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ISOLATION OF UNDEGRADED POLYSOMES FROM RADISH COTYLEDONS: USE OF PROTEINASE K AND CYCLOHEXIMIDE

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Abstract—A procedure for isolating undegraded polysomes from radish cotyledons is described. The current method for plant polysome preparation using buffers of high pH and high ionic strength was unable to prevent the breakdown of large polysomes occurring during the extraction. Proteinase K was very efficient in protecting polysomes from degradation. However, this yielded a high monosome content in the preparations. When proteinase K was combined with cycloheximide, a more satisfactory recovery of polysomes was achieved. This procedure could facilitate investigations of the *in vitro* protein synthesis activity and *m*RNA isolation in tissues possessing a high ribonuclease content.

INTRODUCTION

It is well known that the recovery of large polysomes is difficult because of the degradation of polysomal *m*RNA (by endogenous ribonucleases) which occurs during the extraction [1–4]. The breakdown of polysomes in the presence of trace amounts of ribonuclease is the basis for an assay for endoribonuclease activity which will detect picograms of enzyme [5]. A number of methods have been described to prevent such degradation either in animal or plant tissues. Most of these procedures consist of inhibiting ribonuclease activity by using buffers of high pH and ionic strength [6, 7], bentonite [8, 9], EDTA [10, 11], yeast RNA [2, 12 13] or high Mg^{2+} concentration [12]. Diethyl pyrocarbonate is a powerful inactivator of ribonucleases [14] but its side effects limit its utilization [3, 15]. An alternative is extraction at very low temperature in glycerol [16]. The work reported here was designed to eliminate ribonucleases rather than to minimize their activity. This was achieved by means of proteinase K which has been used successfully for isolating undegraded *m*RNA [17] and DNA [18]. The effect of cycloheximide upon stabilization of polysomes was examined.

RESULTS AND DISCUSSION

When polysomes of radish cotyledons were extracted in high pH and low ionic strength buffer (buffer A) [3], a typical degraded profile was observed (Fig. 1a): the monosome peak was higher than that of dimer, the latter being higher than that of trimer and so on. Such a pattern indicates that a breakdown of large polysomes occurs during the extraction [5, 12]. In order to reduce ribonuclease activity, which is known to be responsible for *m*RNA degradation [1, 4, 5], extractions were then carried out in high ionic strength buffer as suggested by several authors [6, 7, 19]. Fig. 1b shows the polysome profile obtained when buffer B (0.2 M Tris–HCl, pH 8.5, 0.4 M KCl) was used. Comparison between Fig. 1a and 1b shows that in high ionic strength buffer the breakdown of polysomes is reduced. However, an unsatisfactory recovery of large polysomes was obtained although this method gave good results when applied to seedlings of barley [6], pea [20, 21] and soybean [22]. This is likely to be due to the higher ribonuclease content in radish cotyledons. Recent data obtained in this laboratory confirm this hypothesis (to be published elsewhere).

Triton X-100 is necessary for recovering total polysomes, i.e. free and membrane-bound polysomes [19, 20], but unfortunately, detergent treatment releases membrane-bound ribonucleases as well [20]. The following experiment illustrates the importance of the cellular fractionation procedure with regard to the ribonuclease localisation. Polysomes were extracted in buffer of high ionic strength lacking Triton X-100 (buffer C) and the homogenate was centrifuged at 8000 *g* for 10 min (instead of 30 000 *g* for 15 min). Polysomes were then pelleted through sucrose pads as in the other extractions. Analysis of the free ribosome pellet on a sucrose gradient revealed a marked drop of large polysomes (Fig. 1c). This could be explained by a high ribonuclease content of the 8000 *g* supernatant and therefore a significant amount of membrane-bound ribonucleases can be pelleted at 30 000 *g*; moreover, ribonucleases may also be released from mitochondria [23]. It is likely that the addition of detergent in the post mitochondrial supernatant would reduce the release of bound ribonucleases, but the presence of detergent in the grinding medium has been shown to be essential for the recovery of membrane-bound polysomes in high yield [12, 24].

