Purification of multiple functional leaf-actin isoforms from *Phaseolus vulgaris* L.

Claudia DÍAZ-CAMINO and Marco A. VILLANUEVA¹

Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, U.N.A.M., Apartado Postal 510–3, Cuernavaca, Morelos 62250, México

Plant actins show diversity in their gene sequences, protein isovariants and tissue distribution in eukaryotes. Besides general difficulties with the isolation of proteins from plant material (i.e. the presence of a cell wall and high proteolytic activity), the actin concentration in any vegetative plant tissue is much lower than in cytoplasmic animal tissues. In this study, we adapted a deoxyribonuclease I–Sephadex affinity purification scheme and we were able to enrich and isolate multiple functional plant actin isovariants from common bean leaves (*Phaseolus vulgaris*). Urea (4 M) elution proved that the DNase I column was able to bind at least eight actin isoforms with pI values ranging from 5.5 to 5.9, as observed by two-dimensional Western blots. Three of the

most acidic actin isoforms, with pI values of ≈ 5.6 – 5.7 , were eluted partially with 0.75 M urea. The purified actin was also able to bind leaf and rabbit muscle profilin, phalloidin and DNase I. Moreover, the protein could polymerize into filaments that contained the main isoforms eluted from the column. The average actin recovery using this procedure was ≈ 4 – $8 \mu\text{g}$ from 20 g of fresh tissue, of which at least 80% was able to form filaments. This is the first report of the purification of multiple plant-actin isoforms that are functional by the criteria of both binding to other ligands and polymerization.

Key words: DNase I–Sephadex, isovariants, phalloidin.

INTRODUCTION

Actin is a key cellular protein that is capable of self-assembly into dynamic filamentous structures. These microfilaments are fundamental for eukaryotic function and, due to their importance, are tightly regulated within these cells. The presence of cytoplasmic actin has been established in most major plant cell types [1] and it is now generally accepted that filamentous actin (F-actin) participates in cytoplasmic streaming, organelle and nuclear positioning, cellular morphogenesis and cell division. Reorganization of the actin cytoskeleton in eukaryotic cells is regulated at various levels and by a number of physiological parameters including salts, pH and a wide variety of actin-binding proteins (ABPs) that modulate the behaviour of both F-actin and globular actin (G-actin). In animal cells, different ABPs have been identified that have the ability to cross-link into tight bundles or loose networks, cap ends, and sever and even depolymerize filaments [2]. However, only a few ABPs have been identified in higher plants, and little is known about the regulation of actin dynamics in plant cells.

Like animal actins, plant actins display individual isovariants that may have particular functions and/or expression in certain cell types [3,4]. Plant actins are also encoded by multigene families and, although single plant species have more divergent actin genes than do individual animal species, the sites for actin interaction with itself and other regulatory proteins appear to be highly conserved among all eukaryotes [4,5].

Most physicochemical studies of the few plant ABPs so far described have used heterologous components and provide compelling evidence that plant ABPs are indeed functional [6–8]. However, minor variations in the sequences of the interactive sites of heterologous ligands may give rise to misinterpretation of the results. Thus further understanding of plant actin function *in vivo* and *in vitro* and its functional similarities with other eukaryotic microfilament systems will require a detailed analysis

of ABP–actin interactions with homologous components [9]. Hence, the importance of the availability of functional plant actin. Native actin can be purified from eukaryotic cells by various combinations of methods that include cycles of assembly/disassembly, anion exchange, gel filtration and DNase I or profilin affinity chromatography [5,10]. However, the concentration of actin in any given vegetative plant tissue is significantly lower than in animal cytoplasmic tissues and traditional methods of polymerization/depolymerization cycles have been unsuccessful. Therefore, studies reporting actin isolation from plants are limited [11] and most have used alternative methods based precisely on the assumption that conserved actin-binding sites exist on the molecule for interaction with other proteins. Some of these studies have included DNase I and profilin affinity chromatography [9,12,13], and most have been only partially successful. This is mostly due to the fact that very few have shown activity of the isolated actin and none have reported the number of isoforms obtained in the preparation. In this work we have used both actin-binding assays and phalloidin-induced polymerization to demonstrate that at least eight actin isoforms can be isolated from the *Phaseolus vulgaris* leaf. This purified actin was capable of interacting with *P. vulgaris* profilin, bovine DNase I and phalloidin, and also of polymerizing into microfilaments. These microfilaments contained all the actin isoforms from the common bean originally eluted from the DNase I column.

