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REQUIREMENTS FOR EXTRACTION OF POLYRIBOSOMES FROM LYOPHILISED PEEL TISSUE OF CLIMACTERIC PEAR

A. G. DROUET and C. J. R. HARTMANN

Laboratoire de Physiologie de la Maturation et de la Sénescence, Université d'Orléans, 45017 Orleans Cedex, France

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Abstract—Profiles of polyribosomes have been obtained from lyophilised peel tissue of climacteric pear (*Pyrus communis* cv Passe-Crassane) isolated in various buffers. Messenger RNA chains bearing up to 7 ribosomes (heptamers) were resolved and exhibited the highest absorption peak. High vacuolar concentrations of phenolics and acids, which are major obstacles in extracting fruit polyribosomes, were circumvented with the use of polyethylene glycol, insoluble polyvinylpyrrolidone (Polyclar AT), extraction at low temperature and high ionic strength buffer. Addition of Ca^{2+} to the extracting medium precipitated polysomes but ethyleneglycol bis(2-aminoethylether) tetraacetic acid (EGTA), a divalent cation chelator with a high affinity for Ca^{2+}, increases the proportion of polyribosomes.

INTRODUCTION

Since the initial finding of Hulme [1, 2] showing an increase in protein nitrogen content of apples at the beginning of the climacteric phase, it is now well established that enhanced protein synthesis may be linked to maturation [3, 4]. Polyribosomal profiles allow estimation of the rate of protein synthesis, although considerable polyribosome degradation due to endogenous ribonuclease can occur during isolation [5, 6]. In the case of the pear fruit, Romani and his collaborators studied characteristics of the ribosome isolated from the pulp tissue, with a special interest in responses to ionizing radiation or temperature stresses [7, 8]. However, many interesting changes also occur in the peel and, before studying changes in polyribosomal profiles during maturation, we have studied the requirements for undegraded polyribosome profiles obtained from lyophilised peel tissue, since lyophilised material offers several advantages. In this study we used peel from climacteric fruit.

RESULTS AND DISCUSSION

Susceptibility to RNase

The data in Fig. 1A show the particle distribution patterns of polyribosome in a 15–60% linear sucrose gradient. *E. coli* ribosomes and subunits (70S, 50S and 30S) were used as gradient markers. The monosomes (m) were considered to be principally the 80S type; *ca* 15% of the total profile area consisted of monosomes in routine extractions. A visible shoulder on the left of the monosome peak was attributed to ribosomal subunits present in the extract on the basis of results in which no Mg^{2+} was added to the extraction medium. The polymers are presumably various numbers of ribosomes attached to *m*RNA, and sediment as dimers [2], trimers [3] through heptamers, up to aggregates which sediment more rapidly and are not resolved on the gradients.

Figs. 1B and 1C show the effect of RNase treatment on polyribosomes and its effects on particle distribution. The control sample (stable) is a typical profile obtained by the extraction procedure used as outlined in the Experimental. Unstable polyribosome preparations are typified by the RNase-treated samples. When resuspended polyribosomes were treated with 10 ng (Fig. 1B) or 300 ng (Fig. 1C) of pancreatic RNase for 10 min at 20° and then cooled to 0° prior to layering on the linear gradient, the absorbance of large polysomes size decreased so that the polysome size class of maximum absorbance became the dimer. This progressive shift to smaller polysome size with increasing levels of RNase supports the hypothesis that the polyribosomes are linked by *m*RNA rather than protein–protein association. Instability is reflected by an increase of these lighter particles and for convenience, stability can be related to the amount of dimer. Stability of polysome can be numerically illustrated either by the ratio of the maximum *A* 254 nm of the hexamer and dimer peaks [9] or by the ratio *R* of >5 mers area to ≤5 mers area (monosomes and subunits not being included in these measurements) [6].

