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The Isolation of Actin from Pea Roots by DNase I Affinity Chromatography[1]

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ABSTRACT

Native actin can be isolated from pea (*Pisum sativum* L.) roots by DNase I affinity chromatography, but the resulting yields and quality of actin are variable. By use of two assays for actin, a DNase I inhibition assay and a gel scanning assay, we identified several factors that increased actin yield. ATP is required for the actin in crude pea root extracts to bind to immobilized DNase I. Low amounts of ATP are hydrolyzed rapidly by an endogenous ATPase in the extract, and the actin then irreversibly loses the ability to bind to DNase I. High ATP concentrations (5–10 mM) or inhibition of the ATPase (with 10 mM pyrophosphate) are required for pea actin to retain DNase I binding ability. When adequate amounts of ATP are present, actin binding from the extract is further enhanced by basic pH, formamide, and soluble polyvinylpyrrolidone. Once actin is bound to the DNase I-agarose and washed free of extract, high ATP concentrations are not required to keep actin bound. Actin eluted from the DNase I-agarose with formamide retained its ability to polymerize into filaments with the addition of KCl and Mg^{2+}. The advantages and disadvantages of this procedure and its application to other plant materials are discussed.

Actin or actin-like proteins are a component of most, if not all eukaryotic cells (28). In vascular plants, actin gene families and gene transcripts have been identified (22), and the protein itself has been detected with rhodamine phalloidin (27) and with various anti-actin antibodies (21). However, procedures for the routine isolation of actin from plants have not been developed. Attempts have been made to apply animal actin isolation procedures to plant materials (19, 28), but have been hindered by the lack of convenient assays for actin in crude plant extracts. The most promising actin isolation method is DNase I affinity chromatography (15, 30), which exploits the tight, specific binding of G-actin onto DNase I covalently attached to a support matrix (e.g. cross-linked, beaded agarose). The procedure consists of incubating an extract with the DNase I agarose to allow the actin to bind, washing off proteins that do not bind, eluting the actin with a chaotropic agent, removing the chaotropic agent, and concentrating the actin. It was used in modified forms by Kursanov et al. (10) on cow parsnips and Villanueva et al. (29) on soybean protoplasts.

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We attempted to isolate actin from pea (*Pisum sativum* L.) roots with DNase I affinity chromatography, but our yields were variable and the purified actin was not always able to polymerize. To optimize this procedure, we found two assays, a DNase I inhibition assay (14) and a gel scanning assay (3), that could measure relative yields of actin in crude root homogenates and in DNase I-agarose eluates, respectively. These allowed us to identify factors that affect the yield of actin isolated from pea roots by DNase I affinity chromatography.

MATERIALS AND METHODS

Pea plants (*Pisum sativum* L. cv Progress #9, Agway, Syracuse, NY) were grown in vermiculite in growth chambers (24°C day, 18°C night, 12-h day). Roots were cut from 5- to 8-d-old seedlings (secondary roots less than 2 cm long), washed free of vermiculite, blotted dry on paper towels, and weighed. All other procedures involving pea extracts or proteins were performed at 4°C or on ice, unless otherwise specified.

Stock solutions of Grade II Na_2ATP (Sigma Chemical Co.), EDTA, and EGTA were adjusted to pH 7 to 7.5 with NaOH. Na_2ATP concentrations were adjusted using a millimolar extinction coefficient of $\epsilon_{259} = 15.4\ mM^{-1}\ cm^{-1}$. A vanadate stock solution, prepared from V_2O_5 as described by Gallagher and Leonard (6), was a gift of Margherita De Biasi (Cornell University). Pyrophosphate stock solutions were prepared from the tetrasodium salt and adjusted to pH 8.0 with HCl. Formamide (non-ACS grade), soluble PVP (mol wt 40,000), polymerized calf thymus DNA (Type I), and prestained SDS mol wt standards (catalog #SDS-7B) were obtained from Sigma. DNase I, grade II, was from Boehringer (Indianapolis, IN). CNBr-activated Sepharose Cl-4B (Pharmacia, Piscataway, NJ) was prepared by the method of Kohn and Wilchek (9) and coupled to DNase I following the procedure of Lazarides and Lindberg (15). The DNase I-agarose was washed before each use with 40% (v/v) formamide in 25 mM Tris·HCl, pH 7.5, and 5 mM $CaCl_2$ for 20 min to remove traces of pancreatic or pea actin previously bound to the DNase I. The DNase I-agarose bound between 300 and 500 μg of muscle actin/mL, depending upon the batch and its age. DNase I-agarose was stored in 5 mM Tris·HCl, pH 7.5, 0.5 mM $CaCl_2$, and 0.02% NaN_3.

