Purification and Characterization of a 70-Kilodalton Polyadenylate-Binding Protein from Pea (Pisum sativum)¹

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ABSTRACT

A polyadenylate-binding protein (PABP) was purified from cell-free extracts prepared from pea seedlings (Pisum sativum) by ammonium sulfate precipitation and Affi-Gel Blue and polyadenylate-Sepharose 4B affinity chromatography. The final preparation from polyadenylate-Sepharose 4B columns contained a single 70-kilodalton polypeptide with high polyadenylate-binding activity. The purified protein was active over a broad range of ionic strengths and showed temperature and pH optima of 37°C and pH 6.5, respectively. Specificity studies indicated that the pea PABP was most active with polyadenylic acids, showed some activity with polyguanylic acid, and did not bind to polycytidylic acid. Moreover, longer polyadenylate molecules were bound more effectively than shorter ones. Because these properties are similar to PABPs isolated from other sources, we conclude that we have identified, purified, and characterized a plant PABP analogous to those described in yeast and animal systems.

In yeast, PABPs are essential because mutants that lack a functional PABP gene are not viable (17, 18). The requirement for PABP is related to its role in initiation of mRNA translation; PABPs are apparently involved in recruitment of 60S subunits during translation initiation. In yeast, PABPs are also involved in mRNA turnover, because they are required for poly(A) tail shortening of cytoplasmic RNAs (17). Others have suggested that PABPs also play other roles in regulating mRNA stability (4) and mRNA translocation from the nucleus to the cytoplasm (15).

Posttranscriptional events are being increasingly recognized as important for regulation of gene expression in plants (see, for example, refs. 9, 21, 22, 24). Because PABPs are needed for two crucial posttranscriptional aspects of gene expression in yeast, an understanding of plant PABPs should lend insight into posttranscriptional processes in plants. At present, there is no reported information about PABPs from plant cells. Here, we report the purification of a plant PABP from cell-free extracts of pea (Pisum sativum) seedlings by affinity chromatography and a study of the binding properties of the purified protein.

MATERIALS AND METHODS

Substrates

Poly(A)₄₀₀₀ (ICN Biochemicals, average M, approximately 193,000) was 5'-end labeled with [γ-³²P]ATP (NEN DuPont) and T₄ polynucleotide kinase (BRL Life Technologies) as described by Chaconas and van de Sande (6). For one preparation, the size distribution of the end-labeled material was checked on a sequencing gel and was found to correspond to that described by the manufacturer (average length of 400 nts, distribution of <30 nts to about 1000 nts). Poly(A)₅₀ and poly(A)₄₄ were synthesized using T₄ RNA polymerase (BRL Life Technologies [7]). The templates for these reactions were synthetic oligodeoxynucleotides (Fig. 1) carrying a T₄ RNA polymerase promoter followed by 10 and 24 adenines, respectively. For each transcription, equal amounts (250 ng) of each complementary oligonucleotide were mixed in 0.5 mL of water, heated to 85°C for 5 min, and cooled to 26°C at 2°C/min; 2 µL was used in a 50-µL transcription reaction using standard conditions (1). After DNAse treatment, the products were purified using messenger affinity paper (25) and quantitated spectrophotometrically. Other polynucleotides (poly(C), poly(G)), and poly(A)₄₀₀₀₀, each with average

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² Abbreviations: PABP, polyadenylate-binding protein; poly(A), polyadenylic acid; poly(G), polyguanylic acid; poly(C), polycytidylic acid; poly(U), polyuridylic acid; VRC, vanadyl ribonucleoside complex; nt, nucleotide.
A. template for poly[A]24

\[
\begin{align*}
5' & \text{TAATACGACTCACTATAGG} & 3' \\
3' & \text{ATATGCTGAGTGATATACTCC} & 5'
\end{align*}
\]

\[
\text{T7 RNA polymerase}
\]

\[
\begin{align*}
5' & \text{GAAAAAAAAAAAAAAAAAAAAAAAAA} & 3' \\
3' & \text{AAAAAAAAAAAAAAAAAA} & 5'
\end{align*}
\]

B. template for poly[A]10

\[
\begin{align*}
5' & \text{TAATACGACTCACTATAGG} & 3' \\
3' & \text{ATATGCTGAGTGATATACTCC} & 5'
\end{align*}
\]

\[
\text{T7 RNA polymerase}
\]

\[
\begin{align*}
5' & \text{GAAAAAAAAAAAA} & 3' \\
3' & \text{AAAAAAAAAA} & 5'
\end{align*}
\]

Figure 1. Structures of the templates used to produce poly(A)10 (A) and poly(A)24 (B). The nt sequences of the synthetic oligonucleotides used for each reaction are shown, as are the predicted transcription products.

lengths of approximately 10,000 nts) were obtained from Boehringer Mannheim.