MATERIALS AND METHODS

Materials

DNase I from bovine pancreas was purchased from Calbiochem (San Diego, CA, U.S.A.) and rabbit skeletal-muscle actin was purchased from Cytoskeleton (Denver, CO, U.S.A.). EDTA-free protease-inhibitor-cocktail tablets (Complete®) and EGTA were

Abbreviations used: ABP, actin-binding protein; DTT, dithiothreitol; F-actin, filamentous actin; G-actin, globular actin; PLP, poly-L-proline; 2D, two-dimensional.

¹ To whom correspondence should be addressed (e-mail marco@ibt.unam.mx).

purchased from Boehringer Mannheim (Mannheim, Germany). Alexa-594 phalloidin was kindly given by Dr. Federico Sánchez (Instituto de Biotecnología, Cuernavaca, México) and was from Molecular Probes (Eugene, OR, U.S.A.). Bradford protein assay reagent was purchased from Bio-Rad (Hercules, CA, U.S.A.). All other chemicals were from Sigma (St Louis, MO, U.S.A.) or were of reagent grade.

DNase I and poly-L-proline (PLP) were conjugated to cyanogen bromide-activated Sepharose (CNBr-Sepharose) according to the methods of Schafer [14]. Briefly, DNase I or PLP were coupled to CNBr-Sepharose overnight at 4 °C with agitation on a rocking platform. The coupling buffer was 0.1 NaHCO₃, pH 8.5, 0.5 M NaCl. The suspension was packed into a column (10 ml) and washed with 10-ml aliquots of coupling buffer and wash buffer (0.1 M sodium acetate, pH 4.0, 0.5 M NaCl) five times sequentially, ending with a wash of 25 ml of TTBS (0.5 M NaCl, 20 mM Tris/HCl, pH 8.0, 0.05 % Tween-20). Finally, the column was equilibrated in buffer G (see under 'Solutions', below).

Antibodies

Anti-(calf thymus actin) polyclonal antibodies were kindly given by Dr. John L. Wang (Michigan State University, East Lansing, MI, U.S.A.) and have been shown to cross-react with plant actin [13,15]. Monoclonal anti-actin antibodies, N350, peroxidase-conjugated anti-mouse antibodies and substrates for enhanced chemiluminescence (ECL[®]) were purchased from Amersham (Arlington Heights, IL, U.S.A.) and horseradish peroxidase-conjugated anti-rabbit antibodies were purchased from Zymed (San Francisco, CA, U.S.A.).

Anti-DNase I antibodies were raised by immunizing male Balb/c mice with four intraperitoneal injections of 5 µg of bovine DNase I at 2–3-week intervals. The first injection was in complete Freund's adjuvant, and the remaining injections were in incomplete Freund's adjuvant. The resulting immune serum was tested for anti-DNase I antibody by Western immunoblotting assays. The serum with the highest titre was selected and stored in aliquots at –70 °C until used.

Solutions

Buffer A was modified from Ren et al. [9], and contained 2 mM Tris/HCl, pH 8.0, 0.2 mM CaCl₂, 0.2 mM Na₂ATP, 30 mM NaPP₁, 1 tablet of Complete[®]/100 ml, 0.25 mM dithiothreitol (DTT) and 0.005 % NaN₃. Buffer B was composed of 2 mM Tris, pH 8.0, 0.2 mM CaCl₂, 0.2 mM Na₂ATP, 0.25 mM DTT, 0.6 M NaCl and 0.005 % NaN₃. Buffer C included 2 mM Tris, pH 8.0, 2 mM EGTA, 0.2 mM Na₂ATP, 0.25 mM DTT and 0.005 % NaN₃. Buffer F contained 5 mM Tris, pH 8.0, 5 mM MgCl₂, 0.1 M KCl, 0.5 mM Na₂ATP, 0.25 mM DTT and 0.005 % NaN₃. Buffer G included 2 mM Tris, pH 8.0, 0.2 mM CaCl₂, 0.2 mM Na₂ATP, 0.25 mM DTT and 0.005 % NaN₃.

