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Dissociation of Polysome Aggregates by Protease K[1]

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ABSTRACT

Apparent large size-classes of zein-synthesizing polysomes from developing kernels of *Zea mays* L. were converted to smaller polysomes after treatment with Protease K. The reduction in polysome size was not a result of ribonuclease activity, inasmuch as the enzyme did not affect the free polysomes or the size of the mRNA from the membrane-bound polysomes. High concentrations of $MgCl_2$ in polysome buffer inhibited ribonuclease activity and appeared to cause protein interaction between nascent zein polypeptides. Although Protease K inhibited the polysome's capacity for protein synthesis, it was a useful reagent for determining if polysomes were aggregated by protein.

Zein, the major storage protein in maize, is an ethanol-soluble protein localized in structures called protein bodies (10). In normal maize this protein is separated into two major components of approximately 19,000 and 22,800 mol wt by SDS-polyacrylamide gel electrophoresis. The "high lysine" *opaque-2* mutant, which has approximately a 60% reduction in zein content, is deficient in the larger of these two components (7).

We reported that undegraded membrane-bound polysomes which synthesized zein *in vitro* can be isolated from developing kernels of normal maize homogenized in a high pH, high ionic strength buffer (5). The mg concentration in the buffer was a critical factor for recovery of undegraded polysomes. Purified polysomes revealed several distinct size-classes when resolved after sucrose density gradient centrifugation. The smallest contained eight ribosomes, but the larger, major size-classes sedimented too deep in the gradient to be resolved. These large polysomes did not appear to be artifacts since they had high A_{260}/A_{280} ratios, were susceptible to RNase activity, and revealed no membrane contamination when observed with the electron microscope. The amount of these large polysomes correlated with the level of zein synthesis *in vivo*, and they synthesized the major zein proteins when added to an *in vitro* protein-synthesizing system (2, 3). The zein mRNA isolated from the membrane-bound polysomes had a correspondingly large mol wt (6).

Very few large polysomes were isolated from the *opaque-2* mutant at any stage of development, which correlated with a decreased synthesis of zein *in vivo* as well as *in vitro* (2, 3). These results suggested a quantitative as well as qualitative relationship between polysome size and zein synthesis.

Although our initial experiments indicated that these large polysomes were susceptible to RNase activity and were not formed as a result of membrane contamination, they did not rule out the possibility that smaller polysomes were aggregated by interaction of small proteins such as nascent polypeptides. The availability of a proteolytic enzyme free of ribonuclease activity (9) prompted us to test it as a means of detecting protein aggregation of polysomes.

MATERIALS AND METHODS

Isolation and Protease K Treatment of Polyribosomes. Kernels from the maize (*Zea mays* L.) inbred lines W22 or Illinois High Protein were frozen in liquid N_2 22 days after pollination and stored at −80 C (5). After homogenizing kernels in buffer A (0.2 M tris-HCl [pH 8.5], 0.2 M sucrose, 60 mM KCl, 50 mM $MgCl_2$, and 1 mM dithiothreitol), free and initially membrane-bound polysomes were isolated as previously reported (5, 6). Polysomes from *Blastocladiella emersonii* were provided by S. Johnson of the Department of Biology, Purdue University. Polysome pellets were suspended in buffer B (40 mM tris-HCl [pH 8.5], 20 mM KCl, and 10 mM $MgCl_2$) or distilled H_2O. Polysomes were treated with Protease K (Beckman Instruments Inc.) at 20 ng/ml to 200 μg/ml at 4 C or 27 C for varying times. Samples were layered on 150 to 600 mg/ml sucrose gradients, and centrifuged at 189,000*g* for 30 min in a Beckman SW 50.1 rotor (6).

Isolation of Poly(A) RNA from Membrane-bound Polyribosomes. All solutions were autoclaved and glassware was washed with 0.1% diethyl pyrocarbonate and boiled to destroy ribonuclease activity.

Membrane-bound polysomes were suspended in distilled H_2O in the presence and absence of 200 μg/ml Protease K for 2 hr and then dissolved in 10 mM HEPES (pH 7.5), 0.5 M NaCl, 2 mM EDTA, and 0.5% SDS. Two aliquots of 10 ml (20 A_{260} units/ml) were mixed with 0.5 g of oligo(dT)-cellulose (P. L. Biochemical) and the poly(A)-containing RNA was isolated (6).

