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Protocol

Method For the Isolation of High-Quality RNA from Grape Berry Tissues without Contaminating Tannins or Carbohydrates

Catherine Tesniere and Michael E. Vayda*

(CT) INRA Laboratoire de Biochimie Metabolique, INRA, Institut des Produits de la Vigne, 2 Place Viala, 34060 Montpellier cedex 01, France; (MEV) Department of Biochemistry, Microbiology and Molecular Biology, University of Maine, Orono, ME 04469, USA

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Abstract. Grape berries contain compounds that aggregate with and precipitate RNA in the presence of chaotropic agents or phenol. The procedure described here extracts RNA from finely ground tissues using mild denaturants, and selectively precipitates the aggregate-forming material with 30% ethanol. The resulting RNA is suitable for northern blot analysis and translation *in vitro*.

Several methods have been described for the isolation of RNA from fruit tissues (Ku and Romani, 1970; Callahan et al., 1989; Speirs et al., 1984; Grierson et al., 1985; Lay-Yee et al., 1990). Each of these methods includes extraction of tissues with either phenol or high molarity guanidinium salts. Although these techniques yield RNA from a variety of mature fruits, they are not effective for the isolation of RNA from grape berries. Extraction with these compounds results in the

*Correspondence should be addressed to Dr. Catherine Tesniere, Department of Biochemistry, Microbiology and Molecular Biology, University of Maine, Orono, ME 04469 USA (ph: 1-207-581-2815, fax: 1-207-581-2801).
Abbreviations: ATA, aurintricarboxylic acid; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol.

aggregation of RNA with unknown compounds into an insoluble complex. The unidentified components, which strongly absorb light at 270 and 230 nm, interfere with the determination of RNA yield by UV absorption. Further, the aggregating material prevents analysis of RNA by agarose gel electrophoresis; the aggregation is exacerbated by incubations with formaldehyde, formamide or DMSO routinely used to denature RNA secondary structure (Sambrook et al., 1989; Thomas, 1980). Thus, it appears that several agents which alter the hydrophilicity of water promote the aggregation and loss of RNA from grape berries. A recent procedure (Baker et al., 1990) that employs mild denaturants (Hughes and Galau, 1988) yielded grape berry RNA with less contamination, although even this small amount of contaminating material caused gel formation and loss of RNA in the presence of DMSO, formamide or formaldehyde. Our modification of this procedure removes these contaminants by selective precipitation with 30% ethanol. RNA prepared by this procedure is suitable for analysis by northern-blot hybridization and is competent for translation *invitro*, yielding products of greater than 80 kDa. This paper summarizes the results obtained by extraction of grape berries using previously reported procedures and describes the process developed for the isolation of high quality RNA from grape berries.

Materials and Methods

Plant material

Mature grape berries (*Vitis vinifera* L. cv. Chardonnay) were grown in the Clark-Perkins vineyard of Roederer US Inc., Undersun Valley, Mendocino, CA, and harvested in early September, 1990. After harvest, fruits were cut from the stem, washed, frozen in liquid nitrogen and stored at -80°C pending RNA extraction. Tissue was immersed in liquid nitrogen prior to grinding to a fine powder using a Miracle Mill (Markson). Ground tissue was immediately added to homogenation buffer without thawing.

Extraction of RNA

Extraction of RNA from berries using phenol or guanidinium chloride as described previously by Logemann et al., 1987; Shirras and Northcote, 1984; Grierson et al., 1985 and Sambrook et al., 1989, were unsuccessful. All glassware, plasticware and Miracloth (Calbiochem) were autoclaved prior to use. Solutions were prepared from stock solutions by dilution in DEPC-treated distilled water prior to autoclaving. The homogenization buffer of our modified technique includes 200 mM Tris-HCl pH 8.5, 300

mM LiCl, 10 mM Na_2EDTA, 1% (w/v) sodium deoxycholate, 1.5% (w/v) SDS, 1 mM ATA, 5 mM thiourea, 1% (v/v) NP-40 and 10 mM DTT. The latter three components were added as solids to the solution after autoclaving. All manipulations are on ice unless noted.