Assay of Poly(A⁺)-Binding Protein Activity

To measure poly(A)-binding, a filter-binding assay was used. Reaction mixtures (20 μL) included 25 ng [32P]-poly(A)100 (specific activity approximately 1.43 × 10^6 cpm/ng), 10 μg poly(C), and 10 μL of the appropriate extract or column fraction; final concentrations of buffer components (contained in the PABP samples) were 15 mM Tris-HCl (pH 8.0), 0.05 M KCl, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 2.5 mM EDTA, and 7.5 mM β-mercaptoethanol. The reactions were incubated for 15 min at 37°C and stopped by adding 980 μL ice cold stop buffer (60 mM KCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and the diluted reactions were passed through 25-mm nitrocellulose filters (0.4-μm pore size; Schleicher and Schuell). After the filters were washed with 10 mL of the stop buffer, they were air dried and quantitated by liquid scintillation counting. Nonspecific binding to the filters was determined by substituting unlabeled poly(A)10,000 for poly(C). Specific poly(A) binding was defined as the difference of [32P]-poly(A) bound in the presence of poly(C) and poly(A)10,000. One unit of poly(A)-binding protein activity was defined as that activity that retained 1 pg of labeled poly(A)100 on the filters.

In some studies, the pH and ionic strengths were different from the above. To determine pH optima, aliquots of purified PABP were dialyzed against a mixture of 50 mM acetic acid, 50 mM Mes, 0.1 M Tris-HCl containing 0.1 M KCl, 1.0 mM MgCl₂, 1.0 mM CaCl₂, 5.0 mM EDTA, and 15 mM β-mercaptoethanol that had been brought to the appropriate pH with NaOH or acetic acid. In the assay described above, 10 μL of this was used, which resulted in final buffer concentrations of 25 mM acetic acid, 25 mM Mes, 0.05 M Tris-HCl, 0.05 M KCl, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 2.5 mM EDTA, and 7.5 mM β-mercaptoethanol. To assess the poly(A)-binding activity at different ionic strengths, PABP preparations were dialyzed against buffer D without KCl (see below), and the desired ionic strength of each reaction mixture was obtained by adding appropriate quantities of a 2 M KCl solution. To study the effect of temperature on binding activity, reactions were assembled as usual, incubated for 15 min at the appropriate temperature, and processed as described above. To assess the RNA specificity of the pea PABP, poly(C) was replaced with varying concentrations of other polynucleotides.

Protein Determination and Gel Electrophoresis

Protein concentrations were determined with Bio-Rad protein assay reagent as suggested by the manufacturer. Electrophoretic examination of proteins was performed in polyacrylamide gels containing SDS as described by Laemmli (11), except bisacrylamide was replaced with Acrylaide (FMC BioProducts). Polypeptides were detected by silver staining (13). Mol wts were estimated by comparison with silver-stained SDS-PAGE protein standards (Bio-Rad).

Purification of Poly(A)-Binding Activity

Seeds of pea (Pisum sativum) were obtained from Kentucky Garden Supply (Lexington, KY). Plants were grown in the greenhouse for 2 to 3 weeks. Pea seedlings with three to four extended open leaves were used for the following manipulations. Pea seedlings (300 g) were frozen in liquid nitrogen and ground with a Waring blender at high speed. The powder was suspended in 300 mL buffer A (100 mM KCl, 30 mM Tris-HCl [pH 8.0], 1 mM CaCl₂, 1 mM MgCl₂, 5 mM EDTA, 5 mM PMSF, 15 mM β-mercaptoethanol, and 1.5 μg/mL each of leupeptin, chymostatin, and antipain) and passed through four layers of cheesecloth. The filtrate was spun for 10 min at 10,000g at 4°C and the supernatant then filtered through one layer of Miracloth. All subsequent purification steps were carried out at 4°C.