Since the method used for young leaves [21, 25] and epicotyls [20, 26] was inadequate for cotyledons, attempts were made to overcome this difficulty. Diethyl

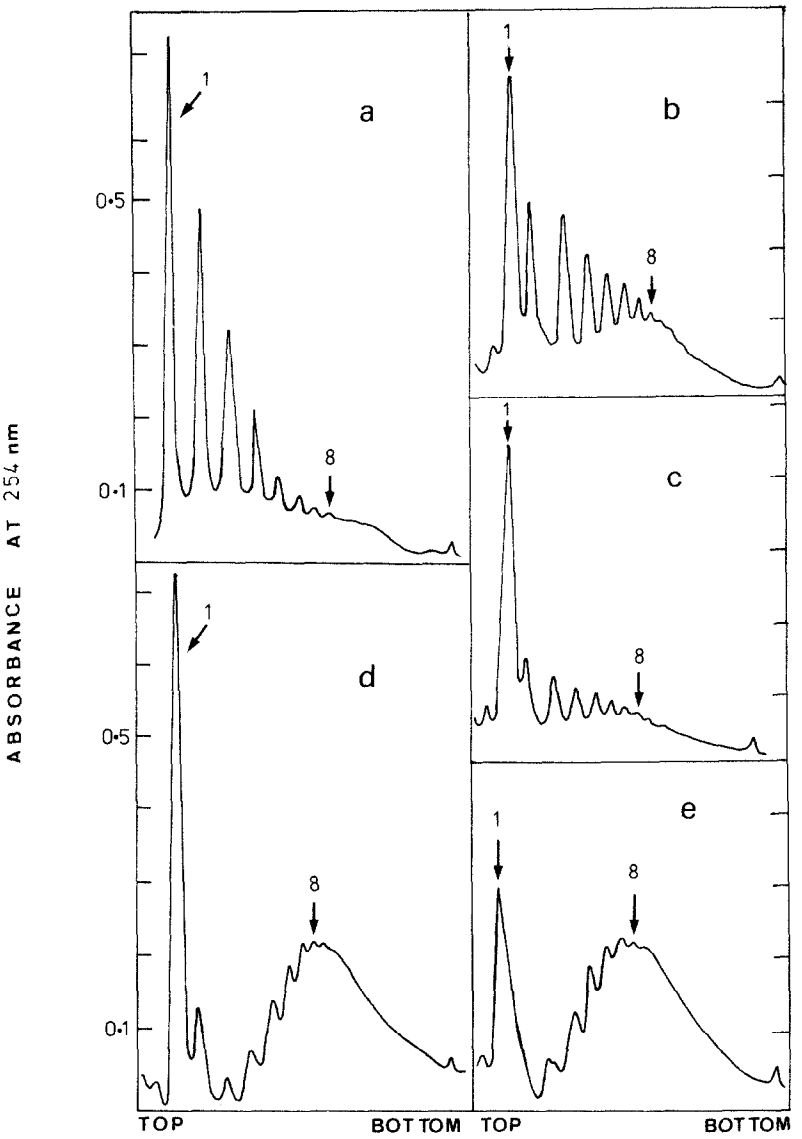

Fig. 1. Sucrose gradient analysis of polysomes. Total polysomes were extracted in (a) buffer of low ionic strength (buffer A); (b) buffer of high ionic strength (buffer B); (d) buffer containing proteinase K (buffer D); (e) buffer containing proteinase K and cycloheximide (buffer E). Free polysomes were prepared from the 8000 g supernatant in buffer C (c). Arrows denote the respective positions of monosomes (1) and polysomes of size class 8 (8). Profiles represent 2–4 separate experiments.

pyrocarbonate has been widely used for polysome preparation [3, 15, 27, 28]. The advantages and disadvantages of diethyl pyrocarbonate have been reviewed by Ehrenberg *et al.* [14] and three main difficulties are encountered when using it, i.e. (a) acidification of the homogenate, (b) dissociation of ribosomes into subunits, (c) decrease of amino acid incorporation *in vitro* by polysomes. In order to avoid these problems, we decided to try another strong ribonuclease inactivator—proteinase K [17, 29].