Reagents

Liquid nitrogen
Homogenization buffer: 200 mM Tris-HCl pH 8.5, 300 mM LiCl, 10 mM Na_2EDTA, 1% (w/v) sodium deoxycholate, 1.5% (w/v) SDS, 1 mM ATA, 5 mM thiourea, 1% (v/v) NP-40 and 10 mM DTT
crystalline CsCl and 5.7 M CsCl containing 10 mM Tris-HCl pH 7.5 and 10 mM Na_2EDTA
70% and 95% ethanol
DEPC-treated distilled water
3 M LiCl
2.5 M sodium acetate buffer, adjusted to pH 5.5 with acetic acid

Optimized procedure

- Weigh tissue and freeze in liquid nitrogen.
- Grind tissue to a fine powder using a Miracle Mill (Markson Scientific Products).
- Quickly add 25 mL of chilled (4°C) homogenation buffer per 10 g of ground, frozen berry tissue, and mix by inversion in an Oak Ridge-type centrifuge tube for 5 min after tissue appears thawed[1].
- Centrifuge the mixture at 12,000 x g for 15 min at 4°C to pellet cell debris.
- Filter the supernatant fraction through two layers of Miracloth and collect in an Oak Ridge-type centrifuge tube.
- Dissolve CsCl into the supernatant to a final concentration of 0.2 g CsCl per mL of filtered homogenate.
- Layer the ~29 mL homogenate over a 10-mL cushion of 5.7 M CsCl containing 10 mM Tris-HCl (pH 7.5) and 10 mM Na_2EDTA in a Beckman Ultraclear Quickseal tube, and centrifuge for 20 hr at 40,000 rpm in a Beckman 55.2 Ti rotor at 20°C.
- Remove the supernatant by aspiration with a pasteur pipette or syringe and discard.
- Wash the pellet containing RNA with 5 mL of chilled 70% ethanol, briefly centrifuge at 12,000 x g, and then air dry.
- Resuspend the clear RNA pellet in 1 mL DEPC-treated water and reprecipitate by addition of 0.2 mL of 3 M LiCl (0.5 M final conc.) and 2.5 mL of 95% ethanol and incubate at -80°C for at least 1 hr, or at -20°C overnight.
- Recover the LiCl-induced RNA precipitate by centrifugation at

12,000 x g for 30 min at 4 °C and successively wash pellet 3 times with 1 mL 2.5 M sodium acetate (pH 5.5) and once with 70% ethanol.

- Centrifuge at 12,000 x g for 15 min at 4 °C and discard the supernatant.
- Resuspend the pellet in 100 µL of DEPC-treated dH_2O.
- Selectively remove the contaminating viscous components by slow addition of 95% ethanol to a final concentration of 30% (v/v).
- Centrifuge at 12,000 x g for 10 min. The supernatant should contain RNA with a peak absorption of 258 nm, an A_{260}/A_{280} ratio of 2.0, and only minor absorption of light at 230 nm. The gelatinous pellet exhibits a slight pink hue.

Northern blots

Total grape berry RNA after selective precipitation of contaminants with 30% ethanol was resolved through a 1.5 % agarose gel containing 2.2 M formaldehyde, blotted to a nylon membrane (Hybond, Amersham) in 20X SSPE, and hybridized 16 hr as described previously (Sambrook et al., 1989; Butler et al., 1990).

***In vitro* translation**

Total RNA isolated from grape berries was used as template (5 µg) for translation *in vitro* using rabbit reticulocyte lysate as described by the supplier (NEN). The [^{35}S]-methionine-labeled polypeptides were resolved by electrophoresis through a 12% SDS-PAGE denaturing gel (Tesniere and Robin, 1991; Laemmli, 1970). Prior to loading on the gel, samples were incubated for 15 min at 37°C with 20 mU RNAase A (Sigma) to degrade [^{35}S]-methionyl-tRNA complexes that otherwise migrate through the stacking gel and obscure high molecular weight proteins at the top of the separating gel. Polypeptides were detected by fluorography using En^{3}Hance (NEN), and Kodak X/AR 5 film

Note

1. A modest increase (less than 2-fold) in RNA yield, is obtained by incubating the mixture at 65 °C for 10 min.

Results

All attempts to extract RNA from grape berries using phenol or guanidinium chloride resulted in poor yields. In each case, an aggregate formed in the presence of these denaturing agents. The aqueous phase, recovered after phenol extraction and RNA precipitation, exhibited a

Table I. Absorbance Characteristics and Yields of Grape Berry RNA Obtained by Various Extraction Methods. All values are the mean of at least three independent isolation attempts.

