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The Gelsolin/Fragmin Family Protein Identified in the Higher Plant *Mimosa pudica*

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***Mimosa pudica* L. rapidly closes its leaves and bends its petioles downward when mechanically stimulated. It has been suggested that the actin cytoskeleton is involved in the bending motion since both cytochalasin B and phalloidin inhibit the motion. In order to clarify the mechanism by which the actin cytoskeleton functions in the motion, we attempted to find actin-modulating proteins in the *M. pudica* plant by DNase I-affinity column chromatography. The EGTA-eluate from the DNase I column contained proteins with apparent molecular masses of 90- and 42-kDa. The 42-kDa band consisted of two closely migrating components: the slower migrating component was actin while the faster migrating components was a distinct protein. The eluate showed an activity to sever actin filaments and to enhance the rate of polymerization of actin, both in a Ca^{2+}-dependent manner. Microsequencing of the faster migrating 42-kDa protein revealed its similarity to proteins in the gelsolin/fragmin family. Our results provide the first biochemical evidence for the presence in a higher plant of a gelsolin/fragmin family actin-modulating protein that severs actin filament in a Ca^{2+}-dependent manner.**

Key words: actin, actin-modulating protein, F-actin severing protein, gelsolin/fragmin, *Mimosa pudica*.

Mimosa pudica L. rapidly closes its leaves and bends its petioles in response to mechanical, electrical or thermal stimulation. The bending occurs at the pulvinus, which is a motor organ located at the base of the petiole or the leaf. The response to the stimuli of parenchyma cells of the pulvinus has mainly been studied in relation to membrane potential, changes in turgor, or changes in ion flux (*1–6*). It is thought that the sudden loss of turgor pressure in the ventral region of the pulvinus results in the rapid bending of petioles. Previous investigations have explained the pathway of the seismonastic movement as follow: the action potential, which is generated at the stimulated site, is transmitted to the parenchyma cells of the pulvinus through the xylem and the phloem. This action potential induces the transport of ions, such as K^+, Cl^-, and H^+, through the membranes of the parenchyma cells. The migration of these ions causes a change in the osmotic potential of the cells, which then causes a rapid change in turgor of these cells. The difference in the turgor pressure between the dorsal and ventral sides of the pulvinous results in the bending movement (*7, 8*).

Calcium ions are considered to play a role as a second messenger in various signaling processes, not only in animal cells but also in plant cells, *e.g.*, stomatal movements and circadian movements in nyctinastic plant. These movements are turgor-mediated, and induced by light and dark (*9, 10*). In the parenchyma cells of the pulvinus of *M. pudica*, Ca ions, which are contained in both the vacuole and the apoplast, are thought to migrate to the cytoplasm during the bending movement. Thus, Ca ions may also be involved in the bending motion of *Mimosa* leaves and petioles (*11*)

Actin is a fundamental element of the cytoskeleton of both muscle and nonmuscle cells, and plays a crucial role in cell morphology, motility, and cytokinesis. In plant cells, the actin cytoskeleton is also involved in various phenomena such as cell growth, mitosis and cytoplasmic streaming (*12–14*). Fleurat-Lessard *et al.* (*15*) have reported that cytochalasin B and phalloidin, which affect actin filaments, inhibit the bending movement of *M. pudica*. We have also confirmed that treatment with cytochalasin D arrests the bending movement of the *Mimosa* plant, while colchicine and propyzamide, both of which disrupt microtubules, have no effect (unpublished data). These results suggest that actin filaments are involved in the bending movement However, little is known about how the actin filaments are involved in this movement. Recently, we reported that the bending motion of the *Mimosa* plant is correlated with reduced tyrosine phosphorylation of actin in the puluvinus (*16*). Furthermore, phenylarsine oxide, a specific inhibitor of protein–tyrosine phosphatase, inhibits both the dephosphorylation of actin and the bending of the petiole. The

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Abbreviations: 2D-PAGE, two dimensional polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol, pI, isoelectric point; PVDF, polyvinilidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

decrease in the level of actin phosphorylation may alter the assembly properties of actin, and then regulate the organization of the actin cytoskeleton during bending.

