Phytochemistry 1975, Vol 14, pp 2563–2567 Pergamon Press Printed in England

EXTRACTION AND PURIFICATION OF *t*RNA FROM FRUIT TISSUES

ROGER J. ROMANI, BONITA V. SPROULE, IRVIN J. METTLER and SUE E. TUSKES
Department of Pomology, University of California, Davis, California, U S A

(*Revised received* 31 *May*, 1975)

Key Word Index—Fruit tissues, transfer RNA, aminoacyl-*t*RNA synthetases, acidic tissues

Abstract—Readily measurable yields of undamaged *t*RNA were obtained from tomato, pear and apple fruits by phenolic extraction at pH 8·8, removal of interfering alcohol insoluble substances by precipitation with 0·2 volumes of *iso*-PrOH and final purification by DEAE chromatography. Various other commonly used extraction and purification procedures were tested and found to be less effective Active synthetases were isolated from acidic fruit tissues by adequate control of pH and maceration in the frozen state. After acylation with a radioactively labelled amino acid, fruit isoacceptor *t*RNA species were separated by reverse phase chromatography.

INTRODUCTION

The involvement of *t*RNA in the translational control of protein synthesis [1–3] has led to the proposal [4] that *t*RNA and *t*RNA synthetases may play a key role in controlling senescent processes Evidence in support of this hypothesis has been recently reviewed by Andron and Strehler [5].

Determinant plants and certain plant organs provide suitable systems for the study of cellular senescence [6] Work has already appeared that establishes a relationship between changes in some *t*RNA species and the senescence of soybean cotyledons [7,8] and wheat leaves [9]. However, similar investigations have not been conducted with fruit tissues whose well-characterized ripening and senescent phase has been especially useful in studies of senescence [10,11]. The absence of such investigations may be attributed, in part, to difficulties encountered in extracting functional *t*RNA and *t*RNA synthetases from acidic fruit tissues. That the successful extraction of *t*RNA from plant tissues requires modification of existing techniques is attested to in the comprehensive review of Lea and Norris [12] and in the discussion of applicable methodology be Vanderhoef *et al* [13]. This paper describes the further development of methods specifically required for the isolation of active *t*RNA from tomato, pear, and apple tissues. A preliminary report has appeared [14].

RESULTS AND DISCUSSION

As noted by Goren [15] the extraction of RNA from fruit tissue is complicated by several factors, notably (1) low levels of RNA, (2) high levels of RNase and (3) interference from alcohol insoluble substances (AIS). The difficulties are compounded when one attempts to extract functional *t*RNA (capable of being aminoacylated) from especially acidic fruit. Tomato fruit wall and septal tissues were chosen as test material for the development of extraction techniques since these tissues present the several deterrents enumerated above. Moreover, tomato fruits are conveniently available throughout the year and display a climacteric ripening (ageing) sequence [16] in common with many other fruit species.

In an empirical testing of several phenolic extraction procedures derived from the method of Zubay [17], only two resulted in functional *t*RNA, viz. the sodium naphthalene–1,5-disulphonate procedure of Hastings and Kirby [18],

Table 1. Relationship between conservation of *r*RNA and functional status of the *t*RNA

Extraction method	*r*RNA/*t*RNA	Chargeability* CPM/OD *t*RNA
Phenol pH 7.4	0.27	762
Phenol pH 8.8	4.03	13000
Na$_2$ naphthalene-1,5-disulphonate	1.83	10200

*Chargeability determined after DEAE purification

and the procedure of Kelmers *et al* [19]. As evident in Table 1, the latter proved successful only when the recommended pH 7.5 was raised to 8.8—a step suggested by the low pH maxima of most RNases and the known stability of *t*RNA at pHs as high as 10. Alkaline conditions (pH 8–8.8) have also been shown to facilitate the extraction of polyribosomes [20] and total RNA from plant tissues [15].