Method	A_{260}/A_{280}	A_{260}/A_{230}	RNAYield (μg/g wet tissue)
Phenol	0.8	0.3	-*
Phenol + CsCl	0.6	0.2	-*
Guanidinium + CsCl	1.0	0.2	-*
Detergent + CsCl + Phenol	1.8	1.3	4**
Detergent + CsCl	2.0	2.3	20**
Detergent + CsCl + 30% EtOH	2.0	2.6	18

*No peak observed at 260 nm by which to estimate RNA yield.
**Aggregates form in the presence of DMSO, formamide or formaldehyde.

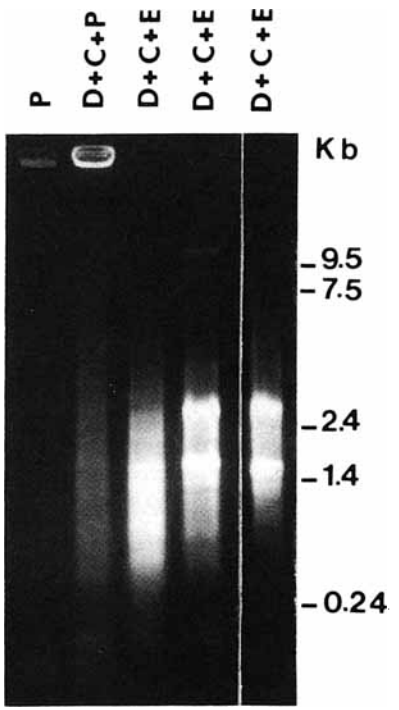

Fig. 1. Gel electrophoretic analysis of RNA extracted by various methods. RNA in samples (5 μg/lane) was resolved under non-denaturing conditions by migration through 1% agarose gels in 90 mM Tris-borate with 2 mM EDTA and 75 ng/mL ethidium bromide. Lanes (left to right) 1, 2 and 3: total grape berry RNA; lane 4: total RNA from potato tuber; Lane 5: total grape berry cell culture RNA. Extraction conditions: C, CsCl; D, detergent; E, ethanol; P, phenol.

absorption profile in the UV with peaks at 270 and 230 nm, and relatively little absorption at 260 nm, precluding determination of RNA yield. Further, the A_{260}/A_{280} ratio of these samples was 0.8 which is aberrantly low (Table 1). Centrifugation through a cushion of 5.7 M CsCl did not remove the contaminating substances (Table I). When precipitated with 0.5 M LiCl and 70% ethanol, contaminating material was not removed by washing with 2.5 M sodium acetate (pH 5.5), which has been reported to selectively remove charged oligosaccharides that coprecipitate with potato tuber RNA (Shirras and Northcote, 1984; Butler and Vayda, 1990). However, the grape berry RNA pellet was resuspended with difficulty, and was poorly resolved by electrophoresis through native agarose gels because aggregated material was trapped in the well (Fig. 1). Extraction of berries with mild detergents (Hughes and Galau, 1988) prior to centrifugation through a CsCl cushion (Baker et al., 1990) yielded RNA with substantially less contaminants. However, subsequent phenol extraction resulted in a nearly insoluble pellet as well, with loss of RNA (Table I). Further, attempts to denature the RNA secondary structure for northern-blot analysis resulted in a gelatinous mass that could not be drawn through micropipettes. Thus, even the small amount of contaminating material was sufficient to aggregate RNA and thwart analysis by gel electrophoresis because significant and variable amounts of ethidium-bromide stained material remained in the sample well (Fig. 1). Thus, none of the reported procedures yielded RNA that could be reproducibly analyzed by gel electrophoresis, nor was this RNA efficiently utilized for translation *invitro* by rabbit reticulocyte ribosomes (data not shown).

We determined that the contaminating material could be selectively precipitated by low concentrations (10 to 40%) of ethanol at room temperature. The optimal extraction of contaminant and minimal loss of RNA was achieved by addition of 95% ethanol to a final concentration of 30% (Table I). At 20°C, the contaminating material formed a gelatinous pellet, whereas greater than 90% of the RNA remained in the supernatant. All of the RNA was reproducibly resolved by gel electrophoresis with no detectable loss retained in the sample well (Fig. 1). Individual RNA species were resolved by probe hybridization to northern blots of either formaldehyde gels (Fig. 2) or glyoxal gels (data not shown). The single bands obtained by hybridization with ^{32}P-labeled aldolase or alcohol dehydrogenase cDNA indicate that little degradation of the RNA had occurred in the extraction process (Fig. 2 panels B and C, respectively). Further, this RNA is biologically active: these RNA samples were efficiently translated *in vitro* by rabbit reticulocyte ribosomes yielding