To this cell-free extract, 14.4 g/100 mL of ammonium sulfate (25% saturation) was added, and the suspension was stirred for 30 min and centrifuged at 10,000g for 10 min. The pellet was discarded, and 23.3 g/100 mL of ammonium sulfate (60% saturation) was added to the supernatant. The suspension was stirred for 60 min and then centrifuged at 10,000g for 10 min. The resulting protein pellet was collected by centrifugation, dissolved in 100 mL buffer B (buffer A without leupeptin, chymostatin, and antipain), and dialyzed against 4 L of the same buffer for 14 h. The resulting preparation was clarified by centrifugation at 10,000g for 10 min and loaded onto a 50-mL Affi-Gel Blue column (Bio-Rad) at a flow rate
Table I. Purification of poly(A)-Binding Protein from Pea Seedlings

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein μg (\times 10^{-6})</th>
<th>Total Activity (\times 10^{-4}) units</th>
<th>Specific Activity units/μg protein</th>
<th>Purification Factor fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>2.200</td>
<td>1.11</td>
<td>0.50</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>1.049</td>
<td>108</td>
<td>103</td>
<td>206</td>
</tr>
<tr>
<td>Affi-Gel Blue</td>
<td>7.43</td>
<td>53.6</td>
<td>7,211</td>
<td>14,422</td>
</tr>
<tr>
<td>poly(A)-Sepharose</td>
<td>0.108</td>
<td>6.37</td>
<td>59.051</td>
<td>116.102</td>
</tr>
</tbody>
</table>

*One unit represents the amount of protein that binds 1 pg of poly(A)\(_{100}\) under standard conditions.

of 50 mL/h. The column was washed with 100 mL buffer B, 200 mL buffer B containing 4 M NaCl, and 100 mL buffer B. PABP activity was eluted with buffer B with 2 M guanidine hydrochloride. Fractions (5 mL) were collected and 0.5 mL aliquots dialyzed against buffer B and assayed for protein content and poly(A)-binding activity. Fractions with poly(A)-binding activity were pooled and dialyzed against buffer C (buffer B with 1 mM EDTA) for 14 h.

The resulting preparation was clarified by centrifugation at 10,000g for 10 min, and poly(C) (Boehringer Mannheim) and VRC (BRL Life Technologies) were added to 0.5 mg/mL and 10 mM, respectively. This solution was passed two times over a 2 mL poly(A)-Sepharose 4B column (Sigma) at a flow rate of 50 mL/h at room temperature and the flow-through discarded. The column was then washed at 50 mL/h with the following: 5 mL buffer C with 5 mM VRC, 5 mL buffer C with 5 mM VRC and 1 mg/mL poly(C), 15 mL buffer C with 0.5 mg/mL poly(C), and 10 mL buffer C. PABP activity was eluted with buffer C containing 1 M urea and 2 M LiCl at a flow rate of 30 mL/h. Fractions (1 mL) were collected, dialyzed against buffer D (buffer C with 5% glycerol and no PMSF), and assayed for PABP activity. Fractions with PABP activity were stored at −80°C.

In a preliminary study, oligo(dT)-cellulose chromatography was used as a purification step for PABPs. In this experiment, the pooled 2 M guanidine HCl elution from an Affi-Gel Blue column was dialyzed against buffer C and clarified by centrifugation. Poly(C) and poly(A)\(_{1000}\) were added to 100 and 1 μg/mL, respectively, and the mixture was incubated at room temperature for 10 min. This was then loaded onto a 4-mL oligo(dT)-cellulose column at 4°C at a flow rate of 30 mL/min. The column was washed with 30 mL of buffer C, 15 mL of buffer C containing 0.3 M NaCl and 1 mg/mL poly(C), and 15 mL buffer C. PABPs were then eluted with 5 mM Tris-HCl (pH 8.0) containing 5 mM NaCl; for this, the elution buffer was heated to 45°C and the column eluted at room temperature.