Muscle actin for controls and standards was isolated from rabbit back muscle by the method of Pardee and Spudich (25). The concentration of muscle G-actin and DNase I solutions were determined from their absorbances using extinc-

tion coefficients of $\epsilon_{290\ nm}$ = 0.63 mL mg^{-1} cm^{-1} and $\epsilon_{280\ nm}$ =1.11 mL mg^{-1} cm^{-1}, respectively. Other protein concentrations were determined from TCA/desoxycholate precipitated samples by the method of Lowry et al. (18) as modified by Larson et al. (12), using BSA as a standard. With this assay, a 1 mg mL^{-1} BSA solution gave the same absorbance as an equal volume of 1 mg mL^{-1} muscle G-actin.

DNase I Inhibition Assay

Pea roots were cut into small pieces with scissors and submerged in 3.2 mL g^{-1} fresh weight of solution containing 5 mM Tris·HCl, pH 8, 0.5 mM $CaCl_2$, and 0.5 mM 2-mercaptoethanol. Roots were homogenized with a Polytron (Brinkmann, Westbury, NY) for 6 to 10 s at 70% power. The pH of the homogenate was immediately adjusted to pH 8 with 2 M Tris base (about 50 μmol g^{-1} fresh weight), and the homogenate was filtered through three layers of Miracloth (Calbiochem). The filtrate was rapidly aliquoted to tubes of test solutions, usually 4 mL filtrate to 1 mL test solution. DNase I inhibitory activity in extracts was measured by a modification of the method of Laub et al. (14). In this assay, 25 μL of pea extract were pipetted into a microfuge tube containing 12 μL of 0.09 mg mL^{-1} DNase I (in 50 mM Tris·HCl, pH 8, 0.1 mM $CaCl_2$, and 0.05 mM diisopropylfluorophosphate) and mixed with the pipet tip. Within 10 s, 0.6 mL of DNA solution (A_{260} = 0.3 in 25 mM Tris·HCl, pH 8, 0.2 mM $CaCl_2$, 2 mM MgCl, and 0.4 μg mL^{-1} ethidium bromide at 23°C) were pipetted into the tube. The contents were transferred into a fluorometer cuvette, the ethidium bromide was excited at 521 nm, and its emission was measured between 580 and 650 nm with an EG&G PARC model 1460 optical multichannel analyzer (Princeton, NJ). The fluorescence intensity was integrated over 0.5-s intervals every second for 30 s. The rate of DNA hydrolysis was taken to be the maximum rate of intensity loss over a 14-s period, as calculated by linear regression. Data points were discarded if the correlation coefficient for the calculated rate was less than 0.9.

Without extract, the rate of DNA hydrolysis was proportional to the amount of DNase I in the range of 0.3 to 1.1 μg. With extract containing 7.5 mM ATP, the percent inhibition of 1.1 μg of DNase I was proportional to the amount of extract in the range of 15 to 25 μL, with 25 μL inhibiting 50%. Approximately 0.6 μg of muscle G-actin inhibited 1 μg of the Boehringer DNase I.

DNase I Binding Assay—Determination of Relative Yields

Pea root homogenate was aliquoted to tubes of test solutions as described above, but without filtering through Miracloth. The tubes were centrifuged for 2 min at 24,000*g*. Ten minutes after homogenization, each supernatant was aliquoted to three 1.2-mL microtest tubes containing DNase I-agarose. The volume ratio of supernatant to DNase I-agarose was about 6:1, such that the agarose would always be much less than half saturated. As controls, two tubes were not loaded with supernatant, and two tubes received 0.6 mL of supernatant with 7.5 mM ATP plus 2 μL of 4 mg mL^{-1} G-actin. The tubes were capped, and the DNase I-agarose was suspended in the supernatant by shaking. The tubes were inverted every few seconds for 20 to 30 min and then centrifuged at 300*g* for 30 s to sediment the DNase I-agarose. The resulting supernatant was removed with the aid of an eight-channel, 1-mL repeating pipetter. The DNase I-agarose was washed twice with 0.2 M NH_4Cl in wash buffer (25 mM Tris, pH 8, 0.5 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol, and 1 mM Na·ATP), twice with 1 M KCl in wash buffer, and twice with wash buffer alone. DNase I-agarose was resuspended in the wash solutions and then centrifuged as above. After the final wash, buffer above the sedimented DNase I-agarose was carefully removed, and a volume of wash buffer with 80% (v/v) formamide equal to the DNase I-agarose volume was added to each tube and vortexed. The tubes were vortexed occasionally over 20 min and then centrifuged at 350*g* for 5 min. The supernatants of replicate tubes were combined and then prepared for and run on 1.5-mm thick discontinuous SDS polyacrylamide gels (11). Gels were stained with Coomassie blue (24) with extended staining times of 2 to 3 d. The total A_{595} of each spot was measured with a Gilford model 2410 gel scanner (Oberlin, OH). The absorbance was proportional to the amount of actin loaded for roughly 0.13 to 2 μg of isolated muscle actin.

