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Identification of stathmin-like proteins in plants

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Abstract – Stathmin-like proteins were identified in broad bean, green bean, pea, soybean, and cowpea extracts by western blotting after SDS PAGE and native isoelectric focusing (IEF). The subunit molecular mass of these proteins ranged from 14-23 kDa in leaf, root and stem tissues, but higher molecular mass forms were observed in germinated seed (cotyledon) extracts. After IEF, multiple forms of stathmin-like proteins were observed in different tissues from all plant extracts. In general, the pIs ranged from 3.5 to 5.0, although each tissue showed a unique IEF pattern of stathmin-like proteins. Stathmin-like proteins in plants appear to be similar to mammalian stathmin in size, pI, and immunological determinants. © Elsevier, Paris

Isoelectric point / molecular mass / Stathmin / plants / tissues

BCIP, 5-bromo-4-chloro-3-indoyl-phosphate / IEF, isoelectric point / NBT, nitro blue tetrazolium chloride / PVDF, polyvinylidene difluoride

1. INTRODUCTION

Stathmin is a 19 kDa, cytosolic protein that also is known as pp17 or prosolin, p18, Op18, P19, pp20, pp21 and pp23 and 19-K [18]. Four major phosphorylation sites are present in the N-terminal portion of stathmin, including sites phosphorylated by a Ca^{2+}/calmodulin kinase (CaMK IV/type GR)[11], mitogen activated protein (MAP) kinases [3] and cyclin-dependent protein kinases [3]. Using a two hybrid screening system, Maucuer et al. [16] also have found that stathmin interacts with a novel serine-threonine kinase designated KIS. Phosphorylation by these various kinases, and perhaps additional kinases, produces multiple phosphorylated forms of stathmin [3]. Stathmin phosphorylation increases as cells progress toward the G2-M phases of the cell cycle [10], and inhibition of stathmin phosphorylation leads to accumulation of cells in the G2-M phases [8, 11]. Cell-cycle defects subsequent to disruption of stathmin phosphorylation are interesting because Belmont and Mitchison [1] have shown that stathmin interacts with tubulin dimers and increases the catastrophe rate of microtubules [2]. Moreover, serine to alanine substitutions enhance stathmin's microtubule-destabilizing actions and substitution of aspartate for serine 63 inhibits these actions [5, 13]. Therefore, phosphorylation may regulate some of stathmin's cell-cycle effects by altering its microtubule destabilizing activity.

Stathmin appears to interact with several proteins. Stathmin's microtubule-destabilizing actions involve interaction between stathmin and tubulin dimers. Maucuer et al. [16] have reported that stathmin interacts with two coiled-coil proteins, cc1 and cc2. Cc2 appears to be a fragment of the tsg 101 gene, a putative tumor susceptibility gene [9]. Stathmin probably associates with these proteins via an alpha helical domain that is predicted to participate in coiled-coil protein-protein interactions [16]. Finally, it has been suggested that stathmin interacts with a novel protein kinase and a hsp 70 protein [16].

Stathmin structure, and perhaps function, is highly conserved. Human stathmin differs from the rat protein by only one amino acid [14] while Xenopus stathmin is 79% identical to the rat protein [15]. Furthermore, antisera generated against rat stathmin recognize stathmin-like proteins in tissues from a wide variety of vertebrates [6], and an antiserum generated against a synthetic stathmin peptide recognizes stathmin-like proteins in invertebrates (unpublished data) and in mung bean leaves [4].

The presence of stathmin in plants is of considerable interest, because it suggests that the protein plays

an integral role in pathways that have been highly conserved over time. To define further the role of stathmin in plants, we have examined the forms of stathmin-like proteins in germinated seeds or cotyledons, roots, stems, and leaves from a variety of plants. Stathmin-like proteins were analysed by western blotting after SDS PAGE and native isoelectric focusing.