Actin purification from leaves of *P. vulgaris*

Leaves (21 days old) were collected from *P. vulgaris* and stored at –70 °C until homogenization. The leaf-actin purification method presented here was adapted from a combination of those reported by Maekawa et al. [16] and Ren et al. [9] as follows: a 20-g sample of frozen leaves was ground in a mortar with 200 ml of ice-cold buffer A for 15 min. All subsequent steps were

performed at 4 °C. The homogenate was then centrifuged at 20400 g for 15 min, and the resulting supernatant was applied to a DNase I-Sepharose affinity column (10 ml bed vol.) pre-equilibrated with buffer G. After the sample was loaded, the column was washed sequentially with 20 vols. of buffer G supplemented with a 0.01 % final concentration of Triton X-100, 20 vols. of buffer G, 3 vols. of buffer B and 3 vols. of buffer C. The column was washed with 1.5 bed vols. of 0.75 M urea in buffer G and elution was completed with 1.5 bed vols. of 4 M urea in buffer G. At this step, the eluant was allowed to drop into a beaker containing an excess of 250 ml of buffer G. The protein solution was then concentrated in an ultrafiltration cell (Amicon, Beverly, MA, U.S.A.) to 1 ml and washed with 50 vols. of buffer G for SDS/PAGE, two-dimensional (2D) SDS/PAGE, immunoblotting and binding assays, or with buffer F for induction of polymerization.

The protein concentrations from the fractions obtained were determined using the modified Bradford assay with IgG as a standard.

SDS/PAGE, 2D PAGE and immunoblotting

Samples were concentrated with 10 % (v/v) trichloroacetic acid, electrophoresed on 10 % polyacrylamide gels, analysed by SDS/PAGE according to Laemmli [17] and stained with Ag²⁺ (Bio-Rad silver-stain kit) or transferred on to nitrocellulose [18]. For analysis by 2D PAGE, the samples in Laemmli's sample buffer were diluted in a 1:1 ratio in O'Farrell's [19] lysis buffer (9.5 M urea, 5 % β-mercaptoethanol, 2 % Nonidet P-40, 1.6 % ampholytes, pH 4.5–6.5, and 0.4 % ampholytes, pH 3.5–10) [20]. The samples were then run by isoelectric focusing in the first dimension followed by SDS/PAGE in the second dimension according to O'Farrell [19] and transferred to nitrocellulose [18]. Actin was detected using the anti-(calf thymus actin) polyclonal or the N350 monoclonal antibodies as the primary antibodies and the corresponding horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgGs as the secondary antibodies. The antigen-antibody complex was detected by chemiluminescence using the ECL kit. In some cases, plant actin polymerized *in vitro* (see below) was analysed by 2D PAGE and immunoblotting with the anti-(calf thymus actin) polyclonal antibodies.

Binding assays

All subsequent procedures were performed at 4 °C unless noted otherwise. Leaf profilin was isolated according to the procedure of Ren et al. [9] with some modifications. The non-bound material passing through the DNase I-affinity column and containing leaf profilin was loaded on a PLP-Sepharose column (2 ml) pre-equilibrated with buffer G. The column was then washed sequentially with 20 bed vols. of 0.01 % Triton X-100 in buffer G, 20 vols. of buffer G alone, 3 bed vols. of 1 M KCl in buffer G, 3 vols. of 2 M urea in buffer G and 2 bed vols. of 4 M urea in buffer G followed by an extensive final wash with buffer G.

To demonstrate specific interaction with G-actin, PLP-Sepharose beads bound with leaf profilin resistant to the 4 M urea wash and DNase I-Sepharose beads were incubated with the purified plant-actin fraction or with rabbit skeletal-muscle actin for 12 h. Supernatants were removed and the beads were washed three times with buffer G. The material bound to the beads was released by boiling in Laemmli's sample buffer and analysed by SDS/PAGE and by immunoblotting assays.