Variation due to differences in staining between gels in a single experiment or in overall yield between identical experiments was reduced by the linear scaling method of Anderson et al. (3). Samples were loaded onto gels in three replicate sets, with each sample loaded once within a set, and each set on one gel. The total absorbance of all the bands in a set was scaled to 1 by dividing each absorbance by the sum of absorbances of its set. Corresponding scaled absorbances in the three sets were then averaged. Averages from independent experiments were averaged and the SES determined using n = the number of independent experiments. Because muscle actin standards were not run within each set, absolute yields could not be calculated. However, in each experiment, one test supernatant was prepared with 7.5 mM ATP in G-buffer without other additives. To facilitate comparisons between experiments, all averages and SES were normalized to this value.

The significance of differences between average yields for different treatments was judged by a modified t test as recommended by Anderson et al. (3).

Phosphate Assays

Rates of phosphate production in pea extracts with 6 mM ATP were determined with the phosphate assay of LeBel et al. (16) as modified by Larson and Jagendorf (13). Pea roots were homogenized as described above, filtered through Miracloth, and four parts filtrate added to 1 part 5× concentrated inhibitor solution in water. At 5-min intervals, aliquots of the extract with inhibitor were assayed. Rates were calculated by linear regression of points where phosphate production was not limited by ATP concentration.

Actin Isolation

Weighed pea roots were cut into small pieces and submerged in 4 mL g^{-1} fresh weight G-buffer containing 0.13 g mL^{-1} soluble PVP, 1 mM ATP, 10% (v/v) formamide, 10 mM

Na·PPi, and about 50 μmol g^{-1} fresh weight Tris base. The Tris base was added to give a pH of between 7.5 and 8.5 after homogenization. The ATP and formamide were added immediately before the roots were homogenized with a Polytron for 10 s at 70% power. The homogenate was filtered through several layers of Miracloth into an Erlenmeyer flask with 1 mL of DNase I·Sepharose for every 20 to 25 mL of pea extract. The Erlenmeyer was placed on a rotary shaker for 20 to 30 min, and then the mixture was poured into a 50-mL plastic filter funnel with three layers of Miracloth replacing a 25-mm diameter filter. The DNase I-agarose in the funnel was washed with 50-mL volumes of 0.2 M NH_4Cl in wash buffer, then 1 M KCl in wash buffer, and wash buffer alone. The moist DNase I-agarose was transferred to 2-mL microcentrifuge tubes, centrifuged briefly, and the supernatant was carefully removed. A volume of wash buffer containing 60% (v/v) formamide and 35% (v/v) glycerol equal to the volume of moist DNase I-agarose was mixed into each tube, giving a final formamide concentration of about 40%. After 10 min of incubation with occasional mixing, the tube was centrifuged, and the supernatant was removed and replaced with wash buffer containing 40% formamide and 20% glycerol for another 10-min incubation. Meanwhile, each mL of the first formamide supernatant was gel-filtered through 10 mL of Sephadex G-25 fine into Hepes-G buffer (5 mM Hepes·KOH, pH 7.5, with 0.5 mM each of $CaCl_2$, 2-mercaptoethanol, and ATP). The eluate containing excluded molecules was collected and concentrated in Centricon-30 tubes (Amicon, Danvers, MA). The DNase I-agarose was sedimented again, and the second formamide supernatant was gel-filtered like the first and added to the Centricon tubes.

To the G-actin concentrate and Centricon tube rinse solution (20 μL of Hepes·G buffer), glycerol and ATP were added to give final concentrations of 20% and 1 mM, respectively. Actin was polymerized by adding one-ninth volume of 0.5 M KCl and 50 mM $MgCl_2$ in Hepes·G buffer. The total time elapsed between homogenization and polymerization was often less than 2 h when working with small amounts of DNase I-agarose (e.g. 1 mL). Filaments were negatively stained and observed by transmission EM as described before (2). The proportion of the purified actin capable of polymerizing was judged by adding phalloidin to the actin with KCl and Mg^{2+}, and electrophoresing it on a native gel (basically a Laemmli gel [11]) without SDS [2]). After electrophoresis, the gel was stained with Coomassie blue (24). Any actin incapable of polymerization electrophoresed as a high-mobility monomer, whereas low-mobility, phalloidin-stabilized polymers remained in the stacking gel.

RESULTS

Pea Root Extracts Require ATP to Retain DNase I Inhibitory Activity

Pea root extracts prepared in G-buffer without ATP lost most of their ability to inhibit DNase I in 10 to 30 min (Fig. 1). Extracts with 5 to 10 mM ATP added immediately after homogenization retained nearly all of their initial DNase I inhibitory activity (Fig. 1). The ATP used here was neutralized with Tris and contained some phosphate, but neither Tris nor phosphate replaced ATP in preventing the loss of DNase I inhibitory activity (data not shown). The presence of from 2 to 10 mM $CaCl_2$ in the homogenate also had no effect on the loss of inhibitory activity (data not shown).