2. RESULTS

Stathmin-like proteins in plants share similar biochemical properties with mammalian stathmins, but the plant stathmin-like proteins also have unique properties. Like mammalian stathmins, plant stathmin-like proteins remain soluble in aqueous solutions even after being heated at 90-100 °C for 10 min. This heat treatment removes some of the pigments, carbohydrates and proteins in plant extracts, but probably does not achieve the same level of purification as in mammalian extracts. Unlike mammalian extracts, many heat soluble proteins remain in the supernatants of heat treated plant extracts. When analysed by SDS PAGE, many of these heat soluble plant proteins have different molecular masses than stathmin-like proteins. As judged by protein staining of concentrated extracts after SDS PAGE, heat treatment produces much less stathmin enrichment in plants. We have also observed that plant stathmin-like proteins cannot be consistently precipitated using trichloroacetic acid (TCA), and variable amounts of these proteins pass through nitrocellulose membranes during electrophoretic transfer for western blotting. In some cases, there seems to be differential transfer of some stathmin-like forms through nitrocellulose membranes under a variety of transfer conditions.

2.1. SDS PAGE and western blotting

When heat-treated and concentrated extracts from a variety of plants were probed with an antibody specific for rat stathmin, all plants were found to contain one or more proteins immunologically related to stathmin. Heat-treated extracts were obtained from germinated seeds, leaves, roots, and stems in each variety examined. Immunoblots of proteins from broad beans, cowpeas, green beans, pea, and soybeans are shown in *figure 1*. With few exceptions, extracts from all plants contained 12-23 kDa proteins that cross reacted with the anti-stathmin antibodies. Often, multiple stathmin-like proteins were present. Stathmin-like proteins could not be detected in concentrated crude extracts not subjected to heat treatment. Part of this problem may be related to the increase in pigments, proteins, carbohydrates, and other plant material that leads to smearing and lane deformation in SDS PAGE and in western blotting (data not shown). Attempts to detect stathmin-like proteins in crude extracts that were not heat treated or concentrated were also unsuccessful, suggesting that the abundance of stathmin-like proteins in plants may be low. Alternatively, the antibody may have a lower affinity for plant stathmin-like proteins.

Extracts from broad bean leaf, root, and stem extracts showed two major forms of stathmin-like proteins with an apparent molecular mass of 20 kDa and 16-17 kDa (*figure 1* A, lanes 2-4). In contrast, germinated seed extracts (*figure 1* A, lane 1) contained only minor amounts of these forms. The major immunostained protein in germinated seeds migrated with an apparent molecular mass of 38 kDa.

To demonstrate the specificity of our stathmin generated antibodies, broad bean stem samples, containing two stathmin-like isoforms, were subjected to SDS PAGE and western blotting. Samples were treated with serum 3038 antibody, nonspecific antibodies against broad bean tyrosinase, affinity purified antibodies isolated from serum 3038, and Dr Sobel's antibodies against residues 15-27 of rat stathmin (*figure 1* F, lanes 1-4). No cross-reactions were observed using incubations without added antibody (data not shown) or with an antibody against broad bean tyrosinase (lane 2). Preincubation of serum 3038 with the synthetic peptide did not reduce the levels of immunostaining appreciably in broad bean stem stathmin-like proteins (data not shown). Although this data would have added further evidence for our antibody specificity, we may not have had enough peptide to saturate the antibody and did not have enough peptide available to carry out other competition experiments. In any case, the affinity purified antibodies against residues 32-44 and antibodies against residues 15-27 of rat stathmin identified the same two proteins as serum 3038 antibodies and demonstrated the specificity of serum 3038.

Strong immunostaining of stathmin-like proteins was observed in green bean root and leaf extracts (*figure 1* B, lanes 2, 3), whereas weak immunostaining was observed in germinated seed and stem extracts (*figure 1* B, lanes 1, 4). Germinated seed and stem extracts contained weakly stained stathmin forms with molecular masses of 14 and 8 kDa. Leaf extracts

(*figure 1* B, lane 2) contained a major protein with a molecular mass of 6 kDa, and minor forms with masses of 23, 19, 14, and 12 kDa. Root extracts (*figure 1* B, lane 3) showed a major protein with a molecular mass of 19 kDa and minor proteins of 21 kDa and 14 kDa.