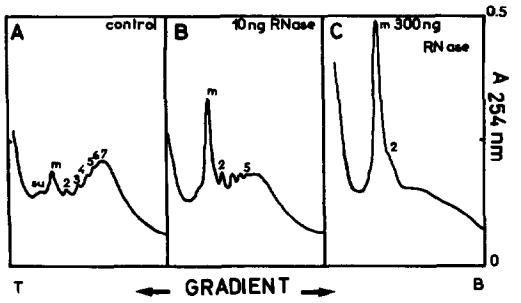

Fig. 1. Polysome degradation by RNase. Polysomes were extracted as described in the Experimental and following resuspension, were incubated for 10 min at 25° with or without (control) added RNase and centrifuged 50 min on SW 60 gradients at 50 000 rpm. A: Control without RNase; B: 10 ng of RNase/0.2 ml; C: 300 ng RNase/0.2 ml of suspension buffer. Subunits are designated su, monosomes m and polymer peaks as 2, 3, 4, 5, 6 and 7 for dimers, trimers... Gradient top: T and bottom: B.

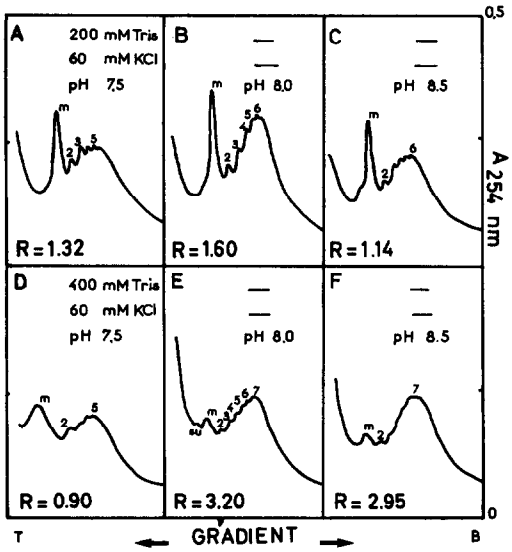

Fig. 2. Effects of pH and concentration of buffer on sucrose density gradient profiles of polysomes. Polysomes were isolated as described in the Experimental except that pH and buffer concentration were varied as indicated. Components are labelled as in Fig. 1.

Influence of pH and buffer concentration on polysome recovery and stability

Polysomes extracted in 0.1 and 0.2 M Tris (Figs. 2A–C) had higher absorbance, but profiles showed decreased RNase degradation when polysomes are prepared with 0.4 M Tris (Figs. 2D–F). The increased stability of polysomes extracted in 0.4 M Tris is numerically illustrated by the higher ratios R of >5 mers to $\leqslant 5$ mers area (Fig. 2). At buffer concentrations of 0.1 and 0.2 M, a drop from the starting extraction pH value of 0.2–0.4 of a unit was normally observed after removal of the polyribosomes. This pH change, attributed to the acidic nature of the cell contents and inadequate buffer capacity of the extraction medium, was reduced by sufficient 1 M KOH to neutralize the vacuolar sap after cell breakage. At the buffer concentration of 0.4 M used, no drop from the starting extraction pH was observed. Recovery of polysomes, as measured by an increase in the polysome class size of maximum peak height, improved when the pH of the extraction medium increased from 7.5 to 8 and decreased between 8 and 8.5. The ratios R involving large polyribosomes (>5 mers to $\leqslant 5$ mers) gave the same results. High pH and high concentration of Tris inhibited polysome degradation as reported in studies with different tissues [9–11]. Thus, the extraction medium for subsequent experiments consisted of 0.4 M Tris at pH 8.

KCl requirements in polyribosome extraction

Polysome recovery from tobacco leaves has been improved by increasing the concentration of KCl [6]. The amount of monomer and dimer decreased as the KCl concentration was increased to 0.2 M in the extraction medium: (Tris–HCl pH 8, 0.2 M). As the lighter particle content of profiles decreased, a concomitant decrease in larger particles was also observed (Table 1). The heptamer band was the last class of particles consistently resolved by the method employed. Polysome degradation was reduced when KCl was increased up to 0.2 M but a lower total yield of ribosomes from the tissue (less area under the curve) was observed. When the extraction medium consisted of 0.4 M Tris pH 8, recovery of large polysomes was not improved when KCl was increased from 0.06 to 0.2 M. High concentrations of KCl were partially effective in preventing the loss of higher polymerized states of polyribosomes from our tissue at Tris concentrations below 0.4 M, although without any effect on high ionic strength buffer. Thus 0.06 M KCl was used in routine extractions.