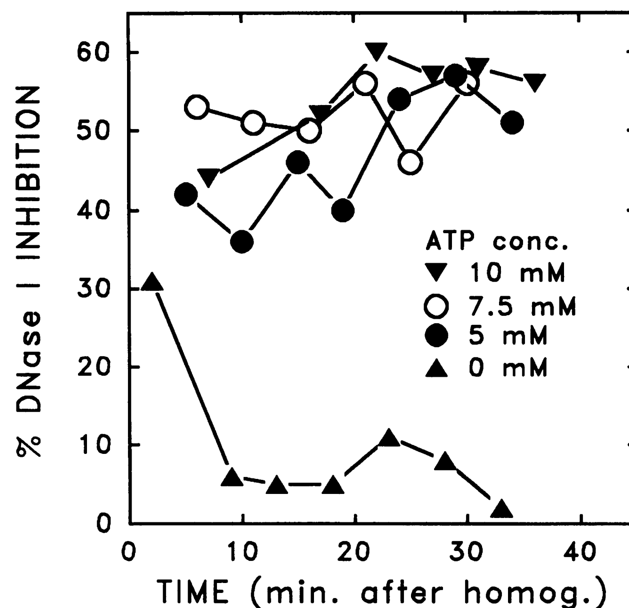

Figure 1. Inhibition of DNase I by pea extracts with different initial ATP concentrations. Pea roots were homogenized in G-buffer without ATP (as described in "Materials and Methods"), and ATP was added to give the concentrations shown. Thirty microliters of extract was added to 7 μL of 0.1 mg mL^{-1} DNase I.

When DNase I was mixed with the pea extract immediately after homogenization instead of just before each assay, activity was initially inhibited. Without ATP, this inhibition was lost within 30 min, whereas the inhibition was maintained if ATP was present (Fig. 2). Inhibitory activity was not fully restored by the addition of ATP 15 min after homogenization (Fig. 2).

Pyrophosphate Reduces the Loss of Inhibitory Activity at Low ATP Concentrations

The pH of pea root extracts dropped by more than 1 unit only when large amounts of ATP were present (data not shown), suggesting that a significant ATP hydrolysis activity was present. When ATP hydrolysis activity at 4°C was monitored by measuring the total phosphate concentration over time, extracts containing 6 mM ATP produced phosphate at a rate of about 0.6 μmol min^{-1} mL^{-1} homogenate. The total phosphate produced amounted to almost two phosphates for every ATP originally present. We attempted to inhibit this ATP hydrolysis activity by adding various potential inhibitors at concentrations known to inhibit some ATPases in plants. Several inhibitors had little effect: 0.2 mM Na vanadate/50 mM KCl, 10 mM $NaNO_3$, and 4 mM NH_4 molybdate all reduced the ATPase rate by less than 20%. But 10% formamide reduced the rate by almost 50%, and 20 mM PPi and 10 mM EDTA reduced the ATPase rate by 93 and 97%,

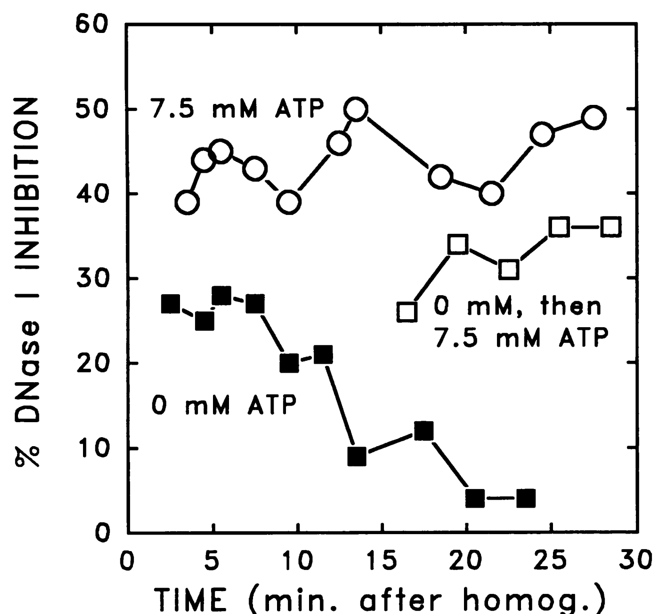

Figure 2. Restoration of DNase I inhibition by ATP. Pea roots were homogenized and immediately added to DNase I, and then ATP was added as shown. DNase I inhibition was assayed as described in "Materials and Methods," but without the addition of DNase I before each assay. Fifteen minutes after homogenization, concentrated ATP was added to part of the DNase I extract mix lacking ATP to give a concentration of 7.5 mM. The equivalent of 17 μL of extract and 13 μL of 0.09 mg mL^{-1} DNase I was added to the DNA solution for each assay.