Extracts from pea leaf and root contained several immunologically stained stathmin-like proteins (*figure 1* C, lane 1, 2). The major protein identified in leaf extracts (lane 1) migrated with a molecular mass of 17 kDa. Minor forms of 20, 14, and 12 kDa were also observed. In contrast, root extracts (lane 2) contained a major 14 kDa form and minor forms at 17 and 12 kDa. Stathmin-like proteins with faint immunostaining were identified in cotyledon and stem extracts and had molecular masses of 17 and 14 kDa (lanes 1 and 4). Stem extracts also contained a minor form of 12 kDa.

A major protein with an apparent molecular mass of 19 kDa was immunostained in soybean extracts from germinated seeds, leaves, roots, and stems (*figure 1* D, lanes 1-4 respectively). Soybean extracts also contained another immunostained protein with an estimated molecular mass of 16-17 kDa. The staining intensity of these two bands appeared to be similar among the various tissues examined.

Proteins from cowpea germinated seeds, leaves, and stems cross-reacted with stathmin antibodies (*figure 1* E, lanes 1-4 respectively). Germinated seed extracts (lane 1) contained two proteins with molecular masses of 33 kDa and 17 kDa. Leaf extracts (lane 2) contained a weakly stained protein of 17 kDa while stem extracts (lane 3) contained immunostained proteins with molecular masses of 21, 17, and 14 kDa. We were not able to identify any stathmin-like proteins in root extracts.

2.2. Native IEF and western blotting

Using standard protein IEF markers, we have determined pIs for the major proteins cross-reacting with