Other requirements for extraction of fruit polyribosomes

Other components used for the isolation of fruit enzymes [12] were found to be necessary for the isolation of undegraded polyribosomes. Mercaptoethanol (11 mM) polyethylene glycol: PEG (3%) and insoluble polyvinylpyrrolidone: Polyclar AT (10%) increased recovery of total A 254 nm material but polysome recovery or stability was not improved by using higher concentrations of these components. $MgCl_2$ (35 mM) yielded the greatest total amount of material on the gradient. In addition, polyribosomes extracted in potassium phosphate buffer at all pHs and concentrations tested showed evidence of RNase degradation.

Degradation of polysomes by Ca^{2+} and effect of EGTA

Addition of Ca^{2+} to polysome extracts of pea was reported to degrade polysomes by activating RNase [5]. Polysome degradation was stimulated by 7 mM Ca^{2+} and 0.1 M Ca^{2+} when polysomes were extracted in 0.2 M Tris pH 8. Polysomes extracted in 0.1 M Tris showed evidence of RNase degradation but 5 mM EGTA reduced polysome degradation. Because EGTA is known to chelate metallic cations, we can postulate that divalent cations in fruit tissues might activate RNase when polyribosomes were extracted in lower ionic

Table 1. Distribution of polyribosomes obtained from peel tisuse of pear ground in a medium buffered with Tris–HCl at varying buffer and KCl concentration

Extraction	Relative area of polysome profiles				
	Total polysomes	$\leqslant 5$ mers	>5 mers	$\frac{>5 \text{ mers}}{\leqslant 5 \text{ mers}}$	$\frac{A254 \text{ 6 mers}}{A254 \text{ 2 mers}}$
	area	area	area	ratio = R	ratio
0.2 M Tris pH 8 0 M KCl	1.71	0.90	0.81	0.90	1.04
0.2 M Tris pH 8 0.06 M KCl	0.98	0.45	0.53	1.18	1.18
0.2 M Tris pH 8 0.2 M KCl	0.78	0.35	0.43	1.23	1.34

strength buffers. High ionic strength of Tris used henceforth appeared to inhibit Ca^{2+} activated polysome degradation.

EXPERIMENTAL

Material. Pears (*Pyrus communis*, cv Passe-Crassane) harvested at the beginning of November, stored at 0° during 12 weeks, ripened subsequently at 15° and extracted at the climacteric maximum.

Polysome extraction procedure. Peel tissues were frozen in liquid N_2 and lyophilised. Extraction was performed on 5 g of pulverized material by homogenizing in 25 ml of cold extraction soln (buffer A: Tris 0.4 M pH 8; mercaptoethanol 11 mM; PEG 3%; KCl 60 mM; $MgCl_2$ 35 mM; sucrose 6% and 2.5 g of Polyclar). The resulting brei was clarified by centrifugation for 10 min at 15 000 *g* and the supernatant was gently layered over 4 ml of 1.2 M sucrose (in buffer B: 10 mM KH_2PO_4 pH 8, 10 mM $MgCl_2$) and spun for 90 min at 105 000 *g* (average) in the R_{50} rotor of a Spinco Model L ultracentrifuge. The pellet was rinsed gently and resuspended in 0.3 ml of buffer B by means of a close fitting Teflon pestle. Aliquots, (usually 0.2 ml) of resuspended polyribosomes were layered on linear (0.15–0.6 g/ml) sucrose gradients in buffer C: (40 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$; 20 mM KCl) and spun for 45 min at 50 000 rpm in a SW 60 rotor. The gradients were prepared by layering 1.9 ml of sucrose at 0.62 g/ml in cellulose nitrate tubes followed by 1.9 ml of sucrose at 0.13 g/ml and equilibrated for 4 hr at 2° [13].

All operations were conducted at 0–4°. After density gradient centrifugation, the contents of the tubes were analysed with an ISCO model 185 density gradient fractionator attached to an ISCO model UA5 absorbance monitor. The areas in different regions of the polysomal profiles were measured. The different regions were: subunits, monosomes, polyribosomes sedimenting faster than pentamers. Equilibrated blank gradients were always monitored in order to report the baseline for each figure, and the area below is excluded from calculations.

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