Other factors that may regulate the actin cytoskeleton during the bending movement are actin-modulating proteins. It is possible that these proteins change the organization of the actin cytoskeleton in parenchyma cells of the pulvinus in response to intracellular signals, which leads to the bending movement. While various kinds of actin-modulating proteins have been identified in yeasts invertebrates, and vertebrates, only a few have been identified in higher plants. Thus, it would be valuable to find novel actin-modulating proteins in higher plants in order to reveal the pathway that regulates the actin cytoskeleton during the bending movement.

In this study, we searched for actin-modulating proteins in the *Mimosa* plant. We obtained a fraction containing an F-actin-severing activity by DNase I affinity column chromatography The main component of this fraction has an amino acid sequence homologous to gelsolin/fragmin family proteins.

MATERIALS AND METHODS

Plant Material—Mimosa pudica L was grown in a greenhouse. We used approximately 2-week-old plants to obtain tissue extracts. The plants were fully grown to be able to respond to various stimuli.

Affinity Chromatography with a DNase I Column—DNase I (Boehringer Mannheim, grade II, Federal Republic of Germany) was coupled to Affi-Gel 10 (Bio-Rad Labs., Richmond, CA, USA) according to the manufacturer's instructions.

Whole *M. pudica* plants were washed with de-ionized water and homogenized in 0.1 M Tris-HCl (pH 8.0), 0.4 M sorbitol, 32 mg/ml polyvinyl pyrrolidone, 0.5 mM $CaCl_2$, 50 mM NaF, 0.5 mM ATP, 5 mM 2-mercaptoethanol (2-ME), 10% (v/v) glycerol, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 0.05% (w/v) NaN_3 with a glass homogenizer The homogenate was centrifuged at 10,000 ×*g* for 40 min. The supernatant was filtered through a 0 45 μm membrane filter and then applied to a DNase I column pre-equilibrated with 0.1 M Tris-HCl (pH 8.0) and 0.5 mM $CaCl_2$ The column was then washed with 50 column volumes of 0.1 M Tris-HCl (pH 7.5), 0.5 mM $CaCl_2$, 0.1 mM ATP, and 10% glycerol. Bound proteins were eluted successively with 0.1 M Tris-HCl (pH 7.5), 5 mM EGTA, 0.1 mM ATP, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 0.05% NaN_3, and the same solution containing 3 M urea. Temperatures were kept at 4°C throughout the above procedures. After analysis by SDS-PAGE, the EGTA-eluate containing a 90-kDa protein and 42-kDa doublet-proteins was pooled and used for the following assays.

Preparation of Actin—Rabbit skeletal muscle G-actin was prepared by the method of Spudich and Watt (*17*), and further purified by gel filtration on a Sephadex G-100 column pre-equilibrated with 2 mM Tris-HCl (pH 8.0), 0.1 mM ATP, 0.1 mM $CaCl_2$, and 1 mM 2-ME. F-actin was obtained by the addition of 0.1 M KCl to the G-actin solution, followed by incubation for 3 h at 20°C.

Sedimentation Analysis of the EGTA-Fraction with F-Actin—The EGTA-eluate was concentrated with Centricon 10 (Amicon, Beverly, MA, USA) and diluted ten-fold with 5 mM Hepes-KOH (pH 7.2), 0 1 mM DTT, 50 μM $MgCl_2$, 0.1 mM ATP, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 0.05% NaN_3. This procedure was repeated two more times and the sample was finally concentrated to an appropriate protein concentration. The concentrated EGTA-eluate was mixed with F-actin (final concentrations: EGTA-eluate, 2 μg/ml; F-actin, 0.2 mg/ml) and incubated for 30 min at 20°C in the presence of 0.5 mM $CaCl_2$ or 0.5 mM EGTA. Finally the mixture was centrifuged for 2 h at 130,000 ×*g* and the resultant supernatant and pellet fractions were analyzed by SDS-PAGE. The gel was first stained with Coomassie Brilliant Blue and then digitally photographed using a CCD camera. NIH Image software (National Institutes of Health, Bethesda, MD) was used for both image capturing and quantitation of bands on the gel.

Electron Microscopy—The EGTA-eluate and F-actin (final concentrations, 10 μg/ml and 0.2 mg/ml, respectively) were mixed and incubated for 6 min at 20°C in the presence of 0.2 mM $CaCl_2$ or 2 mM EGTA. The sample was then mounted on a carbon-coated formvar grid, stained with 1.5% (w/v) uranyl acetate, and examined with a Carl Zeiss LEO 906 transmission electron microscope.