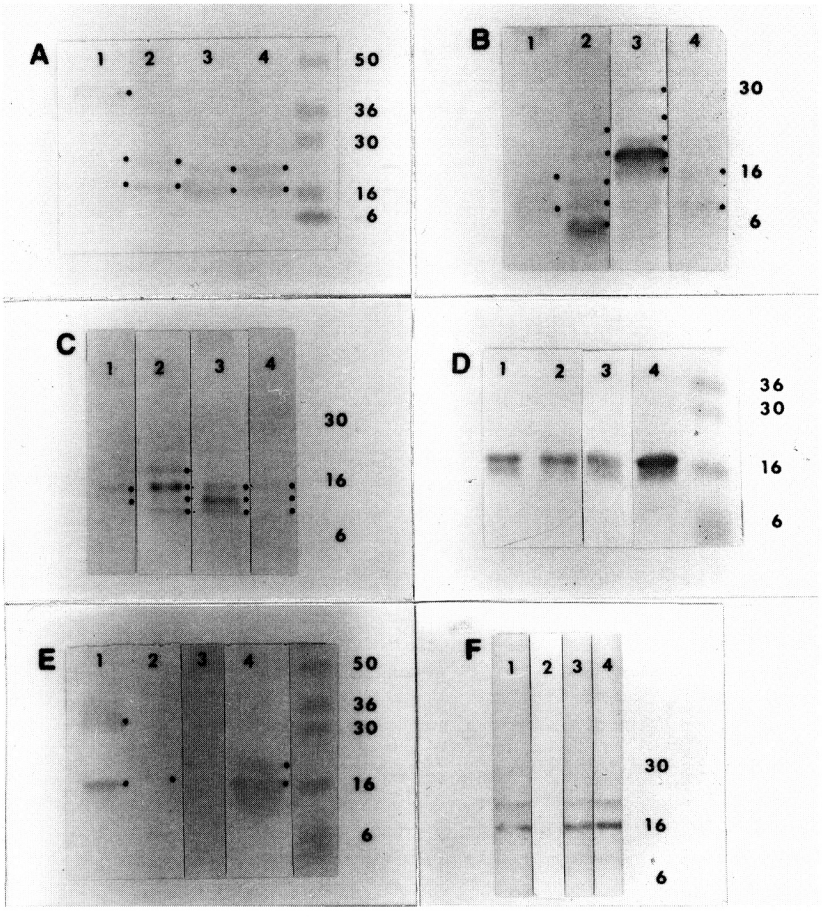

Figure 1. Identification of stathmin-like proteins after SDS PAGE and western blotting. **A**, broad bean extracts; **B**, green bean extracts; **C**, pea extracts; **D**, soybean extracts; **E**, cowpea extracts: lanes 1, germinated seed; 2, leaf; 3, stem; 4, root; **F**, broad bean stem extracts probed with 1, serum 3038 antibody; 2, antibodies against broad bean tyrosinase; 3, affinity purified antibody from serum 3038; 4, antibody against residues 15-27 of rat stathmin (A. Sobel). SDS PAGE and western blotting was carried out as described in Methods. Dots represent visible immunostained bands on the blots. Numbers refer to prestained molecular mass markers in kDa.

anti-stathmin antibodies. In general, samples from different plants and plant tissues showed multiple forms of stathmin-like proteins after native IEF and western blotting. Most major immunostained stathmin-like proteins had pIs between 3.5 and 4.5 although a few forms were detected with pI values higher than 4.5.

IEF analysis of proteins from different broad bean tissues showed variations in the patterns of stathmin or stathmin-like proteins (*figure 2* A). Germinated seed extracts (lane 1) contained three major stathmin forms with pIs of 4.3, 4.4, and 4.55. Some of these forms were also present in leaf, root, and stem extracts. Leaf extracts (lane 2) showed four major stathmin-like forms, three of which exhibited low pIs of 3.7, 3.75, and 3.85. The other major protein had a pI of 4.4. Root extracts (lane 3) also contained several immunostained proteins with pIs similar to those in leaf extracts. Two additional immunostained proteins with pIs of 4.0 and 4.2 were also observed. The stathmin-like forms identified in stem extracts (lane 4) encompassed much of the pI range found in other broad bean tissue extracts, but forms with higher pIs (4.7, 4.75, 4.9 and 5.0) also were found.

Multiple forms of stathmin-like proteins were also observed in green bean extracts (*figure 2* B, lanes 1-4). One major and two minor forms were found in leaf extracts (lane 2) with pIs of 3.5, 3.75, and 4.15, respectively. Five stathmin-like forms were observed in root extracts (lane 3). These forms showed pIs of 3.67, 3.7, 3.75, 3.9, and 4.35. Stem extracts (lane 4) contained three forms of stathmin-like proteins with pIs of 3.7, 3.8, and 4.0. One very faint immunostained protein with a pI of 4.1 was found in germinated seed extracts (lane 1).

Extracts from cowpeas germinated seeds, leaves, and stems showed stathmin-like protein forms with pIs lower than 4.5 (*figure 2* B, lanes 6-8). The major forms of stathmin-like proteins observed in cotyledon extracts (lane 6) had pIs of 3.55, 3.6, 3.63, 3.65 and 4. Other minor bands of immunostaining were also present. Little or no immunostaining was observed in leaf extracts (lane 7), an observation consistent with SDS PAGE data. Four major pI forms of stathmin-like proteins found in stem extracts (lane 8) had pIs of 3.6, 3.65, 3.8 and 3.85.

Unique patterns of stathmin-like proteins were also found in different soybean tissues (*figure 2* C). Two major forms of stathmin-like proteins with pIs of 4.1 and 4.2, and two minor forms with pIs of 4.4 and 4.45 were found in germinated seed extracts (lane 1). Leaf extracts contained four major forms with pIs of 4.2, 4.4, 4.45, and 4.95. Several forms of stathmin-like proteins were observed in root extracts (lane 3). Two of these forms showed low pIs of 3.85 and 3.9, while two others showed pIs of 4.45 and 4.55. Several minor forms with pIs greater than pH 4.6 were also observed.