Methylated albumin-Kieselguhr (MAK) chromatography [21], used to quantitate the products of the extraction procedure, revealed a correlation between the conservation of *r*RNA and chargeability of the *t*RNA (Table 1). The conservation of *r*RNA proved a sensitive and useful index to adequate protection of *t*RNA. As seen in Table 2, both the yield of *t*RNA and conservation of *r*RNA are maximized at or near pH 8.8. After undergoing the purification steps outlined below, the yield of *t*RNA was roughly 1.5 mg per 100 g tomato wall and septal tissue.

As noted by Vanderhoef *et al.* [13], *t*RNA extracted by the phenolic procedure must be purified further to maximize aminoacylation. This is especially true when detergent is used to increase extraction efficiency. Several known purification procedures were tested. As evidenced by the MAK profiles of the resultant "purified" product (Fig. 1) and the yield and chargeability data

Table 2. Yield of unpurified *t*RNA and conservation of tomato *r*RNA as a function of extraction pH

	Yield (µg)*		
pH	*t*RNA	*r*RNA	*r*RNA/*t*RNA
6.5	98	123	1.25
7.0	135	250	1.84
8.0	163	366	2.26
8.8	167	450	2.72
9.2	185	405	2.20

* These quantities of RNA were extracted from 10 g of tomato tissue

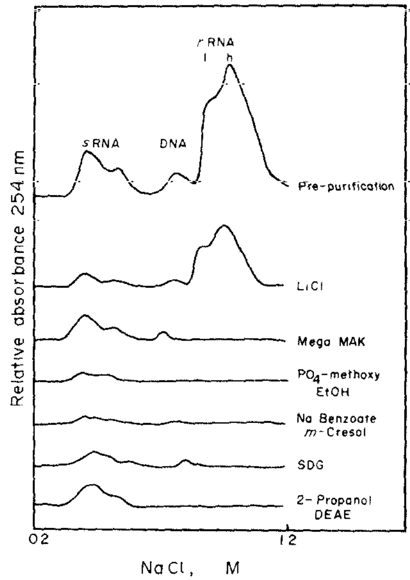

Fig. 1. MAK chromatographic profiles of tomato nucleic acids found in the alcoholic precipitate after phenolic extraction (pre-purification) and after partial purification by several different procedures including use of LiCl [30]; large column (mega) MAK chromatography [21]; PO$_4$-2-methoxyethanol [22]; sodium benzoate *m*-cresol [18]; sucrose density gradient centrifugation (SDG), and *iso*-PrOH (0.2 vol) fractionation followed by DEAE chromatography.

(Table 3), all methods have serious drawbacks. Among the 3 procedures that yield some functional tomato fruit *t*RNA, the Na-benzoate *m*-cresol procedure [18] and the PO$_4$-2-methoxyethanol method [22] result in excessive loss of *t*RNA. DEAE-cellulose chromatography is clearly the superior method, but DNA and other AIS that coprecipitate with the RNA interfere and make it difficult, and often impossible, to use DEAE.

Sein *et al* [23] describe a *iso*-PrOH fractionation step designed to remove interfering substances from crude extracts of rat liver *t*RNA. However, the recommended use of 0.54 vols of *iso*-PrOH not only removed a major portion of

Table 3. Recovery, purity and chargeability of tomato *t*RNA as a result of various purification procedures

Purification procedure	*t*RNA recovered	Chargeability CPM/OD *t*RNA
iso-PrOH DEAE	45	13000
LiCl	34	0
Density gradient	18	0
Mega-MAK	50	190
Na benzoate *m*-cresol	20	1920
PO$_4$-2 methoxyethanol	20	975

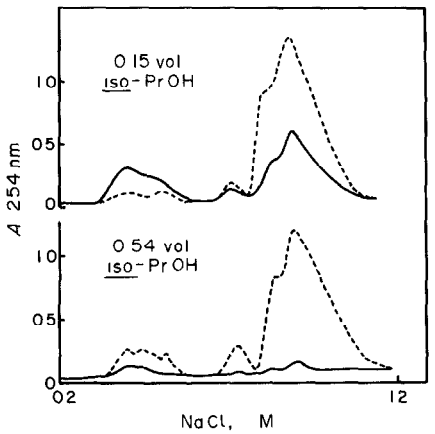

Fig 2 MAK chromatographic profiles of the tomato nucleic acids remaining (——) and discarded (– – – –) after fractionation with either 0 15 or 0 54 vol of *iso*-PrOH