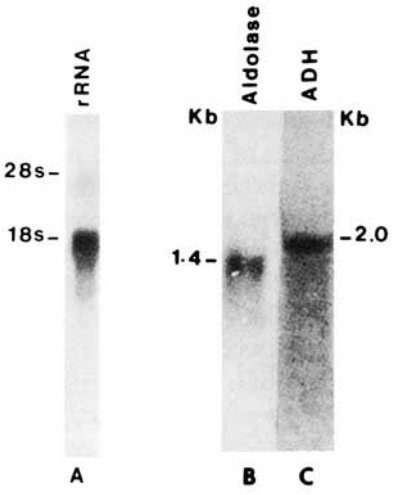

Fig. 2. Autoradiogram of northern blots probed by hybridization with [^{32}P]-labeled cDNA. Panel A: hybridization with labeled 28S and 18S rDNA from potato tuber (10 μg grape RNA per lane). Panel B: hybridization with labeled aldolase cDNA from maize (20 μg grape RNA per lane). Panel C: hybridization with labeled alcohol dehydrogenase cDNA from petunia (20 μg grape RNA per lane). Sizes indicated are relative to marker RNAs of known length (BRL).

numerous, distinct [^{35}S]-methionine-labeled products ranging in molecular mass from less than 10 kDa to greater than 80 kDa (Fig. 3). The incorporation of label into TCA-precipitable products was 2 to 5 fold over background using 5 μg of total grape berry RNA. Thus, the procedure described here selectively removes the contaminating substances which previously prevented RNA isolation from grape berries, without significant loss of material or biological activity.

Discussion

We have succeeded in purifying reasonable quantities (0.2 to 0.3 mg RNA per 10 g wet wt. tissue) of RNA from grape berries without contaminating substances which interfere with assay of RNA. Procedures which employ organic solvents weakly miscible in water, such as phenol, or chaotropic agents, such as guanidinium chloride, cause aggregation of the contaminating substances with RNA (Table I, Fig. 1). This aggregation was observed in the only other reported isolation attempt of RNA from grape tissues, using leaves (Newbury and Possingham, 1977). These investigators concluded that the contaminants which absorbed strongly at 280 and 230 nm were complexed tannins, because of their mobility in a TLC plate assay.

We conclude from our observations that substances such as phenol, guanidinium chloride, DMSO, formaldehyde and formamide cause precipitation of the contaminant. These substances alter the hydrophilicity of the solvent causing weakly soluble materials to fall out of

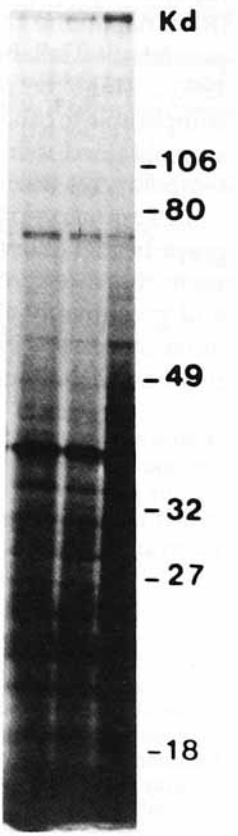

Fig. 3. Translation products of purified grape berry RNA. Translation products were electrophoresed directly (*c*), or incubated at 30° for an additional 6 h (*6*) or 24 h (*24*).

solution. These substances apparently bind to or trap RNA in the precipitation process. By contrast, detergents which in low concentration effect denaturation of proteins and membranes by association with hydrophobic domains do not cause aggregate formation with RNA. The modest success reported by Newbury and Possingham (1977) was probably due to their use of 5% SDS in the extraction buffer. The chaotropic reagent thiourea, used in our procedure, is in low concentration, 5 mM, that apparently does not disrupt solvent structure, and does not promote aggregate formation. The behavior of the interfering substances in various solutions implies that the contaminating material is water soluble but with considerable hydrophobic character, such as tannins. Nevertheless, charged carbohydrates also may be present in the aggregate because the viscous material can be partially solubilized by incubation with pectinase (data not shown).