Assay of Actin Polymerization—The EGTA-eluate was dialyzed against 5 mM Hepes-KOH (pH 7.2), 0.1 mM DTT and 50 μM $MgCl_2$, concentrated with a Centricon 10, and mixed with G-actin (final concentrations, approximately 2 μg/ml and 0.2 mg/ml, respectively). The actin was then polymerized by adding KCl in the presence of 0 1 mM $CaCl_2$ or 0.1 mM EGTA. The polymerization process was monitored by measuring light scattering at 450 nm with a Hitachi F-4500 spectrofluorometer.

Electrophoreses, Immunoblotting, and Amino Acid Sequence—SDS-PAGE was carried out according to Laemmli (*18*), using 12.5% (w/v) polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue or a Silver Staining Kit (Wako Pure Chemical Industries, Osaka).

Two-dimensional gel electrophoresis (2D-PAGE) was carried out according to O'Farrell (*19*) as modified by Mikawa *et al.* (*20*). Isoelectric focusing was carried out at 100 V for 1 h, and subsequently at 600 V for 4 h in one dimensional gels containing 2% (v/v) Ampholine (pH 3.5–10:pH 5–7 = 1:4) (Pharmacia, Uppsala, Sweden). After SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and probed with anti-actin monoclonal antibody N350 (Amersham, Buckinghamshire, UK). The secondary antibody used was horseradish peroxidase–conjugated sheep anti–mouse IgG (Amersham). Signals to the secondary antibody were detected with an enhanced chemiluminescent detection system (ECL, Amersham)

For analysis of the partial amino acid sequence, the EGTA-eluate was subjected to two dimensional PAGE (2D-PAGE) and then transferred to a PVDF membrane. The membrane was stained with 0.1% Ponceau S and the protein spot was cut out. Proteins in the spot were digested with endoproteinase Lys C (Boehringer Mannheim), and the resultant peptides were purified by HPLC. Partial amino acid sequences of the peptides were obtained using a 473A gas-phase amino acid sequencer (Applied Biosystems, Norwalk, CT, USA).

Protein Concentration—Protein concentrations were determined by the method of Bradford (*21*) using BSA as a standard.

RESULTS

Partial Purification of Actin-Modulating Proteins by DNase I Affinity Column Chromatography—Actin-modulating proteins in *M. pudica* were analyzed by DNase I column chromatography. Since calcium ions have been thought to act as second messengers in response to stimuli in *M. pudica*, we searched for Ca^{2+}-dependent actin-modulating proteins. The *Mimosa* plant extract was loaded onto a DNase I column in the presence of Ca^{2+}. Proteins bound to DNase I were sequentially eluted with EGTA-containing solution and 3 M urea. Two closely migrating proteins of 42 kDa and one of 90 kDa were detected by SDS-PAGE analysis in the fractions eluted by the EGTA-containing solution (EGTA-eluate) The ratio of the two 42-kDa proteins was always approximately 1:1 as shown in Fig. 1B. The 90-kDa component was prone to degrade: it degraded during dialysis or concentration of the fraction (data not shown). The fraction eluted with 3 M urea (Urea-eluate) was composed mainly of a 42-kDa protein (Fig. 1).

Since the apparent masses of the two 42-kDa proteins in the EGTA-eluate and the 42-kDa protein in the Urea-eluate were close to each other, we analyzed these proteins by 2D-PAGE. In the EGTA-eluate, two 42-kDa spots with pI values of 5.6 and 5 8 were detected, which corresponded to the slower migrating 42-kDa protein and the faster migrating 42-kDa protein in the SDS-PAGE, respectively (Fig. 2a). In the Urea-eluate, two 42-kDa components with pI values of 5.6 and 5.4 were detected (Fig. 2b). The mixture of