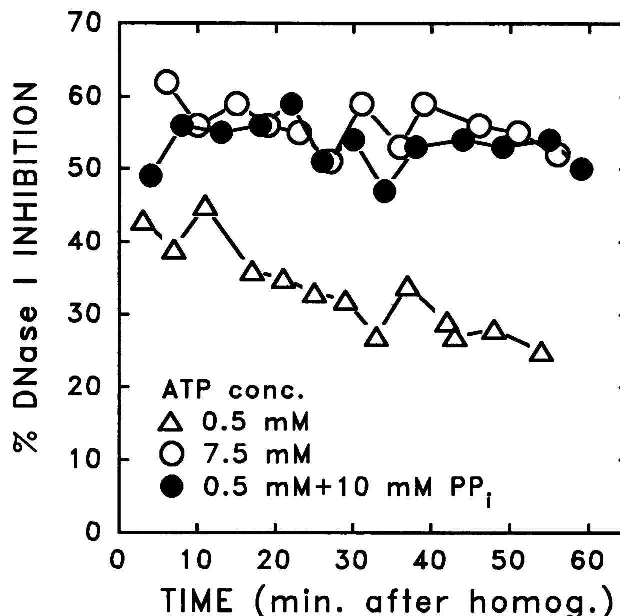

Figure 3. Inhibition of DNase I by extracts with PPi and 0.5 mM ATP. Pea roots were homogenized in G-buffer without ATP and aliquoted, and ATP and PPi were added to give the concentrations shown. Immediately before each assay, 18 μL of extract was added to 12 μL of 0.09 mg mL^{-1} DNase I.

respectively. The ATPase rate was measured in extracts with several concentrations of PPi, and 10 mM PPi, which inhibited 90% of the ATPase activity, was selected for further investigation.

When pea root extracts were prepared with 10 mM PPi and 0.5 mM ATP, they retained DNase I inhibitory activity to the same extent as extracts prepared with 7.5 mM ATP (Fig. 3).

Actin Requires ATP to Bind to Immobilized DNase I in Pea Extracts

More actin binds to DNase I-agarose from pea root extracts initially containing 5 mM or more ATP than from extracts with 2.5 mM or less ATP, as judged from the amounts eluted from DNase I-agarose (Fig. 4). Once actin loses the ability to bind to immobilized DNase I, the addition of ATP to the extract does not restore DNase I binding ability (Table I). The loss of DNase I binding ability was prevented by the addition of 10 mM PPi to pea extracts, even when no ATP was added to the extract (Fig. 4).

Basic pH, Formamide, and PVP Tend to Increase Actin Yield

The pH of the pea extract during incubation with DNase I-agarose had a slight effect on the amount of actin eluted. When the pH of the extract containing 7.5 mM ATP was maintained above pH 8, the amount of actin eluted was 25% greater than extracts with a pH of 6.5 to 7. This tendency

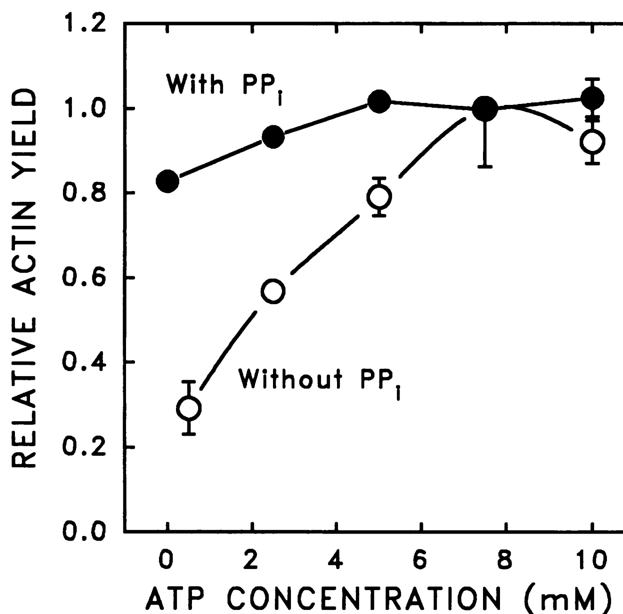

Figure 4. Relative amounts of actin eluted from DNase I-agarose incubated with pea extracts containing different amounts of ATP and PPi. Pea roots were homogenized in G-buffer without ATP and filtered, and then ATP and PPi were added to give the concentrations shown. The amounts of actin binding to DNase I-agarose were determined by elution, electrophoresis, and scanning of gels. Error bars are SES. Data points with and without 10 mM PPi are the average of two and three independent experiments, respectively. The yields with 0.5 and 2.5 mM ATP without PPi are less than all other yields at a significance level of $P < 0.05$.

Table I. *Restoration of the DNase I-Agarose Binding Ability of Actin by ATP*

A pea root extract was prepared as described in "Materials and Methods," but without ATP or PPi. ATP was added to aliquots of this extract at the times given below to make the homogenate 7.5 mM ATP. Aliquots with or without ATP were incubated with DNase I-agarose 2 to 32 min after homogenization (period 1) or from 30 to 60 min after homogenization (period 2). Amounts are averages of two independent experiments. The amount of actin eluted when ATP was added 30 min after homogenization is less than the amount when ATP was always present, at a significance level of $P < 0.05$.