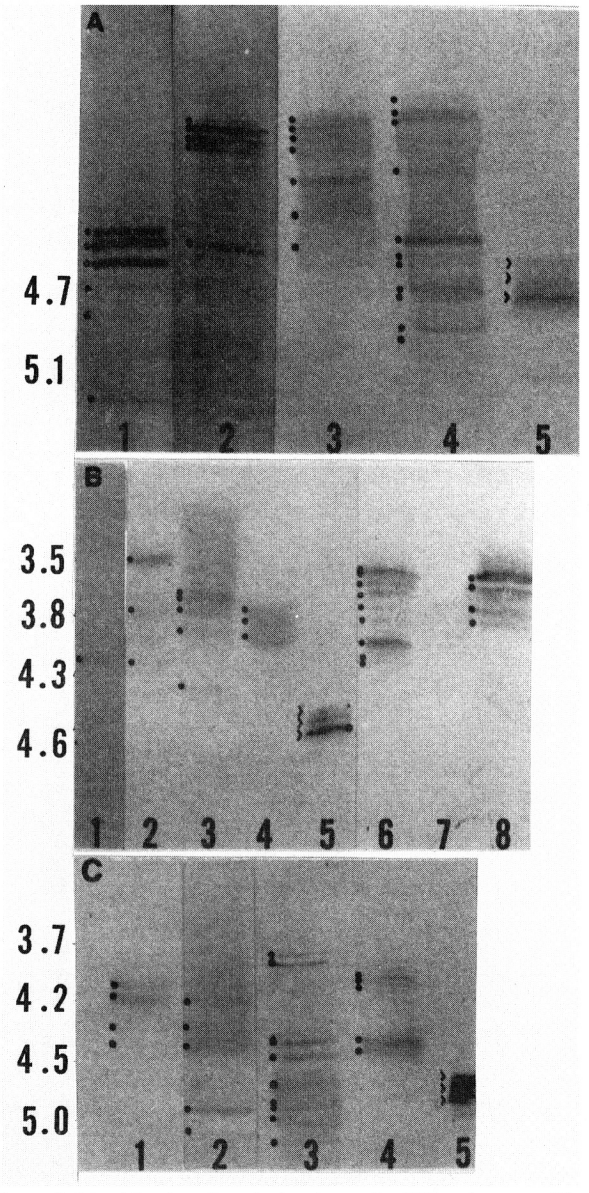

Figure 2. Identification of stathmin after native IEF and western blotting. **A**, broad bean extracts; **B**, cowpea and green bean extracts: lane 1, green bean seed; lane 2, green bean leaf; lane 3, green bean root; lane 4, green bean stem; lane 5, phycocianin; lane 6, cowpea seed; lane 7, cowpea leaf; lane 8, cowpea stem; **C**, soybean extracts: lanes 1, germinated seed; 2, leaf; 3, root; 4, stem; 5, phycocyanin. Native IEF and western blotting was carried out as described in Methods. Dots represent visible immunostained bands on blots.

Stem extracts (lane 4) appeared to contain four major forms with pIs of 4.0, 4.15, 4.45, and 4.50, respectively.

To determine whether pI forms of stathmin-like proteins migrated with molecular masses consistent with stathmin-like proteins (i.e. 18-30 kDa), broad bean germinated seed and soybean root extracts were subjected to IEF, electroeluted from IEF gels, and reanalysed by SDS PAGE and western blotting. Anti-stathmin antisera recognized proteins with molecular masses similar to those found by SDS PAGE and western blotting of samples prior to IEF (data not shown).

3. DISCUSSION

Stathmin-like proteins were identified in cytosolic extracts from plants using an antibody specific for a conserved region of rat stathmin, residues 32-44. The specificity of this antibody was comparable to affinity purified antibody from the same serum and to antibodies against a completely different sequence (residues 15-27) of amino acids in rat stathmin. Although there were a number of heat soluble proteins in the plant extracts, we did not observe any Coomassie blue stained proteins corresponding to the immunologically stained proteins. This latter observation, in conjunction with having to concentrate samples in order to detect stathmin-like proteins, suggests the levels of stathmin-like proteins are low in plants.