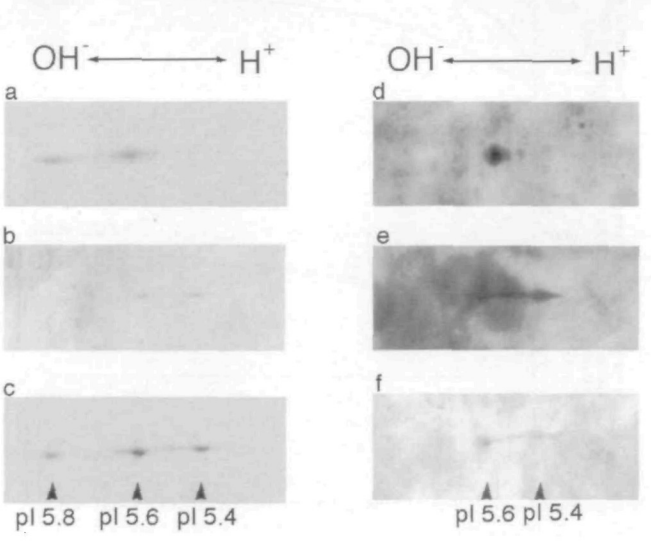

Fig 2 **Two-dimensional PAGE analysis of the EGTA-eluate (a, d), urea-eluate (b, e), and a mixture of these fractions (c, f).** The gels were stained with Coomassie Brilliant Blue (a–c) or immunoblotted with anti-actin antibody (d–f).

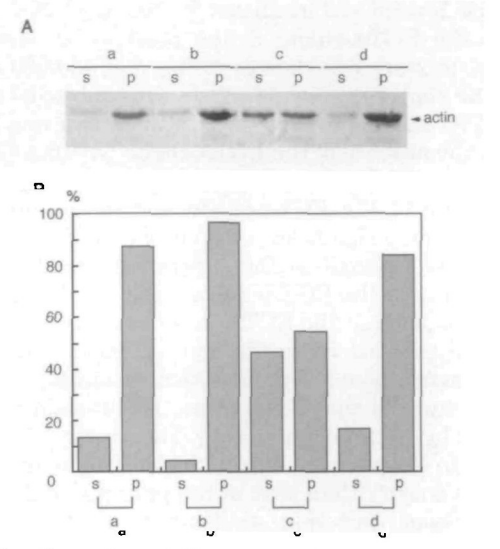

Fig 3 **F-actin sedimentation assay of the EGTA-eluate.** (A) A mixture of F-actin and the EGTA-eluate in the presence of 0 5 mM $CaCl_2$ or 0 5 mM EGTA was centrifuged at 130,000 ×*g* for 2 h The resultant supernatant (s) and pellet (p) were subjected to SDS-PAGE a, actin plus $CaCl_2$ b, actin plus EGTA. c, mixture of actin and the EGTA-eluate plus $CaCl_2$ d, mixture of actin and the EGTA *eluate* plus EGTA. (B) The amounts of actin in the supernatant and pellet, respectively, were estimated by NIH image software, and the percentages of the total actin in samples a, b, c, and d are shown as bars

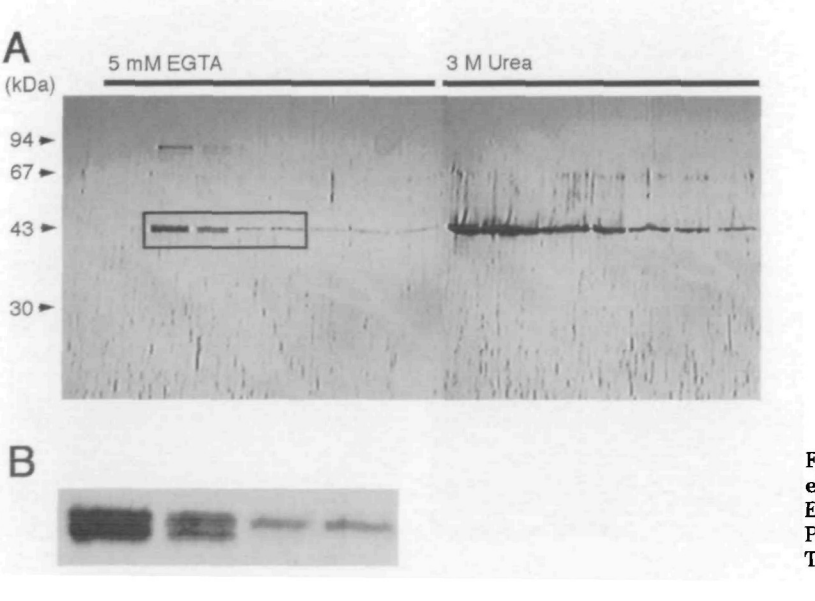

Fig 1 **SDS-PAGE analysis of the DNase I column eluates.** Proteins were eluted successively with 5 mM EGTA and 3 M urea, and then examined by 12 5% SDS-PAGE (A). The boxed region in (A) is enlarged in (B) The proteins were silver-stained.