ATP Additions	DNase I Incubation Period	Relative Amount of Actin Eluted (±SE)
ATP added immediately after homogenization	1	1.0 ± 0.04
	2	1.1 ± 0.10
ATP never added	1	0.53 ± 0.17
	2	0.08 ± 0.02
ATP added 30 min after homogenization	2	0.13 ± 0.01

remained when 10 mM PPi was present in the extract with 0.5 mM ATP.

The addition of 0.6 g g^{-1} fresh weight soluble PVP to pea extracts increased the amount of actin eluted from DNase I-agarose when 7.5 mM ATP was present and PPi was absent (Table II). Formamide increased the amount of actin eluted both in the presence and absence of PPi (Table II).

The Effect of Different Wash Buffers on Yield

Immobilized DNase I, washed free of pea extract, did not need high ATP concentrations to keep actin bound. Aliquots of DNase I-agarose were washed over 60 min in wash buffer containing 0, 1, or 7.5 mM ATP with or without 10 mM EDTA. Then the actin was eluted and the relative amounts determined by the DNase I-binding assay. In the absence of EDTA, the amount of actin retained at the three ATP concentrations was nearly identical. In the presence of EDTA, the amount of actin retained was reduced by half at 0 mM ATP and by one-quarter at 1 or 7.5 mM ATP relative to the amounts in the absence of EDTA.

Pea extract that has been filtered but not centrifuged contains considerable debris, including remains of organelles. A brief but thorough wash with G-buffer was adequate to remove almost all nonactin proteins from the DNase I-agarose. Further washes with 0.2 M NH_4Cl, 1 M KCl, or 20% formamide removed some actin and traces of other proteins, as judged by SDS-PAGE of the concentrated wash solutions. The amount of actin eluting into the wash solutions was greatest with formamide, least with NH_4Cl and KCl, and in all cases much less than the total amount of actin loaded.

Isolated Pea Root Actin Is Capable of Polymerization

Several groups, including our own, have previously shown that pea root actin isolated by DNase I affinity chromatography can polymerize into filaments (2, 10, 29). The presence of PPi in extracts did not affect the ability of the isolated actin to polymerize, as judged by negative staining and transmission EM. Filaments prepared with 1 mM ATP and 10 mM PPi in the extract were indistinguishable in both appearance and quantity from filaments prepared with 7.5 mM ATP and no PPi in the extract.

The proportion of the purified actin that retained the ability to polymerize was judged by stabilizing filaments with phalloidin and electrophoresing them on a native gel. Nearly all of the purified actin was able to polymerize in the presence of Mg^{2+}, KCl, and phalloidin, and was retained in the stacking gel as high mol wt polymers (Fig. 5). Phalloidin was not able to stabilize any filaments when added to solutions of isolated actin without $MgCl_2$ and KCl (Fig. 5).

DISCUSSION

The isolation of a protein by affinity chromatography consists of four basic steps: binding the protein from a crude solution to an immobilized ligand, washing the immobilized ligand-protein free of contaminants, eluting the pure protein, and separating the protein from the eluent. Here, we sought to determine what factors in the first two steps were important for the isolation of pea root actin using DNase I as a ligand.

The success of the first step, binding of actin to DNase I, is proportional to the concentration of native G-actin in the extract. The relative amounts of native G-actin in extracts can be judged by DNase I inhibition assays. The assay of Blikstad et al. (5), which measures DNA hydrolysis by the increase in absorbance at 260 nm, proved unsuitable with crude pea extracts both because of the extract's low actin concentration and high absorbance of phenolic compounds at 260 nm (17). The modified DNase I inhibition assay of Laub et al. (14) overcomes the absorbance problem by measuring DNA hydrolysis by the loss of ethidium bromide fluorescence at 602 nm, where pea root extracts do not absorb or

Table II. *Relative Yields of Actin from DNase I-Agarose Incubated with Pea Extract Containing PVP or Formamide*

Pea roots were homogenized in G-buffer without ATP, and then ATP, PPi, formamide, and PVP were added to give the concentrations shown. The amounts of actin eluting from DNase I-agarose were determined as described in "Materials and Methods." Data points are the average of the number of independent experiments shown in parentheses. Because experiments were not identical, for each experiment scaled yields were normalized to the yield with 7.5 mM ATP and without 10 mM PPi, and were then averaged. The yield with formamide plus PVP is different from the yield without either at a significance level of $P < 0.05$ (see "Materials and Methods").

Formamide/PVP Additions	Relative Amount of Actin Eluted (±SE)	
	With 7.5 mM ATP	With 7.5 mM ATP and 10 mM PPi
No formamide or PVP	Normalized to 1	1.10 ± 0.01 (3)
10% Formamide	1.35 (1)	1.26 (1)
0.6 g g^{-1} Fresh weight PVP	1.44 ± 0.05 (2)	1.13 ± 0.04 (3)
10% Formamide and 0.6 g g^{-1} Fresh weight PVP	n.d.[a]	1.37 ± 0.06 (3)

[a] n.d., Not determined.