Confocal microscopy of purified and plant actin polymerized *in vitro*

Purified leaf actin at 4–8 $\mu\text{g/ml}$ was polymerized in buffer F for 24 h at 4 °C and then incubated overnight with 0.13 μM Alexa-594 phalloidin (final concentration). Polymerized actin was collected by centrifugation at 150 000 *g* at 4 °C for 1 h. The supernatant and the pellet were examined using a Zeiss Axiovert 100 microscope with a Bio-Rad laser-scanning confocal attachment. The images were captured on a computer and printed on Kodacolor Gold 100 ASA film from either single optical sections or compositions of extended Z-series optical sections, added (and interpolated) together using software provided by the microscope manufacturer.

RESULTS

Purification of leaf actin from a DNase I-affinity column

Using a DNase I-affinity column, we adapted a strategy for actin enrichment and purification from plant tissues in which this protein is present at low concentrations. We wanted to test our procedure with leaf tissue from *P. vulgaris* because we have observed that it contains high proteolytic activity (results not shown), and this has been a difficult problem to overcome when actin isolation from plant tissue is attempted. This material is also readily available in our greenhouse. A total leaf homogenate was passed through the DNase I-Sepharose column and the proteins adsorbed on to the column were eluted as described in

Figure 1 Purification of leaf actin from a DNase I-affinity column

(a) Silver-stained gel of a representative actin purification experiment. Lane 1, fraction eluted with the high-ionic-strength buffer B; lane 2, fraction eluted with EGTA-containing buffer C; lane 3, fraction eluted with 0.75 M urea in buffer G; and lane 4, as lane 3 but with 4 M urea. Rabbit skeletal-muscle actin (lane 5) was included as a molecular-mass marker. (b) Immunoblot of an equivalent gel probed with anti-(calf thymus actin) polyclonal antibodies. The blot shows the presence of actin in lanes 3 and 4, which correspond to the fractions eluted with 0.75 and 4 M urea, respectively. The positive control of the immunoblotted 42-kDa polypeptide corresponding to rabbit skeletal-muscle actin (lane 5) is also shown.

Figure 2 Identification of several leaf-actin isoforms

The presence of leaf actin was analysed on a 2D-PAGE gel followed by Western immunoblotting using anti-(calf thymus actin) polyclonal antibodies. (a) Total leaf homogenate, (b) 0.75 M urea wash, (c) 4 M urea-eluted fractions and (d) an interpretative diagram that includes the spot shape, size and pI values of the corresponding isoforms. The arrows in (a) and (c) point towards the main isoforms (pI $\approx$ 5.8 and 5.9). The arrows in (b) indicate the positions of pI $\approx$ 5.6 and 5.7. The arrows in (d) indicate points of real pH values measured from an isoelectric focusing (IEF) gel run in parallel in the same experiment.

the Materials and methods section. The protein composition of the various fractions obtained from the column was analysed by SDS/PAGE (Figure 1a). The same fractions were analysed in parallel by Western immunoblotting with anti-(calf thymus actin) antibodies (Figure 1b). Many polypeptides were released from the column when a wash with a high ionic-strength solution (buffer B) was applied (Figure 1a, lane 1). The following wash with a buffer containing EGTA (buffer C) released most of the remaining proteins from the column, except actin (Figures 1a and 1b, lanes 2). The protein composition from this fraction was similar to the one obtained previously with buffer B. The major contaminating proteins in these fractions had molecular masses of 55, 50 and 40 kDa. The 55- and 50-kDa polypeptides have been shown to be, respectively, the large subunit of ribulose biphosphate carboxylase, and a putative proteolytic fragment derived from it, some of which, due to its high abundance, always remains in the column (C. Díaz-Camino, unpublished work). The other 40-kDa protein might be an ABP but we have failed to demonstrate this fact in many different assays, and it thus remains to be determined. A small amount of actin along with other protein contaminants were released from the column by the subsequent wash with 0.75 M urea (Figures 1a and 1b, lanes 3). When the column was eluted further with a solution containing 4 M urea, this wash resulted in the elution of most of the actin (Figures 1a and 1b, lanes 4), as seen in other procedures