the EGTA-eluate and the Urea-eluate was also analyzed by 2D-PAGE (Fig. 2c). The two pI 5.6 proteins co-migrated. The pI 5.4 protein in the EGTA-eluate and the pI 5.4 and 5.6 proteins in the Urea-eluate were identified as actin by immunoblotting using an anti-actin antibody (Fig. 2, d–f). These data show that the slower migrating band in the EGTA-eluate is actin, and the faster migrating band is a distinct protein.

Approximately 30 μg EGTA-eluate protein and 0.5 mg Urea-eluate protein were obtained from 100 g of whole *M. pudica* plants.

Interaction of the EGTA-Eluate with F-Actin—To examine the ability of the proteins in the EGTA-eluate to act on F-actin, we performed a sedimentation assay. Rabbit skeletal muscle F-actin was incubated for 30 min at 20°C with or without the EGTA-eluate in the presence or absence of Ca^{2+}. The mixture was then centrifuged at 130,000 ×*g* for 2 h, and the supernatants and pellets were subjected to SDS-PAGE (Fig. 3A). Approximately 90% of F-actin was precipitated in the absence of the EGTA-eluate whether Ca^{2+} was present or not. Likewise, 85% of F-actin was precipitated in the presence of the EGTA-eluate and in the absence of Ca^{2+}. However, a significant amount of actin (approximately 45%) was detected in the supernatant fraction in the presence of both the EGTA-eluate and Ca^{2+} (Fig 3B). This result indicates that the EGTA-eluate possesses an activity that depolymerizes or severs actin filaments to produce shorter fragments in a Ca^{2+}-dependent manner.

The mixture of the EGTA-eluate and F-actin was then examined by electron microscopy. The length of the actin filaments in the presence of both the EGTA-eluate and Ca^{2+} was much shorter than that in the presence of the EGTA-eluate without Ca^{2+} (Fig. 4). Thus, it is likely that the EGTA-eluate possesses an activity that severs actin filaments in a Ca^{2+}-dependent manner.

The Effect of the EGTA-Eluate on Actin Polymerization—To probe whether the EGTA-eluate affects actin polymerization or not, the actin polymerization process in the presence of the EGTA-eluate with or without Ca^{2+} was monitored by a light scattering method. In the presence of both the EGTA-eluate and Ca^{2+}, the duration of the lag phase in the actin polymerization process, which corresponds to the nucleation process, was markedly shortened, indicating that the nucleation of the actin polymerization process was accelerated (Fig. 5A). The activity of the EGTA-eluate was depressed in the absence of Ca^{2+}. These results suggest that the EGTA-eluate facilitates the process of actin polymerization by accelerating the nucleation process in the presence of Ca^{2+}

Microsequencing of the 42 kDa Peptide—We analyzed the partial amino acid sequences of fragments of the faster

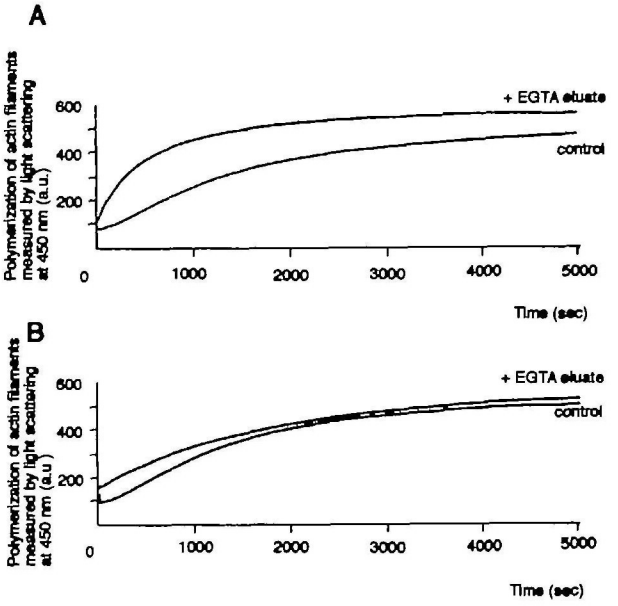

Fig 5. **Effect of the EGTA-eluate on actin polymerization in the presence of 0.2 mM $CaCl_2$ (A) or 0.2 mM EGTA (B).** The control curve shows the polymerization of actin alone with or without Ca^{2+}