The majority of the stathmin-like proteins identified in plants had molecular masses (16-23 kDa) consistent with those of phosphorylated and unphosphorylated forms of stathmin and stathmin-like proteins in mammals. The nature of low molecular mass forms of 6-14 kDa identified in green bean and pea extracts is unclear, but it is possible that these forms resulted from protein modifications or proteolysis during extract preparation. Overall, the results suggest that stathmin-like proteins in plants are similar to mammalian stathmin-like proteins in both size and immunological determinants.

Plant stathmin-like proteins also share another major biochemical property with mammalian stathmin-like proteins. The plant stathmin-like proteins remain soluble when heated to 100 °C. The resistance of mammalian stathmin-like proteins to heat denaturation is believed to be related to the highly charged nature of the proteins [18]. Accordingly, stathmin-like proteins in plants may also be highly charged cytosolic proteins.

As in the case of mammals, plants contain multiple pI forms of stathmin-like proteins. Some of the stathmin-like proteins identified in plants have pIs within the range of pH 4.5-6.2 which is characteristic of mammalian stathmin [3]. However, extracts from many plant tissues also contained more acidic forms. These acidic forms may represent highly-phosphorylated forms of plant stathmin-like proteins, perhaps forms phosphorylated at sites in addition to the four serines commonly found modified in mammalian stathmin [3]. No one pattern of stathmin-like protein forms was characteristic of a specific tissue (i.e. root, stem, leaf or germinated seed) or a particular species of plant. In mammalian tissues, phosphorylation appears to be related in part to growth and differentiation [6, 17]. Accordingly, the highly variable patterns of pI forms in plants may reflect the balance of highly differentiated and growing cells within a particular tissue.

4. METHODS

4.1. Materials. Broad beans (*Vicia faba* L. cv Long pod), cowpeas (*Vigna unguculata* cv Blackeye #5), green beans (*Phaseolus vulgaris* cv Tenderpick), mung beans (*Vigna radiata* L. Wilczek cv Burken), peas (*Pisum sativa* cv Wanda), and soybeans (*Glycine max* L. Moensch) were grown in potting soil. Corn seedlings (*Zea mays* L. Pioneer 3343) were germinated on moist paper in the dark. At harvest, leaves, stems, roots and the remaining germinated seeds were separated and used immediately or stored at −15 °C until used. Nitrocellulose was obtained from BioRad Laboratories. PVDF membranes were obtained from BioRad Laboratories and from Millipore Corporation. Agarose 3-7 and 3-10 IEF gels were purchased from FMC Corporation.

4.2. Antibodies. Four different rabbits were injected with a synthetic peptide corresponding to residues 32-44 in rat stathmin (Multiple Peptide Systems, San Diego, CA). All rabbits produced antibodies that recognized multiple forms of rat stathmin. Some of the antisera also recognized stathmin-like forms in mung bean leaves [4]. Serum from one rabbit (3038) was used in immunoblotting experiments. Affinity purified antibodies from serum 3038 were isolated from affinity columns containing the synthetic peptide (residues 32-44) coupled to Proton columns (Multiple Peptide Systems, San Diego, CA) via primary amine groups. Antibodies against a synthetic peptide corresponding to residues 15-27 of rat stathmin were provided by Andre Sobel(INSERM-CNRS, Paris, France).

4.3. Partial purification of stathmin. Freshly harvested leaves were homogenized in 5-10 volumes of cold (4 °C) 0.05 M Tris (pH 7.5), 0.4 M sorbitol, 1 mM $MgCl_2$, and

5 mM ascorbate in four 2-3 s bursts in a Waring blender. The homogenate was filtered through 4 layers of cheesecloth and 1 layer of Miracloth. The filtrate was then centrifuged at 6 000 × *g* for 10 min at 44 °C to remove intact chloroplasts. The supernatant was made 0.1 M in NaCl and heated for 10 min at 90-100 °C. The solution was cooled on ice and centrifuged at 10 000 × *g* for 20 min at 4 °C. The resulting supernatant was concentrated to 5 ml by ultrafiltration using Amicon YM5 membranes. Further concentration was performed using centricon 10 and microcon ultrafiltration membranes (Amicon Inc., Beverly, MA) or by lyophilization. All samples were concentrated approximately 100-fold and stored in small aliquots at –15 °C.