Figure 3 Leaf profilin and bovine DNase I binding to purified leaf actin

The presence of actin, profilin or DNase I bound to the beads was assessed by Western immunoblotting with anti-(calf thymus actin) (Actin), anti-profilin (Profilin) or anti-DNase I (DNase I) antibodies. (a) Actin–profilin interactions. After passing the leaf homogenate to PLP–Sepharose beads in buffer G followed by immunostaining with anti-profilin antibodies, leaf profilin (lane 1) but not actin (lane 1), was detected bound to the beads. The profilin-bound beads were able to bind actin from leaf (lane 3) and rabbit actin (lane 5), as observed by Western immunoblotting with anti-(calf thymus actin) antibodies. (b) Actin–DNase I interactions. The presence of DNase I bound to the Sepharose beads (lanes 6, 8 and 10) was observed by Western immunoblotting with anti-DNase I antibodies. The DNase I beads were able to bind DNase I-affinity isolated actin from leaf (lane 10) and rabbit muscle actin (lane 8) also detected by Western immunoblotting with anti-(calf thymus actin) antibodies. Positive controls of rabbit muscle (lanes 2 and 7) and leaf actin (lanes 4 and 9) were run in parallel and detected with anti-(calf thymus actin) antibodies as well.

involving DNase I-affinity chromatography [10,12,13,16]. Purified leaf actin co-migrated with rabbit skeletal-muscle actin (Figures 1a and 1b, lanes 5) and had a molecular mass of 42 kDa based on molecular-mass markers. The yield was 4–8 μ g/20 g of fresh tissue, obtained from approximate measurements on scanned gels. Since we know that actin occurs as several isoforms in common bean embryos [15] and in the closely related species soya bean [21], we wanted to check the isoform composition of the purified leaf-actin fraction.

Leaf-actin isoforms

To analyse in more detail the characteristics of actin in the various fractions eluted from the column, we carried out a 2D-PAGE analysis of the total leaf homogenate, the 0.75 and 4 M urea-eluted fractions, followed by Western immunoblotting using polyclonal antibodies against calf thymus actin [13,15]. We observed the presence of two main isoforms in the leaf homogenate with a similar 'spot' shape corresponding to those found in the 4 M urea fraction (Figures 2a and 2c, arrows). These isoforms corresponded to pI values of 5.8 and 5.9. However, we could not obtain a well-resolved isoform pattern, even with the highest amount of protein that we could load in the gels from these crude extracts. Nevertheless, in the fraction eluted with 4 M urea we could detect at least eight isoforms present in leaf tissue, with pI values ranging from 5.5 to 5.9 (Figures 2c and 2d). Interestingly, the wash with 0.75 M urea resulted in a selective and partial elution of three of the most acidic isoforms of leaf actin (pI $\approx$ 5.5–5.7; Figure 2b). Similar results were obtained with the Amersham N350 monoclonal antibody (results not shown). These data showed for the first time that DNase I-affinity column chromatography was an appropriate procedure to purify all the actin isoforms present in leaves of *P. vulgaris* and furthermore that it helped isolate some of them selectively.

Binding of purified leaf actin to leaf profilin and bovine DNase I

The purified leaf actin was evaluated for its ability to interact with other proteins *in vitro*. Actin-binding proteins have been

Figure 4 Purified leaf actin retains its ability to polymerize into filaments

A 150 000- g pellet stained with Alexa-594 phalloidin was placed on microscope slides and viewed by confocal laser microscopy. (a) An optical section from a sample containing sedimented microfilaments. (b) An image from a similar sample but composed of seven optical sections in an extended Z-series that have been added and interpolated. Scale bar, 100 μ m.

shown to behave differently with heterologous sources of actin [4,22]. Since we had a homologous source of the protein we decided to test the interaction between profilin and G-actin from leaves of *P. vulgaris*. Incubation of rabbit and leaf actin on PLP–Sepharose beads that had bound leaf profilin previously showed that leaf actin (Figure 3a, lane 5) was as capable as rabbit skeletal muscle actin (Figure 3a, lane 3) of binding to leaf profilin. The presence of leaf profilin bound to the PLP–Sepharose column was confirmed by immunoblotting (Figure 3a, lanes 1, 3 and 5). Similar results were obtained when leaf actin or rabbit actin were incubated with a DNase I-affinity column (Figure 3b, lanes 8 and 10, respectively). The presence of DNase I covalently bound to the column was assessed by Western immunoblotting with anti-DNase I antibodies produced in our laboratory (Figure 3b, lanes 6, 8 and 10). These results showed that at least some of the purified leaf actin had the ability to renature in areas of the molecule responsible for the interaction with these ABPs in assays *in vitro*.