Polyacrylamide Gel Electrophoresis of RNA. Electrophoresis of RNA was on 9-cm 2.4% polyacrylamide gels containing 0.5% agarose in 36 mM tris, 30 mM NaH_2PO_4, and 1 mM EDTA (pH 7.8), as previously described (6). RNA samples were heated at 60 C for 3 min and rapidly cooled before being applied to gels. Electrophoresis was performed at room temperature for 2.5 hr at 6 mamp/gel, and gels were soaked in distilled H_2O for 1 hr before scanning at 260 nm with a Gilford model 2410 gel scanner.

***In Vitro* Protein Synthesis.** Polysomes were suspended in buffer B with and without 200 μg/ml Protease K and incubated at 4 C for 15 min. Samples were layered over 4 ml of 1 M sucrose in buffer B and centrifuged at 230,000*g* for 90 min in the 65 rotor of a Beckman L5-65 ultracentrifuge.

Pelleted polysomes were suspended in H_2O and added to a standard wheat germ cell-free amino acid-incorporating system (6, 8). The complete system in a final volume of 50 μl contained: 15 μl of wheat germ supernatant, 20 mM HEPES (pH 8.1), 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 40 μg/ml creative phosphokinase, 8 mM creative phosphate, 2.5 mM magnesium acetate, 100 mM KCl, 0.25 μCi of [^{14}C]leucine (Amersham/Searle), 25 μM of 19 unlabeled amino acids, and 8 A_{260} units of polysomes. Hot 5% trichloroacetic acid-insoluble protein was removed by filtration with Whatman GF/A filters and counted in Omnifluor-toluene (New England Nuclear).

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Assay for Endolytic Messenger Ribonuclease Activity. Maize kernels were homogenized in 0.2 M tris-HCl (pH 8.5), 0.2 M sucrose, 60 mM KCl, 0.2 mM $MgCl_2$, and a postribosomal supernatant prepared. Methods for preparing pea polysomes and their use for determining ribonuclease activity have been described (1). Polysome pellets were suspended in water and aliquots incubated in maize extract for 30 min at room temperature after adjusting the $MgCl_2$ concentration to 5, 10, and 30 mM. RNase activity was determined by the conversion of large polysomes to small polysomes (1).

RESULTS

The comparative effects of incubating free and membrane-bound polysomes in 200 μg/ml Protease K for 30 min at 4 C are shown in Figure 1. In the absence of protease, the free polysomes had a large proportion of monosome and a normal distribution of polysomes with the size-class containing 10 ribosomes/mRNA as the size-class of maximum absorbance (Fig. 1A). The size distribution of the polysomes was not altered after protease treatment, although the enzyme caused some dissociation of monosomes into subunits (Fig. 1B). In the absence of protease the membrane-bound polysomes contained a small proportion of monosome, and showed several distinct polysome size-classes (Fig. 1C). The smallest of these contained eight ribosomes and the larger ones sedimented to approximately where polysomes containing 20 ribosomes/mRNA would be expected (3). However, after treatment with Protease K these large polysomes were converted into polysomes bearing eight to nine ribosomes (Fig. 1D). This shift in polysome size was not accompanied by an increase in the proportion of monosomes and small polysomes, indicating that the larger membrane-bound polysome size-classes were specifically affected.

In a similar experiment using polysomes from the water mold *B. emersonii*, Protease K treatment gave the same results as with the maize free polysomes (data not shown).

The conversion of large polysomes to smaller polysomes by Protease K depended on enzyme concentration, temperature, and time. At 4 C the reaction was very rapid at enzyme concentrations above 200 ng/ml. At room temperature sequential conversion of large polysomes to polysomes containing eight ribosomes was detectable by incubating up to 60 min at enzyme concentrations of 20 ng/ml. The alteration in polysome size was immediate at protease concentrations of 200 μg/ml; however incubation for 2 hr at 4 C or 27 C resulted in no further alteration in polysome size or rate of sedimentation.