RESULTS

Purification of Poly(A)-Binding Protein

Our initial attempts to purify PABPs from pea extracts involved adaptations of the methods described previously (16) in which Affi-Gel Blue and oligo(dT)-cellulose chromatographic steps were utilized for rapid affinity purification. However, in our hands, the oligo(dT)-cellulose step invariably yielded preparations with several polynucleotide-binding activities that could not be separated by elutions with different homopolymers. Our initial studies were also confounded by low but persistent levels of nucleases, levels sufficient to cause low yields of material from the oligo(dT)-cellulose columns. For these reasons, we used a poly(A)-Sepharose 4B column as a final chromatographic step. Also, to minimize degradation of the column by residual nucleases, VRC was applied in the loading sample and wash buffer. For this purpose, we determined that 10 mM VRC could be added to extracts with no apparent effect on PABP activity (data not shown).

A summary of the purification of pea PABPs is shown in Table I. Because of high levels of nuclease activity, our initial cell-free extracts had a rather low total amount of PABP activity. Subsequent purification steps removed nucleases in addition to purifying the PABP, leading to what is probably a great overestimation of the purification factor we measure. Based on the total activity present after ammonium sulfate precipitation and the total protein present in the initial cell-free extract, we estimate that the purification reported here represents a 1000-fold enrichment of the protein characterized below.

As a first characterization of our different preparations, the results of each purification step were analyzed by SDS-PAGE. The preparation obtained after poly(A)-Sepharose 4B chromatography contained a single polypeptide with a molecular mass of about 70 kDa (Fig. 2). Because of its chromatographic properties, we tentatively identified this polypeptide as a pea poly(A)-binding protein.

General Properties of the 70-kD PABP

To verify our assignment of the 70-kD polypeptide as a plant analog of poly(A)-binding proteins isolated from other
sources, we characterized the binding properties of the purified protein. Initially, we established pH, temperature, and ionic strength optima. The pH profile of poly(A) binding is shown in Figure 3A. These data indicate that the 70-kD polypeptide had poly(A)-binding activity over a broad pH range, with maximal activity at pH 6.5. Figure 3B shows the temperature profile of the poly(A)-binding activity, revealing an optimum temperature of 37°C and substantial activity between 28 and 50°C. The poly(A)-binding activity in different ionic strengths is shown in Figure 3C. Within the ionic strength range shown, little variation in activity was seen, although marginally higher activity was seen at the lower ionic strengths.

**Figure 3.** Effects of pH, temperature, and ionic strength on poly(A)-binding activity of the purified protein. Poly(A)-binding assays were modified as described in the text. Relative binding activities under different pH values (A), temperatures (B), or KCl concentrations (C) were determined. One hundred percent relative activity represents approximately $1 \times 10^4$ units of PABP activity as defined in "Materials and Methods."

**RNA-Binding Specificity of the 70-kD Polypeptide**

To further test our hypothesis that the purified 70-kD polypeptide noted above is a plant-derived analog of PABPs isolated from other sources, we characterized the binding specificities of the pea polypeptide. In these studies, specificity was assessed by evaluating the ability of other RNAs to inhibit binding of labeled poly(A)$_{10,000}$ by the pea PABP. Our results (Fig. 4) indicate that 50% inhibition of binding of poly(A)$_{10,000}$ in our standard conditions was achieved with a 1.5-fold excess (by weight) of poly(A)$_{2,400}$ as opposed to 25- to 50-fold excess of poly(G). In addition, no inhibition could be seen with poly(C), even at the highest excesses (200-fold) tested. Thus, the purified 70-kD polypeptide had a high degree of specificity for poly(A), could bind poly(G) with a lower affinity, and was not able to bind poly(C).

We also examined the effect of varying the length of polyadenylate on the binding activity of the 70-kD polypeptide. For this experiment, the different quantities of unlabeled poly(A)$_{10,000}$, poly(A)$_{2,400}$, or poly(A)$_{100}$ were used as competitors for labeled poly(A)$_{10,000}$, and the concentration of the 70-kD protein was held constant (Fig. 5). We found that poly(A)$_{10,000}$ inhibited binding to poly(A)$_{10,000}$ with 50% inhibition occurring at a competitor to substrate ratio of about 1.5. In contrast, excesses approaching 30- to 40-fold were needed for 50% inhibition by either of the shorter polyadenylates. These studies indicated that poly(A)$_{10,000}$ was a much more effective competitor for poly(A)$_{10,000}$ than either of the shorter polyadenylates tested and are suggestive of cooperative interactions in the assembly of PABP-polyadenylate complexes.