When extractions were carried out in a medium containing 0.2 mg/ml proteinase K (buffer D), the analysis of polysomes on a sucrose gradient showed a very different pattern (Fig. 1d) from that obtained in the absence of proteinase (Fig. 1b). The maximum absorbance at 254 nm of the polysome area corresponds to *m*RNA bearing 7–8 ribosomes. Fig. 2 depicts the comparative composition in size classes of polysomes extracted in low salt and high salt buffers containing or lacking proteinase K. Only in the presence of proteinase K was the recovery of substantial amounts of polysomes, made of more than 6 ribosomes per *m*RNA, achieved. As judged by the polysome profiles, monosomes were more abundant when proteinase K was added in the extracting medium (cf. Fig. 1b and 1d). Since proteinase K attacks all protein substrates [29], it is possible that it also degrades nascent polypeptide chains in elongation, and consequently it could yield a release of ribosomes as it occurs when polypeptide chains are terminated [30].

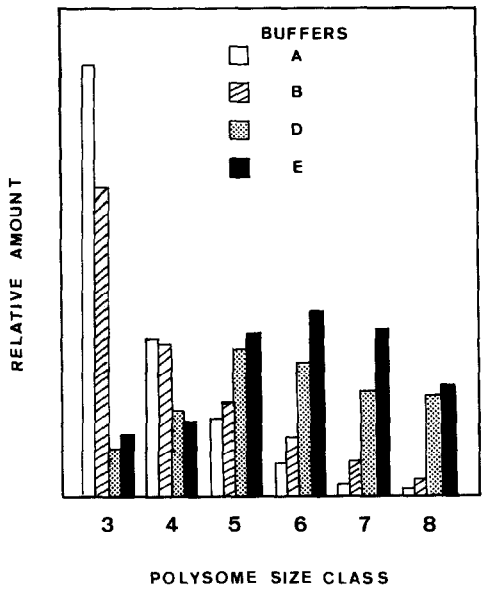

Fig. 2. Size class polysome distribution. Areas under polysome peaks presented in Fig. 1 were determined by planimetry and the values were then divided by the number of ribosomes of the corresponding size class. Results are expressed in arbitrary units.

In developing maize kernels it has been observed that proteinase K converted large membrane-bound polysomes (*ca.* 20 ribosomes per *m*RNA) into smaller ones (8–9 ribosomes per *m*RNA) [31]. The authors reached the conclusion that proteinase K degraded the nascent zein chains and by doing so, prevented the aggregation of polysomes by interactions between the polypeptides in elongation. Although in radish seedlings we did not observe such an effect (i.e. dissociation of polysome aggregates), our data indicate that nascent protein chains are sensitive to proteinase K. An attempt to reduce the monosome proportion of the ribosomal preparation was then made.

Cycloheximide has been previously shown to preserve polyribosome integrity [4, 6, 32]. This possibility was tested by preparing polysomes in the presence of both proteinase and cycloheximide (buffer E). Fig. 1e shows that the monosome peak decreased under such conditions (cf. Fig. 1d and 1e). The size class distribution of polysomes is shown in Fig. 2. An estimation by planimetry gave a ratio of polysomes to monosomes of 4.8 and 2.4 for polysomes extracted in the presence or absence of cycloheximide, respectively. It has been demonstrated that cycloheximide inhibits the transfer of amino acids from *t*RNA to the polypeptide chain in elongation [33, 34]. Therefore, it could be effective in minimizing the running off of ribosomes since the elongation process is stopped. The present data are in agreement with the earlier findings of Wettstein *et al.* [35].

The aim of this work was to isolate undegraded polysomes, rather than to recover the maximum amount of ribosomal material. However, it is important to know whether polysome samples are representative of the *in vivo* situation. The use of heavy sucrose cushions for pelleting ribosomes has been reported to decrease the relative amount of monosomes of polysome preparations [20] but the authors pointed out that this could be, at least in part, an artefact; when using lighter sucrose gradients the apparent large proportion of monosomes could result from polysome degradation by contaminating membrane-bound ribonucleases. Ribosome pelleting through heavy sucrose gradients results in preparations being highly purified and especially free of ribonucleoprotein particles [36, 37], but a 2 hr centrifugation at 160 000 *g* is probably not sufficient to pellet all ribosomes [19, 20, 38]. A short centrifugation time was chosen in the early stages of this work so that polysome degradation by ribonuclease should be reduced; use of proteinase K and cycloheximide allows longer centrifugation time without further degradation.