To determine if protein-synthesizing activity was affected as a result of protease treatment, polysomes were incubated with 200 μg/ml protease K for 10 min and centrifuged through 1 M sucrose. The pelleted polysomes were suspended and added to a wheat germ cell-free protein-synthesizing system. After 15 min incubation the control polysomes increased incorporation 270-fold above background, whereas those treated with protease increased incorporation only 8-fold above background.

In order to determine if mRNA size was altered as a result of protease treatment, the poly(A)-containing RNA was isolated. Polysome pellets were suspended in distilled H_2O with and without 200 μg/ml Protease K and incubated at 4 C for 60 min. The polysomes were then dissolved in SDS buffer containing 0.5 M NaCl and the poly(A)-containing RNA isolated by affinity chromatography using oligo(dT)-cellulose. Figure 2 shows an

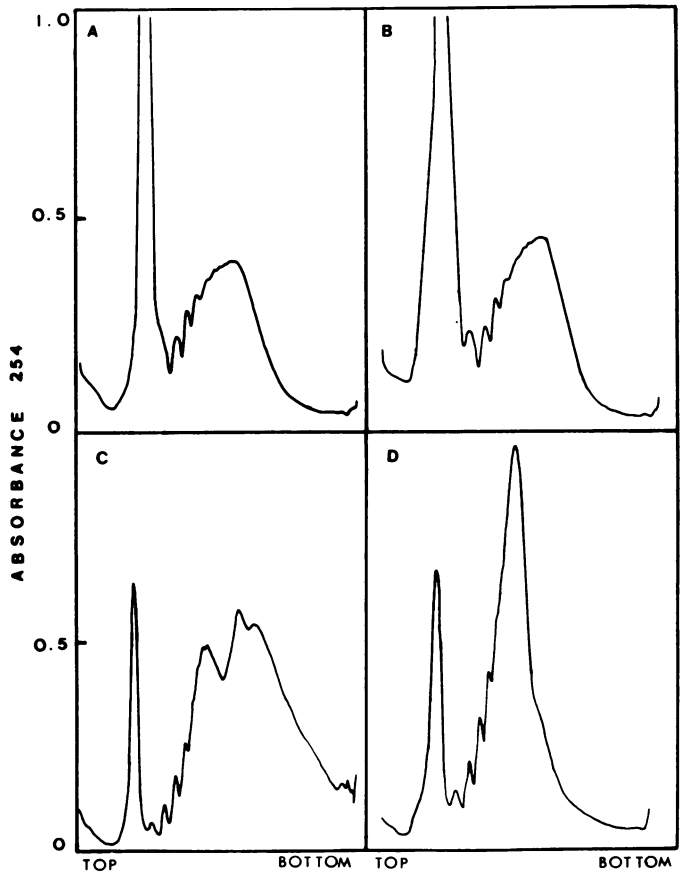

FIG. 1. Effect of Protease K treatment on free and membrane-bound polysomes from maize endosperm. Polysomes were prepared by procedures described under "Materials and Methods" and 0.5-ml samples were incubated 30 min at 4 C in the presence or absence of Protease K. Polysome and Protease K concentrations were: (A) 7.2 O.D./ml free polysomes; (B) 7.2 O.D./ml free polysomes plus 200 μg/ml Protease K; (C) 10 O.D./ml membrane-bound polysomes; (D) 10 O.D./ml membrane-bound polysomes plus 200 μg/ml Protease K.