Fresh or frozen samples of germinated seeds, roots, and stems were ground to a fine powder in liquid nitrogen. The powder was homogenized in 5-10 volumes of 0.05 M Tris (pH 7.5), 0.1 M NaCl, and 5 mM ascorbate for 1 min in an Omni mixer. The homogenate was centrifuged at 6 000 x *g* for 10 min at 4 °C. The supernatants were heated at 90-100 °C for 10 min and processed as described above. The entire procedure for isolating plant stathmin-like proteins is very similar to that used to isolate heat soluble mammalian stathmin.

4.4. Electrophoresis and western blotting. Stathmin samples were boiled for 2-3 min. One quarter volume of 4 × Laemmli sample buffer [7], containing 0.2 M dithioerythritol, was added and the mixture boiled for another 2-3 min. Samples were centrifuged in a microfuge for 5 min to remove insoluble material. SDS-PAGE was carried out according to the method of Laemmli [7] using 11 % polyacrylamide mini-gels. After electrophoresis, proteins in the gels were electroblotted onto nitrocellulose (BioRad, 0.2 μ) backed by PVDF membranes (Millipore Corp., Immobilon P, 0.4 μ) or directly onto PVDF membranes (BioRad, sequencing grade, 0.2 μ). Transfer was carried out in Laemmli electrophoresis buffer, minus SDS but containing 10 % methanol, at 5 V overnight. After blotting, the membranes were rinsed 2 times in distilled water for 30 s and then incubated in 5 % glutaraldehyde for 30 min with shaking. The membranes were rinsed 3 times for 1 min in 0.01 M Tris (pH 7.5), 0.15 M NaCl, 1 % BSA (w/v), 0.02 % sodium azide (w/v) (TBSAB). The membranes were incubated for 60 min in TBSAB buffer containing 5 % nonfat dry milk, washed 3 times for 10 min each in TBSAB, and then incubated overnight in TBSAB containing anti-stathmin peptide antibodies (1:1000 in TBSAB). Unbound antibody was removed from the membranes by 2 × 10 min washes in TBSAB and one 10 min wash in 0.01 M Tris (7.5), 0.15 M NaCl, 0.02 % sodium azide (TBSA). The membranes were then incubated for 2 h with goat anti-rabbit IgG conjugated alkaline phosphatase antibodies (Promega Corp., Madison, WI, 1:7000 in TBSA). Unbound antibody was removed from membranes by 2 × 10 min washes with TBSA and one 10-min wash with 0.01 M Tris (pH 7.5) containing 0.15 M NaCl (TBS). The membranes were then incubated in alkaline phosphatase substrate solution NBT/BCIP. TBSAB, TBSA, and the nonfat-dry milk blocking solution contained 0.5 mM PMSF when first prepared. To inactivate any protease contaminants, the TBSAB and TBSA solutions were also heated for 30 min at 65 °C.

4.5. Native IEF. Concentrated stathmin samples were applied to precast Agarose 3-7 IEF gels (FMC Corp., Rockland, MA) using applicator masks. The samples were subjected to IEF for 1 h at 10 °C [19]. Phycocyanin (pI 4.75) and methyl red (pI 3.5) were used as visual indicators to determine when focusing was completed. The pH gradient was determined using known protein pI markers from BioRad or Pharmacia/LKB. In some cases, 1 cm sections of the agarose gel were removed and soaked in 2 mL of distilled water for 2 h before measuring the pH of the solution. After focusing, proteins in the gel were vacuum blotted onto PVDF membranes (BioRad, sequencing grade, 0.2 u) for 30 min. The membranes were then processed as described above. The dried gels were stained for protein using Coomassie Blue R-250 to detect protein remaining in the gel, an indicator of transfer efficiency.

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