It was consistently noted that 28S ribosomal RNA was underrepresented in the purified RNA. This is unlikely to be due to preferential loss of a particular RNA species, but rather is a characteristic of the maturity stage of the berry. The same observation has been made by Rattapanone et al. (1977) in mature tomato fruits. Further, the relative amount of 28S and 18S ribosomal RNA recovered from potato tubers and from grape cell culture using the modified technique presented here (Fig. 1) was the same as that obtained using the Shirras and Northcote (1984) procedure.

The successful isolation and translation of RNA from grape berry tissues has not been reported previously, presumably due to the interference by the aggregating contaminant. The yield of total RNA, approximately 20 to 30 μg per gram of fresh weight tissue, is lower than that

reported for avocado fruit (Christoffersen et al., 1982) and tomato fruit (Rattanapanone et al., 1977), but is similar to that reported for ripening peach fruit (Callahan et al., 1989) and mature apple fruit (Lay-Yee et al., 1990). Thus, the procedure yields noncontaminated RNA in amounts comparable to other ripening fruits. No significant increase in RNA yield was obtained using higher concentrations of detergents, or increased temperatures during extraction.

In summary, our procedure for the isolation of RNA from mature grape berries allows the isolation and analysis of RNA free from contaminants that interfere with northern-blot hybridization, translation *in vitro* and presumably cDNA cloning. This procedure may be applicable to other systems with high concentrations of tannins and carbohydrates that have been recalcitrant to molecular analyses.

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References

Baker,S.S., C.L. Rugh, and J.C. Kamalay. 1990. RNA and DNA isolation from recalcitrant plant tissues. BioTech. 9:268-272.

Butler, W.O.,L. Cook, and M.E. Vayda. 1990. Hypoxic stress inhibits multiple aspects of the potato tuber wound response. Plant Physiol. 93: 264-270.

Callahan, A., P. Morgens, and E. Walton. 1989. Isolation and *in vitro* translation of RNAs from developing peach fruit. Hort. Sci.24:356-358.

Christoffersen, R.E., E. Warm, and G.G. Laties. 1982. Gene expression during fruit ripening in avocado. Planta 155:52-57.

Grierson, D., A. Slater, and G.A. Tucker. 1985. The appearance of polygalacturonase mRNA in tomatoes: one of a series of changes in gene expression during development and ripening. Planta 163: 263-271.

Hallick, R.B., B.K. Chelm, P.W. Gray, and E.M. Orozo. 1977. Use of aurintricarboxylic acid as inhibitor of nucleases during nucleic acid isolation. Nucleic Acids Res. 4: 3055-3064.

Hughes, D.W. and G. Galau. 1988. Preparation of RNA from cotton leaves and pollen. Plant Mol. Biol. Rep. 64: 253-257.

Ku L.L. and R.J. Romani. 1970. The ribosomes of pear fruit. Their synthesis during the climacteric and the age-related compensatory response to ionizing radiation. Plant Physiol. 45: 401-407.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lay-Yee, M., D. Della-Penna, and G.S. Ross. 1990. Changes in mRNA and protein during ripening in apple fruit (*Malus domestica* Borkh. cv Golden Delicious). Plant Physiol. 94: 850-853.

Logemann, J., J. Schell, and L. Willmitzer. 1986. Improved method for the isolation of RNA from plant tissues. Anal. Biochem. 163: 16-20.

Newbury, H.J., and J.V. Possingham. 1977. Factors affecting the extraction of intact ribonucleic acid from plant tissues containing interfering phenolic compounds. Plant Physiol. 60: 543-547.

Rattanapanone, N., D. Grierson, and M. Stein. 1977. Ribonucleic acid metabolism during the development and ripening of tomato fruits. Phytochem. 16: 629-633.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. In: *Molecular Cloning. A Laboratory Manual.* Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shirras, A. and D. Northcote. 1984. Molecular cloning and characterization of DNA complementary to mRNA from wounded *Solanum tuberosum* tuber tissue. Planta 162: 353-360.

Speirs, J., C.J. Brady, D. Grierson, and E. Lee. 1984. Changes in ribosome organization and messenger RNA abundance in ripening tomato fruits. Aust. J. Plant Physiol. 11: 225-253.

Tesniere, C. and J.P. Robin. 1991. Two-dimensional electrophoresis of the total polypeptides in ripe red grape berries. Electrophoresis (in press).

Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77: 5201-5205.