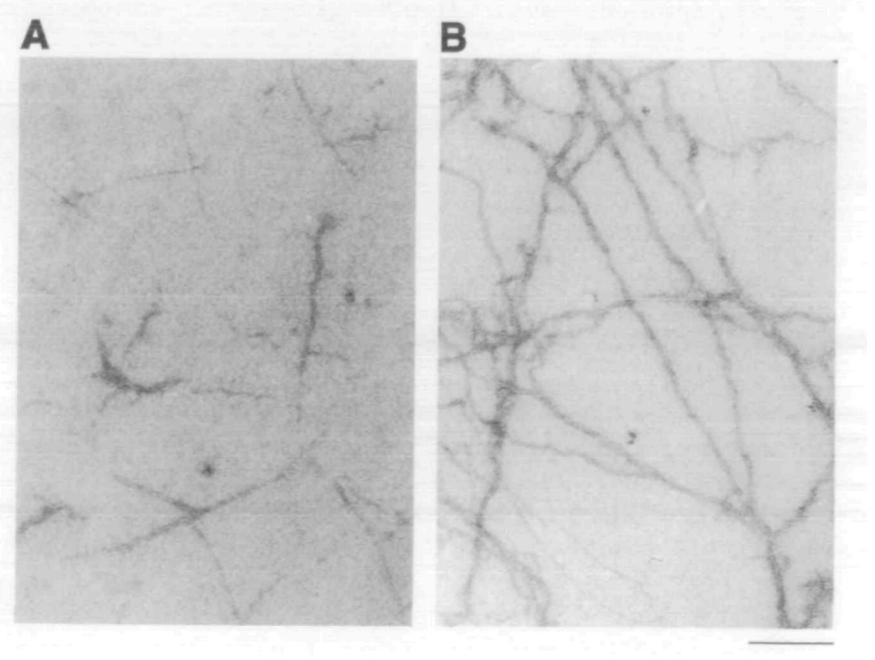

Fig. 4. **Electron micrographs of negatively stained F-actin.** The EGTA-eluate was added to an F-actin solution in the presence of 0 2 mM $CaCl_2$ (A) or 2 mM EGTA (B) The bar indicates 2 μm.

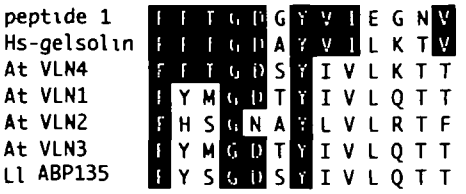

Fig 6 **Sequence similarity between gelsolin/fragmin family proteins and peptide 1 from the faster migrating 42-kDa component in the EGTA-eluate.** Accession numbers are as follows Hs-gelsolin (human), Swiss-Prot P06396, At-villin 4 (*Arabidopsis thaliana*), TrEMBL 065570; At-villin 1, TrEMBL 081643, At-villin 2, TrEMBL 081644, At-villin 3, TrEMBL 081645, Ll-villin ABP-135 (*Lilium longiflorum*), GenBank AF088901

migrating 42-kDa component in the EGTA-eluate, and obtained data for four internal peptides. One of these peptides (peptide 1) shows a high homology with members of the gelsolin/fragmin family of proteins (Fig. 6): it is quite similar to a region in domain S1 of mammalian gelsolin and *Arabidopsis thaliana* villin 4 (*22, 23*). The other three peptides showed no significant similarity to any other proteins. These results suggest that this 42-kDa component belongs to the gelsolin/fragmin family.

DISCUSSION

Gelsolin/fragmin family proteins have been identified from vertebrates, invertebrates, and both acellular and cellular slime molds. These proteins are able to sever actin filaments and cap the barbed end of the actin filament. Both activities lead to an enhancement of the rate of actin polymerization by increasing the number of filament ends and by stabilizing the nucleus for polymerization, respectively. Also, the activities are positively regulated by Ca^{2+} and antagonized by polyphosphoinositides *in vitro* (*24–26*). There are two groups of proteins within the gelsolin/fragmin family distinguished on their molecular structures: one group has a molecular weight around 80K and is considered to have evolved by gene duplication from the other group, which has a molecular weight around 40K. Gelsolin (*22, 26*), villin (*27, 28*), and adseverin (*29*) belong to the former group, while fragmin (*30*), severin (*31*) and the 45K actin filament-severing protein from sea urchin eggs (*32, 33*) belong to the latter group.