The experiment in shown Figure 5 also showed that low concentrations of the shorter polyadenylate molecules reproducibly enhanced the binding of the labeled poly(A)$_{10,000}$ substrate. We cannot explain this result, although it may reflect cooperative interactions between PABP molecules associated with the shorter polyadenylates and unliganded PABPs such

**Figure 4.** RNA-binding specificity of purified PABP. Standard reaction mixtures for PABP activity assay were used except the standard amount of cold poly(C) was replaced with varying amounts of poly(A) (A), poly(G) (O), and poly(C) (■). Original poly(A)-binding activity of the purified protein ($1 \times 10^4$ units) was used in each reaction.
that the unliganded molecules now have a greater avidity for the labeled substrate.

**DISCUSSION**

Although it has been almost two decades since the first reports of PABPs from eukaryotic cells (5) and similar proteins from several sources have been identified and characterized (8, 10, 12, 16, 19, 26), plant PABPs have not been described or characterized. In this work, we have identified a pea protein that has a strong affinity with poly(A). The properties of this protein are similar in many ways to those properties of PABPs isolated from other sources. For example, it has a molecular mass (approximately 70 kD; Fig. 2) similar to PABPs found in yeast (68 kD; 16), rat (75 kD; 19), sea urchin (80 kD; 8), and humans (70 kD; 10). The pea PABP described here is active over broad ranges of ionic strength, retaining 70% or more of its activity over a KCl concentration range between 0.01 and 0.3 M (Fig. 3C). By way of comparison, the *Xenopus* PABP retains 80% or more of its activity over a range of NaCl concentrations between 0.02 and 0.5 M, the yeast protein was 50% active at 0.25 M NaCl as at 0.05 M NaCl, and the protein isolated from HeLa cells retained substantial activity (>50%) at 1.0 M NaCl.

A comparison on polynucleotide-binding properties of the pea protein described here and PABPs isolated from other sources strongly suggests that the 70-kD polypeptide we have purified is analogous to other PABPs. Like the 70-kD polypeptide we have purified, the PABPs from *Xenopus laeviss*, HeLa cells, and yeast were able to bind with poly(A) but not with poly(C) (14, 16, 18, 23). The HeLa cell and *Xenopus* PABPs were also found to bind poly(G) but with lower affinities than with poly(A). With the *Xenopus* PABP, for example, poly(A) was bound with a 10-fold greater affinity than was poly(G) (14). We see a similar difference in affinities for these polynucleotides (Fig. 4).

We find that longer polyadenylate molecules are more effectively bound by the pea PABP than very short molecules (Fig. 5). These results are consistent with a cooperative interaction of PABP subunits bound to long polyadenylate tracts. Such a cooperative interaction would promote the formation of structures noted for PABP-containing polyadenylates in other systems (2, 3) and again suggests that the 70-kD polypeptide identified here is in fact a plant homolog of PABPs from other organisms.

In yeast, there is, in addition to the cytoplasmic 70-kD PABP, a 55-kD nuclear protein that is a proteolytic product of the cytoplasmic PABP (16, 19). We have not seen an analogous protein in our preparations. However, this is not surprising because we used relatively mild conditions to prepare our cell-free extracts, conditions that may not release large quantities of nucleus-localized proteins.

The pea PABP has a temperature optimum of 37°C, and it retains >40% of its activity at 52°C. The physiological significance of this rather high temperature optimum is unclear. However, it is of interest to note that the 73-kD PABP of HeLa cells has been identified as a heat-shock protein (20). It is possible that the pea PABP has an essential role in heat-stressed cells, a role for which there is no distinct heat-shock protein.

In summary, we have purified a 70-kD polypeptide that has RNA-binding characteristics similar to those ascribed to PABPs from other sources. Based on the physical and RNA-binding properties of this protein, we conclude that it is, in fact, a plant poly(A)-binding protein.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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