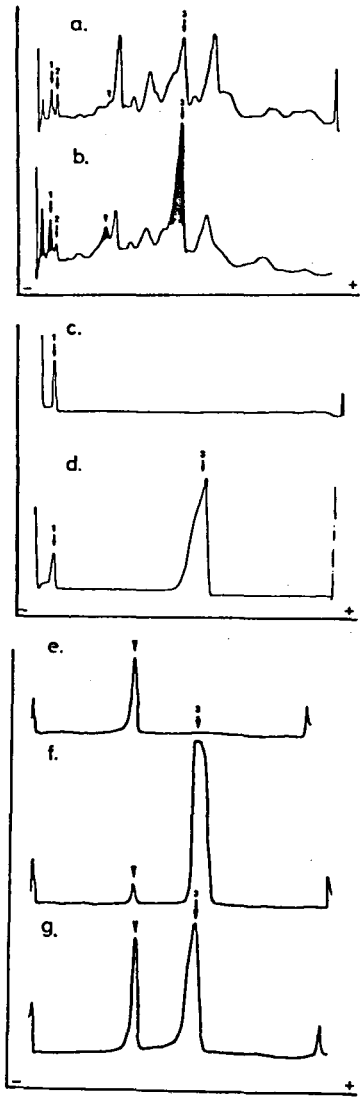


Fig. 29

SDS-polyacrylamide gel electrophoretograms of purified gelation factors, crude and reconstituted gels.

Fig. 28 Ca^{2+}-sensitivity of gelation reconstituted from skeletal muscle actin, actinogelin and filamin. Reaction conditions were similar to those described in "Materials and Methods", except that 400 μg of actin, 13.6 μg of actinogelin (hydroxylapatite fraction) and indicated amount of purified filamin was used in a total volume of 0.2 ml. (□),(○), in the presence of 0.5 mM EGTA and 65 μM $CaCl_2$. (■),(●), in the presence of 115 μM $CaCl_2$.

Fig. 29 SDS-polyacrylamide gel electrophoretograms of purified gelation factors and crude and reconstituted gels. (a) crude extract. (b) gel formed from crude extract. Protein bands which were concentrated in the gel compared to the extract were shadowed. (c) filamin purified from Ehrlich tumor cells. (d) gel formed by reconstitution of 340 μg-actin and 20 μg-filamin. (e) purified actinogelin (Sepharose 6B fraction), 10 μg. (f) gel formed by reconstitution of 352 μg actin and 21 μg of actinogelin. Amount of total protein applied to the gel column was 30 μg. (g) precipitate obtained from the mixture of 40 μg actin and 40 μg of pure actinogelin by centrifugation at 140,000 x g for 3 hrs. Amount of total protein applied to the gel column is 30 μg. Band 1, 2, 3 correspond to filamin, myosin hevy chain and actin, respectively. Actinogelin is marked by an arrowhead.

Table I Inhibition of gelation by Ca^{2+} and its reversal by EGTA

Total Ca^{2+}	Calculated free Ca^{2+}	Gelation Incubation time (min)					
		10	20	35	45	60	75
(mM)	(μM)						
0	0	++	+++	+++		+++	+++
0.5	0.78	+	++		++	++	
0.6	1.17	−	−	−	−	−	−
0.8	2.95→0.06	−*	+++	+++		+++	+++
0.8	2.95→0.06	−	−	−*		+++	+++

Cell-free extract was filtered through a Sephadex G-25 column equilibrated with the extraction medium to remove endogeneous Ca^{2+} and the eluate containing 7.6 mg of protein in 0.4 ml were used. Gelation was performed at *p*H 7.0. Degree of gelation and calculation of free Ca^{2+} concentrations were performed as described in the legend to Fig. 2. *EGTA in a volume of 20 μl was added to a final concentration of 10 mM (free Ca^{2+} concentration falling to 0.06 μM, as shown).

Table II Purification of actinogelin

Purification Steps	Total protein*	Total activity** (Recovery)		Specific activity***			
				+EGTA	$+Ca^{2+}$	EGTA-Ca^{2+}	(Relative purity)
2nd ammonium sulfate	630 mg	189 units	(100%)	0.4	0.1	0.3	(1.0)
DE32	77	215	(114)	4.1	1.3	2.8	(9.3)
hydroxylapatite	16	110	(58)	9.4	2.5	6.9	(23.0)
Sepharose 6B	6.2	57	(30)	13.3	4.1	9.2	(30.5)

* Purification was started from 5,700 mg protein of the cell extracts.

** One unit is defined as amount of actinogelin which precipitate 1 mg of protein (mostly actin) by centrifugation at 49,000 x g for 10 min. It was caluculated from linear phase of calibration curves (Fig. 18). Total activity was calculated from differnce in protein precipitation between 0.5 mM EGTA-containing samples and Ca^{2+}-supplemented (0.05 mM) samples.

*** Specific activity was expressed as units/ mg of protein.

Table III. Ca^{2+}-inhibition of Gelation of Prepolymerized Actin and Actinogelin

Addition to Polomerization Medium	No Further Addition	First Addition	Incubation after 1st addition (min)			Second Addition	Incubation after 2nd addition (min)	
	60 min Incubation		10	20	60		5	60
	Degree of Gelation							
EGTA	-	Actinogelin	++	+++	+++			
Ca^{2+}	-	Actinogelin	-	-	-			
Ca^{2+}	-	Actinogelin	-	-	-	EGTA	+++	+++
Ca^{2+}	-	Actinogelin	-	-	-	Water	-	-

G-actin was polymerized in a buffer containing 20 mM imidazole-HCl(pH 7.2), 50 mM KCl, 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 1 hr at 25°C, when indicated 10 µl of 20 mM EGTA(final concentration of 1 mM) was added. Actinogelin (20 µg) was added in 20 µl solution when indicated. For second addition, 10 ul of EGTA (final concentration of 1 mM) or water was added as indicated. All reactions were carried out at 25°C. Degree of gelation was expressed as described in "Materials ans Methods".

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