Microfilament assembly from DNase I affinity-purified leaf actin

DNase I affinity-purified leaf actin was placed in a polymerization buffer (buffer F) and, after a 24-h incubation period at 4 $^{\circ}$ C, and further incubation with Alexa-594 phalloidin for 12 h at 4 $^{\circ}$ C, its ability to polymerize was tested. After this period, putative polymers formed were sedimented at 150 000 g at 4 $^{\circ}$ C for 1.5 h and the pellet was resuspended in buffer F. Aliquots placed on microscope slides and analysed by confocal microscopy showed a highly enriched fluorescent microfilament network (Figure 4), whereas the supernatant did not show any fluorescent signal (results not shown). Figure 4(a) shows a representative optical section and Figure 4(b) shows the composition from an extended Z-series of seven optical sections that were integrated using software provided by the microscope manufacturer. The polymerized material, as observed from SDS/PAGE analysis of the pellet, was approx. 80% of the total fraction prior to polymerization.

The polymerized leaf actin analysed by 2D PAGE showed a 'spot' shape that appeared to include the main basic isoforms and a 'tail' with some of the acidic isoforms (Figure 5a). The measured pI values of this focused zone ranged from 5.5 to 5.9 (Figure 5b), which suggests that all of the originally isolated isoforms were present in the microfilaments. These results showed that most, if not all, of the isolated isoforms, and the two main

Figure 5 Isoform composition of actin microfilaments

The leaf-actin isoforms in the filaments from a 150 000-*g* pellet were analysed by 2D PAGE and detected by Western immunoblotting using anti-(calf thymus actin) polyclonal antibodies (a). An interpretative diagram (b), which includes the spot shape, size and pI values of the corresponding isoforms, is also shown. IEF, isoelectric focusing.

purified leaf-actin isoforms from bean, were functional and had retained their capacity to interact with phalloidin and polymerize into microfilaments.

DISCUSSION

Microfilaments are recognized as cytoskeletal and contractile elements found in virtually all types of cells. Indeed, actin, the major protein component of these filamentous structures, has been isolated from a variety of animal and non-animal cells [5]. The most widely used approaches for purification are those based on the method of Feuer developed in the late 1940s [5]. Actin can also be purified by directly exploiting its affinity for DNase I [10,23] or by indirectly exploiting its affinity for profilin [5,9]. DNase I forms a high-affinity 1:1 complex with monomeric actin [23] and, when it is covalently linked to a chromatographic support, is extremely practical for the isolation of actin from a variety of eukaryotic tissues [10,12,13,16]. The formation of the strong DNase I-actin complex allows a rapid, single-step isolation of actin from crude extracts that avoids a prolonged exposure to the proteases commonly present in plant tissues.

Although Garrels and Gibson [10] reported the isolation of all three isoforms of animal actin using a DNase I column, the reports on the isolation of plant actin using this approach have not documented the number of isoforms obtainable in this manner. In the only report where O'Farrell's [19] isoelectric focusing technique was used to analyse DNase I-purified plant actin from soya bean suspension cultures, only one isoform was observed [13]. Since actin occurs as several isoforms in soya bean plants and common bean seedlings [15,21], there are several possibilities that might explain this result: (i) the elution with 2 M urea used in that report was insufficient to elute all of the isoforms originally bound to the column; (ii) minor isoforms could not be detected with the particular method used; (iii) expression of only one isoform of actin occurs under conditions of a suspension culture. Although we cannot rule out this last possibility, some of our results support a combination of the first two hypotheses. First, 0.75 M urea could only elute a portion of the more acidic leaf-actin isoforms from the whole population