The total amount of ribosomes of the 30 000 g supernatant after extraction in buffers A, B, D, has been determined. Results are presented in Table 1. When Triton X-100 was added to the extracting media the amount of extractable RNA was *ca* twice that found in the absence of detergent. Our data are in good agreement with recent work on avian liver polysomes which stresses the importance of the detergent in the homogenization step to prevent a considerable loss of polysomes (*ca* 50% as judged by the published profiles) during the first low speed centrifugation [39]. As can be seen from Table 1, other modifications in the composition of buffers did not result in significant changes in ribosome recovery.

To summarize, we have combined the advantages of proteinase K, which acts to preserve the integrity of polysomal *m*RNA [17], and cycloheximide, which has a stabilizing effect on the binding of ribosomes to *m*RNA [4, 32]. Isolation of undegraded polysomes is essential for the knowledge of the *in vivo* state of protein synthetic activity in a given material at a given time; it is also a prerequisite for isolating undegraded polysomal *m*RNA. If ribonuclease activity cannot be strictly controlled during extraction, one can not be sure that changes in the polysomal population under a stimulus, such as light [40], water stress [9] or iron deficiency [41], reflect the true effect of the stimulus. The procedure for isolating polysomes, which is described here, could allow further studies on the developmental changes of the protein synthesis machinery in tissues with a high content in ribonucleases.

Table 1. RNA content of the 30 000 g supernatant

Buffer	Triton in the extracting media	μg RNA/pair of cotyledons
A	+	47
A	–	24
B	+	49
B	–	27
D	+	41
D	–	25

RNA was measured spectrophotometrically assuming an *A* of 1 A unit to correspond to a RNA concentration of 31.7 μg/ml.

EXPERIMENTAL

Plant growth conditions. Seeds of radish (*Raphanus sativus* cv Longue Rave Saumonnée) were surface-sterilized with NaOCl and germinated on moist filter paper in the dark for 36 hr in the dark at 25°. Seedlings were then transferred to continuous standard far-red light [42] for 24 hr at 25°.

Buffers. Buffer A = 50 mM Tris–HCl, pH 8.5; 100 mM KCl; 10 mM $MgCl_2$; 250 mM sucrose; 1% Triton X-100. Buffer B = 200 mM Tris–HCl, pH 8.5; 400 mM KCl; 20 mM $MgCl_2$; 250 mM sucrose; 1% Triton X-100. Buffer C = 200 mM Tris–HCl, pH 8.5; 400 mM KCl; 20 mM $MgCl_2$; 250 mM sucrose. Buffer D = 200 mM Tris–HCl, pH 8.5; 400 mM KCl; 20 mM $MgCl_2$; 250 mM sucrose; 1% Triton X-100; 200 μg/ml proteinase K (Merck). Buffer E = 200 mM Tris–HCl, pH 8.5; 400 mM KCl; 20 mM $MgCl_2$; 250 mM sucrose; 1% Triton X-100; 200 μg/ml proteinase K; 60 μg/ml cycloheximide. Buffer F = 50 mM Tris–HCl, pH 8.5; 100 mM KCl; 10 mM $MgCl_2$.

Preparation of polysomes. Cotyledons (100) were gently ground in an ice-cold mortar in 20 ml buffer (A–E). Extractions were carried out at 1°. The homogenate was filtered through nylon cloth and centrifuged at 30 000 g max and 1° for 15 min. The supernatant was then filtered through glass wool in order to remove lipids which are abundant in this material. Filtrate (20 ml) was layered on a discontinuous sucrose gradient made of 8 ml 2.2 M and 2 M sucrose [37]. Sucrose solns were prepared in the corresponding buffer used for the extraction but which was free of Triton X-100. After centrifugation for 2 hr at 160 000 g max and 1° in a Beckman 60 Ti rotor, the supernatant was removed by aspiration. The ribosome pellet was carefully washed twice and resuspended in 1 ml of buffer F.

Sucrose gradient analysis of polysomes. The resuspended pellets (0.5 ml) was carefully layered onto 10–45% linear sucrose gradients made up in buffer F. The tubes were centrifuged at 116 000 g max and 1° for 150 min in a Beckman SW27 rotor. Gradients were then continuously monitored at 254 nm with an Isco gradient fractionator model 185.

RNA determination. Aliquots of 5 ml of the 30 000 g supernatants were subjected to RNA determination as described in ref. [43].

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