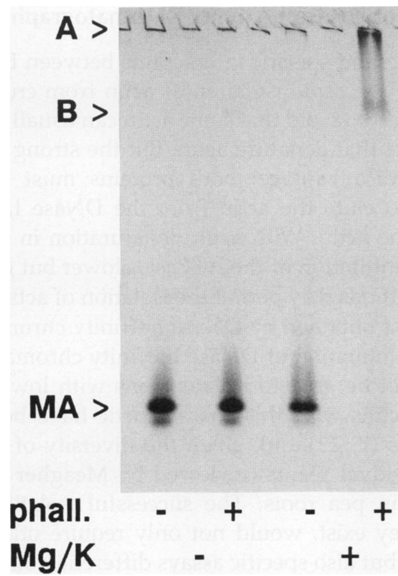

Figure 5. Native gel of purified pea root actin with and without phalloidin. Pea root actin was purified by the actin isolation procedure described in "Materials and Methods." The actin was diluted into Hepes·G buffer with (+) or without (−) KCl and $MgCl_2$ (Mg/K; final concentrations 50 and 5 mM, respectively), and with or without a final phalloidin (phall.) concentration of 190 μM. Arrows indicate the bottom of the sample well (A), the stacking/separating gel interface (B), and the mobility of monomeric rabbit muscle actin (MA).

fluoresce. This assay is rapid and simple, and suitable for detecting major differences in actin concentrations.

Pea root extracts were first prepared with the muscle actin depolymerizing buffer used by Zechel (30), containing low amounts of Ca^{2+} and ATP. These extracts rapidly lost their ability to inhibit DNase I. Muscle actin irreversibly loses DNase I inhibitory activity in the presence of nucleotidase (20) or in EDTA solutions lacking nucleotides (26), suggesting that Ca^{2+} and ATP concentrations in the pea extracts were insufficient. Additional Ca^{2+} in the extract did not prevent the loss of inhibitory activity. ATP was able to maintain inhibitory activity in the extract (Fig. 1) and to partially restore lost activity (Fig. 2), but only when present at high concentrations.

High ATP concentrations were needed not because of an intrinsic requirement of pea actin, but because the ATP was rapidly hydrolyzed. When the hydrolysis of ATP was inhibited with 10 mM PPi, low ATP concentrations were able to maintain actin inhibition of DNase I in the extract (Fig. 3).

Polzar et al. (26) found that even if muscle actin irreversibly lost its bound ATP and Ca^{2+}, it could remain attached to DNase I. This suggested that actin in pea extracts might remain bound to immobilized DNase I even after it had lost its ability to inhibit DNase I. We judged the amount of actin bound to DNase-agarose by eluting the actin, electrophoresing it on an SDS gel, and determining the relative absorbance of the stained actin band. Actin in pea extracts without ATP rapidly and irreversibly lost the ability to bind to DNase I-agarose (Fig. 4; Table I). Either high ATP concentrations or the inhibition of the ATPase were required to obtain relatively high binding (Fig. 4).

Basic pH, formamide, and soluble PVP also increased the amount of actin bound to immobilized DNase I from pea extracts. Although formamide partially inhibits the ATPase, it increased actin binding to DNase I in the presence of 7.5 mM ATP and 10 mM PPi (Table II), suggesting that it acted not by preventing ATP hydrolysis, but by some other mechanism. Formamide and basic pH may act by facilitating the depolymerization of F-actin in the extract. Zechel (30) found that 10% formamide helped to depolymerize HeLa cell actin filaments, and basic pH favors the depolymerization of muscle actin (31).

The significance of formamide and basic pH as depolymerizing agents in pea extracts is not known because the ratio of F- to G-actin in extracts is not known. We attempted to determine this by the method of Blikstad et al. (5), but failed because their recommended depolymerizing solution also substantially inhibited our DNase I. Laub et al. (14) used DNase I itself as a depolymerizing agent, incubating their samples with DNase I for 3 h. When pea extracts were incubated with DNase I for 30 min, inhibition did not increase substantially (Fig. 2), although an increase in G-actin from depolymerization may have been offset by G-actin denaturation.

PVP binds phenolic compounds and probably increased actin binding to DNase I-agarose (Table II) by preventing the phenolic compounds from reacting with and damaging actin (17). PVP does not bind phenolic compounds as well in alkaline solutions (17). Thus, the failure of PVP to increase binding in the presence of PPi may have been due to PPi's inhibition of the ATPase, preventing the pH of the extract from dropping to 7.