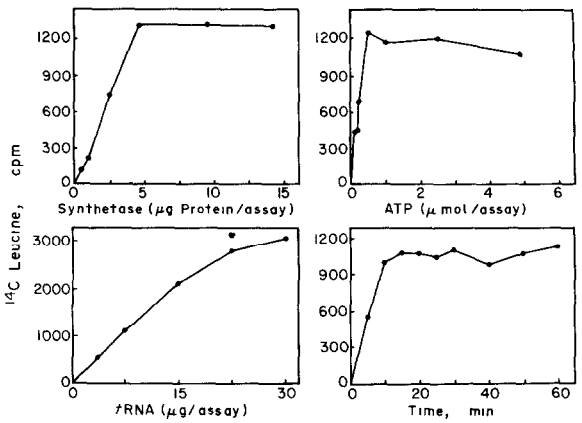

Fig 3 Amino-acylation of tomato *t*RNA with ^{14}C-leucine as a function of synthetase (upper left), ATP (upper right), *t*RNA (lower left) and incubation time (lower right)

the interfering material but also *ca* 80% of the tomato *t*RNA (Fig 2, Table 4). Such a loss is unacceptable when working with the minute amounts of *t*RNA present in fruit tissue. Testing various proportions (Table 4) revealed that 0 15 to 0 2 parts *iso*-PrOH remove a sufficient amount of *r*RNA, DNA, and other AIS to permit DEAE chromatography without incurring large losses of *t*RNA. A nearly similar and equally acceptable fractionation was obtained with the NaOAc procedure recommended by Vanderhoef *et al.* [13]. However, we favor the *iso*-PrOH method as it does not require an additional 12 or more hr for precipitation of the interfering substances.

Extraction of synthetase. As with *t*RNA, it is absolutely essential that tissue acids be counteracted with equivalent amounts of Tris or base. Moreover, grinding of both the frozen tissue and extraction buffer at liquid N_2 temperature assures a co-mingling of cell contents with protective agents, rendering the latter immediately effective upon thawing. Detailed analyses of the extraction procedures were not made. However, by following the precautions noted above and in the experimental section, active and reasonably stable (4–6 weeks at −20°) synthetases were isolated from both tomato and pear fruit

Table 4 Fractionation of *t*RNA with *iso*-PrOH

Vol of *iso*-PrOH	% of original *t*RNA	% of original *t*RNA remaining in "purified" *t*RNA	Purification factor % *t*RNA/% *t*RNA
0	100	100	1
0 15	78	25	3 1
0 25	64	24	2 7
0 35	51	23	2 2
0 45	43	5 3	8 1
0 54	20	2 4	8 4

Aminoacylation and separation of specific tRNA species. Established procedures [19] were found suitable for the acylation of fruit *t*RNA. As is the common practice Mg, ATP, enzyme, and *t*RNA concentrations were adjusted to maximize the functions of each aminoacyl-*t*RNA synthetase. The results of tests with tomato $tRNA^{Leu}$ shown in Fig. 3 display the commonly observed kinetics for these reactions. The stability of acylated *t*RNA with time (lower right graph) implies the absence of deacylase in the enzyme preparation.

Shown in Fig. 4 are reverse-phase chromatography (RPC-6) profiles of tomato $tRNA^{Leu}$, and apple and pear $tRNA^{Lys}$ which had been isolated, purified, and acylated as described above The apple and pear *t*RNA were both charged with pear enzyme. Three iso-acceptor $tRNA^{Leu}$ and 4 $tRNA^{Lys}$ are clearly discernible. The use of a small (5 × 120 mm) column facilitated the identification of *t*RNA species from the equivalent of 10 g of fruit tissue.