originally bound to the DNase I column (Figure 2b). Second, we detected only four isoforms of the isolated leaf actin from the 4 M urea elution when alkaline-phosphatase-conjugated secondary antibodies were used (results not shown); however, when enhanced chemiluminescence was used, we were able to observe at least eight distinct isoforms present on the same blotted membrane (Figure 2a). Ren et al. [9] developed a strategy for purification of functional maize pollen actin that involved the formation of a high-affinity profilin-actin complex bound to a PLP-Sepharose column. They detected a single band on a one-dimensional isoelectric focusing gel, which represented a major actin isovariant with a pI of 5.4, and several minor bands that they could not distinguish between being isoforms or degradation products. In this study, using a DNase I-affinity column, we were able to enrich and isolate, for the first time, all functional plant-actin isoforms originally present in *P. vulgaris* leaves confirmed by 2D PAGE and Western immunoblotting (Figures 2a and 2c), and we observed no degradation products in our 2D blots. The ability of DNase I to bind many actin isoforms as observed in this affinity procedure has several important implications. First, it permits a significant increase in the detection of the isovariant composition. Second, provided that common bean leaves contain high proteolytic activity when the compartmentalization is lost, even in the presence of protease inhibitors, this method also proves useful to minimize proteolysis by minimizing the time of exposure of the actin to the proteases in the total homogenate. Finally, the DNase I-binding site on actin appears to be highly conserved [10]. This is further supported by the fact that DNase I is able to bind to a wide range of actin isoforms ([5,10] and this study), and so this method could be useful for increasing the detection level of the isoform composition in total homogenates from other species.

Although plant-actin isovariants have similar pI values, they have a much wider pI range than animal-actin isovariants in a single species [4,10,21]. In homogenates from soya bean, several major actin isovariants and a few minor isovariants with pI values ranging from 5.1 to 5.8 have been identified [21]. Similar results were obtained when crude extracts from *P. vulgaris* hypocotyls [15], *Petunia* leaf, *Arabidopsis* leaf or maize leaf and root were analysed [21]. In this study, the bean-actin isoforms detected from the purified leaf-actin fraction fell within the reported range (pI 5.5–5.9).

It has been proposed previously that the diverse actin gene types present today in vascular angiosperms arose concomitantly with their numerous cell types, and that they have been conserved throughout evolution because they have unique patterns of gene regulation or encode proteins with unique functions [4,24]. There are at least 5–6 actin genes present in the *P. vulgaris* genome (E. Dantán and F. Sánchez, personal communication). This gene number is consistent with the four actin isoforms previously detected in common bean hypocotyls [15]; however, since we detected at least eight actin isoforms from leaf, it is possible that some of the isovariants from this tissue could have arisen via post-translational modifications. Such modifications on actin have been reported in other systems; for example, actin in *Dictyostelium discoideum* is tyrosine-phosphorylated by stress conditions [25]. In dormant spores, half of the actin is tyrosine-phosphorylated and this seems to be required for maintaining dormancy [26]. In addition to actin, profilin, which has been shown to have the dual function of promoting actin depolymerization [27] and the exchange of ADP-actin to ATP-actin to enhance polymerization [4], can also undergo post-translational modifications on tyrosine residues [28]. Thus post-translational modifications may add versatility to cytoskeletal components and their interactions.

With the method developed here we obtained a relatively low yield, but we also used a small-scale purification scheme (10 ml bed vol. column/run) that could be scaled up. Purified leaf actin was able to interact with leaf profilin, bovine DNase I and phalloidin (Figures 3 and 4). These data proved the functionality and capacity of DNase I affinity-purified leaf actin, to carry out protein–protein interactions and polymerize into filaments (Figure 4). The polymerized microfilaments were essentially identical to those obtained from rabbit muscle actin and stained with fluorescent phalloidin in previous reports [29].

In higher plants, profilins, actin-depolymerization factors and annexins p34 and p35 have been shown to function *in vitro* in a manner similar to their animal and fungal counterparts [6–8]. These reports have contributed significantly to our present understanding of actin interactions with other proteins in plants. Nevertheless, since most of these studies have used the rabbit skeletal muscle α -actin isoform and bacterially expressed plant ABPs [6,8,30], the availability of homologous actin would be an added advantage to the further study of these interactions.

This study was supported by grant IN-201696 from DGAPA-UNAM (Dirección General de Asuntos del Personal Académico-Universidad Nacional Autónoma de México). C.D.-C. was supported by a scholarship from DGAPA-UNAM. We thank Dr. Federico Sánchez for providing some of the reagents used in this study. The Microscopy Facility of the Instituto de Biotecnología and assistance of Xóchitl Alvarado, and the photographic work of Sergio Trujillo are also acknowledged.