Several components of the homogenization buffer had no noticeable effect on actin binding in the presence of 7.5 mM ATP. A variety of serine protease inhibitors (diisopropyl fluorophosphate, PMSF, 4-(2-aminoethyl)-benzenesulfonyl-fluoride, HCl) did not increase binding (data not shown) and were not used in assays because of their toxicity or expense. Neither 5 mM EGTA nor 5 mM Ca^{2+} had any effect on actin binding to DNase I, whereas 5 mM EDTA reduced actin binding by one-third, suggesting that low amounts of Ca^{2+} or Mg^{2+} were sufficient for binding (data not shown).

The second basic step in affinity chromatography is washing the loaded affinity-agarose free of contaminants. We found that a quick, thorough wash with G-buffer was adequate to remove most nonspecifically bound materials, despite the debris in the pea extracts. ATP was not required in the wash buffer to keep actin bound to DNase I if divalent cations were present. Different wash solutions varied mainly in the extent that they washed off actin; the solutions with higher ionic strength washed off less, presumably because of the hydrophobic nature of the bond between the actin and DNase I (8).

The final steps in the DNase I affinity purification of actin are the elution of the actin from the DNase I-agarose, and the separation of the actin from the eluent. We did not specifically attempt to optimize these steps for maximum yield, but we did modify them for convenience. We found that pea actin seemed to elute from DNase I-agarose best

with 40% formamide, as Zechel (30) had found, but we used a batchwise elution procedure to minimize the total eluted volume. We raised the buffer concentration of the wash buffers and elution buffer from 2 to 25 mM to maintain better control over the pH, because total yields seemed to be higher if wash and elution pHs were basic rather than neutral (data not shown).

Once the actin was eluted, we subjected it to gel filtration with Sephadex into a low-salt buffer. Without the glycerol or formamide, the actin solution concentrated rapidly in Centricon tubes and no washing was required. The isolated actin polymerized into filaments after the addition of $MgCl_2$ and KCl, as observed with the electron microscope. Nearly all of the Mg^{2+}- and KCl-treated actin was capable of polymerization when phalloidin was added (Fig. 5).

Yields

The maximum inhibitions of DNase I presented in Figures 1 to 3 suggest that pea roots contain 10 to 20 μg actin mL^{-1} of extract. With total protein in the extract at about 1.5 mg mL^{-1}, actin might comprise around 1% of the extracted protein or 50 to 100 μg g^{-1} fresh weight in pea roots. In one case, where measured, the final yield of gel-filtered, concentrated G-actin was 14 μg g^{-1} fresh weight of roots. If the DNase I assay accurately predicts the amount of plant actin in extracts, the yield was about 15 to 30%. These rough estimates suggest that pea extracts do not contain much G-actin capable of inhibiting DNase I, and that considerable losses occur in the isolation procedure.

Losses could easily result from incomplete binding of actin in the extract to the DNase I-agarose, incomplete elution from the agarose, or loss during gel filtration or concentration. Modifications to the procedure might increase total yield, but might also increase the proportion of partially denatured actin. Further optimization of this actin isolation procedure will require both an assay for total actin and a sensitive assay for actin function.

Application to Other Tissues and Plants

We have used the DNase I affinity chromatography procedure of Zechel (30) to isolate actin from a diverse range of plants, but with limited results (1). The actin isolation method presented here may give better results with those plant materials, but may still need modification. For example, *Chamaecyparis* roots contain more phenolic compounds than pea roots—more than just PVP might be required to obtain reasonable yields of actin. Alternatively, tissues with more actin relative to total protein, and that are low in ATPases, phenolic compounds, and proteases, may produce good yields with Zechel's original procedure. An example of such a tissue is maize pollen, which contains large amounts of actin (4). Maize pollen that we have collected is relatively free of ATPases and phenolics and does not contain large acidic vacuoles. Actin in maize pollen amounts to about 5% of the total protein, as estimated by the DNase I inhibition assay, and gives correspondingly higher yields of purified actin (data not shown).

Limitations of DNase I Affinity Chromatography

The strong and specific interactions between DNase I and actin permit the rapid isolation of actin from crude extracts; the isolation is so rapid that some actin can usually be isolated from extracts that denature actin. But the strong interactions are also a disadvantage; both proteins must be partially denatured to elute the actin from the DNase I, potentially damaging the actin. With actin denaturation in pea extracts reduced by inhibition of the ATPase, slower but gentler actin isolation methods may permit the isolation of actin denatured less than that obtained by DNase I affinity chromatography.

A second limitation of DNase I affinity chromatography is that it cannot be used to isolate actins with low affinity for DNase I. Actins with this characteristic have been isolated from protists (7, 23) and, given the diversity of actin genes within individual plants (reviewed by Meagher [22]), could be present in pea roots. The successful isolation of these actins, if they exist, would not only require other isolation procedures, but also specific assays different from the DNase I inhibition assay used here.

Although DNase I affinity chromatography may not be the ideal method for isolating native actin from plants, it is quick, simple, reproducible, and currently the only method that can potentially be used on a variety of tissues with some success.

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