In plants, P-135-ABP, purified from lily (*Lilium longiflorum*) pollen tubes, has been identified as a villin-like protein on the basis of its primary structure (*34*). However, the ability of P-135-ABP to act on actin is different from that of mammalian villin. The mammalian villin bundles actin filaments at low Ca^{2+} concentrations, while it has actin-filament severing and capping activities at high Ca^{2+} concentrations (*35*). P-135-ABP, on the other hand, bundles actin filaments *in vitro* in both the presence and the absence of Ca^{2+} (*36, 37*), and this activity is inhibited by Ca^{2+}-calmodulin (*38*). Furthermore, no severing or capping activity has been demonstrated for isolated P-135-ABP even at high Ca^{2+} concentrations (*36*). This protein is considered to play a role in pollen tube growth. In addition, four villin isoforms (AtVLNs) have been identified in *Arabidopsis thaliana* using molecular biological techniques (*23*). The biochemical activity of AtVLNs toward actin filaments is not yet known.

The involvement of actin in the bending movement of *M. pudica* has been suggested by the fact that the movement is suppressed by treatment with cytochalasins B or D, or with phalloidin (*15*). In the present study, we found activities that sever actin filaments and accelerate the nucleation of actin polymerization, both in a Ca^{2+}-dependent manner, in the EGTA-eluate from the DNase I affinity column chromatography of *Mimosa* extract. We detected three proteins in this fraction. The immunoblotting test with a monoclonal antibody against actin revealed that the slower migrating 42-kDa protein in SDS-PAGE is actin. The 90-kDa protein was easily degraded so that this component was not detectable when the fraction was assayed for activities that act on actin. A partial amino acid sequence of the faster migrating 42-kDa protein showed similarity to gelsolin/fragmin family proteins These results strongly suggest that the 42-kDa protein belongs to the gelsolin/fragmin family of proteins which sever actin filaments in a Ca^{2+}-dependent manner.

The same ratio of the faster migrating 42-kDa proteins to actin was always detected in the EGTA-eluate. This suggests that these proteins may be eluated as a complex. It has been reported that the gelsolin/fragmin family proteins form a 1:2-complex with actin in the presence of Ca^{2+}, and one of the two actin molecules of the complex strongly binds this family protein so that the 1:1-complex cannot be dissociated even in the presence of EGTA (*39–41*). This complex no longer severs actin filaments, although it retains the barbed end-capping activity. The active protein in the EGTA-eluate shows a weak actin-nucleating activity in the absence of Ca^{2+}, suggesting that such a complex really exists in the eluate. However, the EGTA-eluate showed an F-actin–severing activity in the presence of Ca^{2+}. This might be due to part of the 1:1-complex having dissociated in the eluate.

Although many actin-modulating proteins have been identified in animal cells, only the G-actin-binding protein (profilin), the actin-depolymerizing protein (ADF), and actin-bundling proteins (fimbrin and villin) have been identified in plant tissues (*34, 42–44*). We showed for the first time the presence in plant cells of a protein that has F-actin-severing and nucleating activity. Since it has been suggested that actin filaments are involved in the bending movement of the *Mimosa* plant in response to various stimuli (*15*), and that the Ca ion concentration in the cytoplasm of parenchyma cells may increase during movement (*11*), the gelsolin/fragmin family proteins may play an important role in the bending movement. In addition, another movement of higher plant cells in which actin filaments may function has very recently been reported. The guard cells of the open stomata of *Commelina communis* have long cortical actin filaments. Treatment of the guard cells with Ca ions induces closure of the stomata and, concomitantly, the cortical actin filaments disappear. These phenomena are similar to those induced in these cells by abscisic acid. Conversely, the removal of extracellular Ca ions with EGTA inhibits the abscisic acid-induced change in actin organization (*45*). Thus, Ca^{2+} and the gelsolin/fragmin family proteins may also be involved in stomatal movement.

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