REFERENCES

- Parthasarathy, M. V., Perdue, T. D., Witzmun, A. and Alvernaz, J. (1985) *Am. J. Bot.* **72**, 1318–1323
- Pollard, T. D. and Cooper, J. A. (1986) *Annu. Rev. Biochem.* **55**, 987–1035
- Meagher, R. B. and McLean, B. G. (1990) *Cell Motil. Cytoskeleton* **16**, 164–166
- Meagher, R. B., McKinney, E. C. and Kandasamy, M. K. (1999) *Plant Cell* **11**, 995–1005
- Sheterline, P., Clayton, J. and Sparrow, J. C. (1995) *Protein Profile* **2**, 1–103
- Ballweber, E., Giehl, K., Hannappel, E., Huff, T., Jockusch, B. M. and Mannherz, H. G. (1998) *FEBS Lett.* **425**, 251–255
- Calvert, C. M., Gant, S. J. and Bowles, D. J. (1996) *Plant Cell* **8**, 333–342
- Jiang, C.-J., Weeds, A. G., Khan, S. and Hussey, P. J. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9973–9978
- Ren, H., Gibbon, B. C., Ashworth, S. L., Sherman, D. M., Yuan, M. and Staiger, C. J. (1997) *Plant Cell* **9**, 1445–1457
- Garrels, J. I. and Gibson, W. (1976) *Cell* **9**, 793–805
- Meagher, R. B. and Williamson, R. E. (1994) in *Arabidopsis* (Meyerowitz, E. and Somerville, C., eds.), pp. 1049–1084, Cold Spring Harbor Press, Cold Spring Harbor
- Andersland, J. M. and Parthasarathy, M. V. (1993) *J. Cell Sci.* **104**, 1273–1279
- Villanueva, M. A., Ho, S.-C. and Wang, J. L. (1990) *Arch. Biochem. Biophys.* **277**, 35–41
- Schafer, D. A., Jennings, P. B. and Cooper, J. A. (1998) *Cell Motil. Cytoskeleton* **39**, 166–171
- Villanueva, M. A., Campos, F., Díaz, C., Colmenero-Flores, J. M., Dantán, E., Sánchez, F. and Covarrubias, A. A. (1999) *Planta* **207**, 582–589
- Maekawa, S., Nishida, E., Ohta, Y. and Sakai, H. (1984) *J. Biochem.* **95**, 377–385
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Towbin, J., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- O'Farrell, P. M. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Sinclair, J. and Rickwood, D. (1981) in *Gel Electrophoresis of Proteins* (Hames, B. D. and Rickwood, D., eds.), pp. 189–218, IRL Press, Washington DC
- McLean, B. G., Huang, S., McKinney, E. C. and Meagher, R. B. (1990) *Cell Motil. Cytoskeleton* **17**, 276–290
- Rubenstein, P. A. (1990) *BioEssays* **12**, 309–315
- Lazarides, E. and Lindberg, U. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4742–4746
- Meagher, R. B. (1991) *Int. Rev. Cytol.* **125**, 139–163
- Jungbluth, A., Eckerskon, C., Gerish, G., Lottpeich, F., Stocker, S. and Schweiger, A. (1995) *FEBS Lett.* **375**, 87–90
- Kishi, Y., Clements, C., Mahadeo, D. C., Cotter, D. A. and Sameshima, M. (1998) *J. Cell Sci.* **111**, 2923–2932
- Staiger, C. J., Yuang, M., Valenta, R., Shaw, P. J., Warn, R. M. and Lloyd, C. W. (1994) *Curr. Biol.* **4**, 215–219
- Guillén, G., Valdés-Lpez, V., Noguez, R., Olivares, J., Rodríguez-Zapata, L. C., Pérez, H., Vidali, L., Villanueva, M. A. and Sánchez, F. (1999) *Plant J.* **19**, 497–508
- Grabski, S., Arnoys, E., Busch, B. and Schindler, M. (1988) *Plant Physiol.* **116**, 279–290
- Giehl, K., Valenta, R., Rothkegel, M., Ronsiek, M., Mannherz, H.-G. and Jockusch, B. M. (1994) *Eur. J. Biochem.* **226**, 681–689

Received 14 May 1999/12 July 1999; accepted 11 August 1999