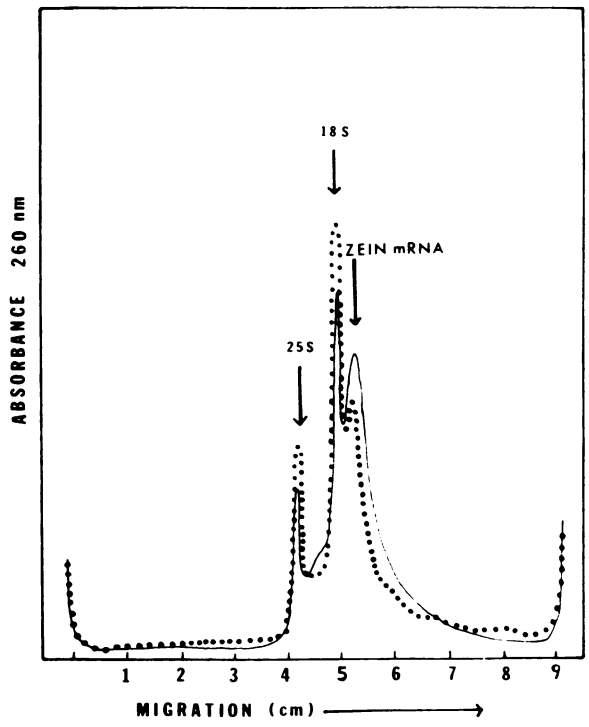

FIG. 2. Determination of zein mRNA mol wt by polyacrylamide gel electrophoresis. Twenty μg of RNA were dissolved in electrophoresis buffer and electrophoresis was on 2.4% polyacrylamide gels. Gels were placed in distilled H_2O for 60 min before scanning at 260 nm. Poly(A) RNA from membrane-bound polysomes with (——) and without (· · ·) Protease K treatment.

analysis of the RNA on agarose-polyacrylamide gels. Although the samples contained some residual ribosomal RNA, both contained a major RNA which was previously shown to be zein mRNA (6). The mRNA had identical mobility on either gel indicating that the alteration of polysome size was not associated with a change in mol wt of the mRNA.

Isolation of polysomes exhibiting a high ratio of large polysomes to small polysomes depended on the $MgCl_2$ concentration of the grinding buffer (5). Proof that $MgCl_2$ inhibits maize endosperm ribonuclease was obtained by incubating pea polysomes in maize postribosomal supernatant. Pea polysomes incubated in buffer B at room temperature for 30 min had a normal distribution of polysome size-classes (Fig. 3A). When the polysomes were incubated in maize extract containing 5 mM $MgCl_2$ (Fig. 3B), the amount of small polysome size-classes such as dimers, trimers, increased, indicating RNase activity. At 10 mM $MgCl_2$ degradation was less extensive (Fig. 3C), and at 30 mM $MgCl_2$ (Fig. 3D) the profile was nearly identical with that of the control.

DISCUSSION

Conversion of large membrane-bound polysomes to polysomes containing eight to nine ribosomes as a result of protease treatment did not result from RNase activity. Polysome degradation is an extremely sensitive assay for RNase activity (1), and Protease K caused no increase in the proportion of small free or membrane-bound polysomes (Fig. 1, B and D). In addition, Protease K had no effect on the size distribution of polysomes from the aquatic water mold *B. emersonii*. The addition of nonproteolytic protein, *e.g.* BSA, had no effect on polysome size distribution (data not shown). The most conclusive evidence that protease conversion of large polysomes to small polysomes was not a result of RNase activity was isolation of mRNAs of identical size in the presence or absence of protease (Fig. 2).

Protease K has proven very useful for isolating mRNAs because it destroys RNase and is active even in the presence of SDS (4, 9). Potentially, the enzyme could hydrolyze polysomes to RNAs and amino acids, although incubation of polysomes in 200 μg/ml Protease K for 2 hr at room temperature did not affect their rate of sedimentation in sucrose gradients. The protein-synthesizing activity of polysomes was reduced by 98% after 10 min incubation in protease at 4 C, however, indicating that ribosomes were rapidly affected.

We previously reported that maize endosperm ribonuclease was essentially inactive in the high pH, high ionic strength polysome buffer (5). Because the proportion of large free and membrane-bound polysomes was greatest when the $MgCl_2$ concentration was 30 to 50 mM, we concluded that Mg reduced ribonuclease activity. Proof that high $MgCl_2$ concentrations inhibit maize RNase was demonstrated by experiments in which pea polysomes were incubated in maize extract containing $MgCl_2$ at varying concentrations. There was less conversion of large polysomes to small polysomes as the $MgCl_2$ concentration was increased, demonstrating that RNase activity was reduced at high $MgCl_2$ concentrations (Fig. 3, B–D). Formation of large polysomes dissociated by protease treatment suggested that the high Mg concentration also caused protein interaction between polysomes. Aggregation occurred when maize membrane-bound polysomes were isolated in the high Mg buffer, but not when maize free polysomes, pea polysomes, or polysomes from the water mold were isolated in this buffer.

Certain evidence suggests that the large polysomes were formed by the interaction of nascent zein polypeptides. Zein contains primarily nonpolar amino acids and is insoluble in aqueous or saline solutions. The appearance of aggregated

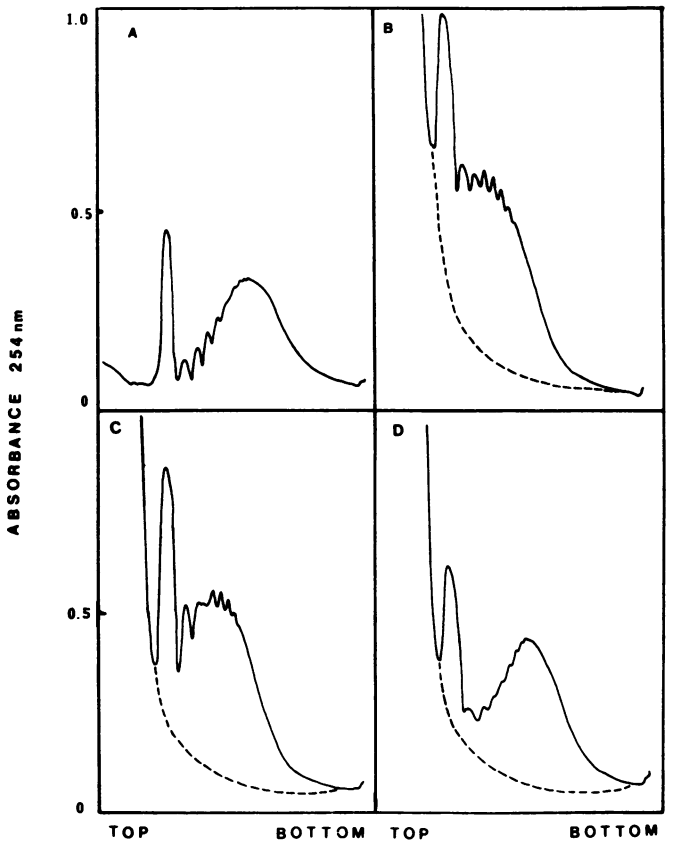

FIG. 3. Inhibition of maize ribonuclease by high concentrations of $MgCl_2$. Pea polysomes were either added to buffer B at room temperature for 30 min and applied directly to a sucrose gradient (A) or incubated for 30 min at room temperature in postribosomal supernatant from maize kernels containing (B) 5, (C) 10, and (D) 30 mM $MgCl_2$.

polysomes correlated with the onset of zein synthesis, and these polysomes synthesized zein when placed in an *in vitro* protein-synthesizing system (2). Aggregation did not occur between membrane-bound polysomes of the *opaque-2* mutant which synthesized less zein *in vivo* and *in vitro* (3). Protease K rapidly hydrolyzed zein into polypeptides soluble in cold trichloroacetic acid (data not shown), so if such aggregation were occurring it would be prevented by the protease. However, it is difficult to explain why discrete size-classes of polysomes were formed if aggregation was due to nonspecific interaction of nascent polypeptides. Regardless of the cause, it is evident that the polysomes do not contain 20 ribosomes as previously reported (3, 5).

Our conclusion that the large membrane-bound polysomes were not formed by aggregation was based upon their high A_{260}/A_{280} ratio, susceptibility to RNase, and freedom from membrane contamination when viewed with the electron microscope (5). These critieria of polysome integrity would be fulfilled even though small polysomes were aggregated by protein. Protease K may therefore prove a useful reagent, in addition to RNase, to verify the size of putative polysomes.

Note Added in Proof. Our conclusion that the polysome aggregates resulted from the interaction of nascent zein polypeptides was also supported by results from experiments in which aggregated polysomes were incubated in puromycin. In 0.5 M KCl buffer at 4 C, 1 mM puromycin, which releases nascent polypeptides from ribosomes, converted the large polysome aggregates into polysomes containing eight to nine ribosomes. At 37 C, puromycin converted all of the polysomes to material sedimenting at 80S or less.

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