CONCLUSION

It is clear from these studies that the methods normally used to isolate *t*RNA and synthetase from mammalian and microbial tissues can, with

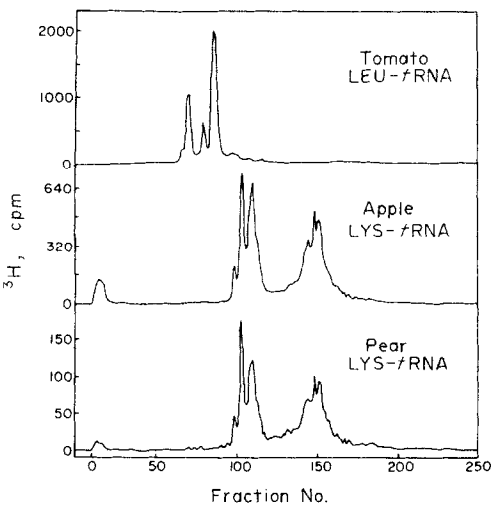

Fig. 4. Iso-acceptors of tomato $tRNA^{leu}$, and apple and pear $tRNA^{lys}$ separated by reverse-phase chromatography. Elution gradients were 0·3 M → 0·75 M NaCl for leucyl-*t*RNA and 0·25 M → 0·85 M NaCl for lysyl-*t*RNA.

some necessary modifications, be used successfully with fruit tissues. In view of the difficulties often encountered in the isolation of active enzymes, RNA, and other cellular components from acidic fruit tissues, we believe the methods described herein should prove advantageous with a variety of other less intractable plant materials. The application of these methods to a study of ripening and aging of tomato fruit is described in the accompanying paper.

EXPERIMENTAL

Tissue. Tomatoes (*Lycopersicon esculentum*, cv Ace), pears (*Pyrus communis*, cv Bartlett or Williams), and apples (*Malus sylvestris*, cv Gravenstein) were obtained from the U.C. Experimental farm or from local markets. Mature green tomatoes were cored and locular contents removed leaving only the walls and septa. Mature but not fully ripened pears and apples were cored and peeled. Fruit tissues were immediately frozen in liquid N_2, broken into small pieces and stored at −78°. Before use, a representative 100 g sample of each tissue was combined with *ca* 100 ml H_2O, macerated in a blendor for 1 min and titrated to pH 8·8 with 1 M Tris (pH 11·1–11·5).

Extraction of tRNA. The procedure is a modification of the phOH methods of Zubay [17] and Kelmers *et al.* [19]. 100 g of pHOH prepared by dissolving white crystalline PhOH in ing of 50 mM Tris–HCl (pH 8·8), 10 mM β-mercaptoethanol (–SH), 1 g SDS, 0·5 g bentonite, additional 1 M Tris to bring the pH of the tissue to 8·8 (based on prior titration) and 300 ml of PhOH prepared by dissolving white crystalline PhOH in 50 mM Tris–HCl, pH 8·8 (88% w/v). The mixture was blended at high speed for 5 min, with 4-ml 1 M $MgCl_2$ added after the first min. Blending was continued at a low speed for 25 min and liquid N_2 added periodically to maintain the mixture at or near room temp. Dry-ice should not be used to control temp for it may result in pH reduction.

After centrifugation of the mixture at 12000 *g* for 10 min, the aq. layer was withdrawn and treated once again with 200 ml of satd phOH by blending at low speed for 10 min. The aq. layers were combined, 0·1 vol of 20% (w/v) KOAc and 2 vol of cold EtOH were added and the contents placed at −20° for 18 hr. The resultant ppt. containing RNA, DNA, and other AIS was collected by centrifugation for 10 min at 10000 *g*.

Purification of tRNA. A modification of the *iso*-PrOH method of Sein *et al.* [23] was used to remove portions of *r*RNA, DNA, and AIS. The ethanolic ppt was first dispersed in cold 0·3 M NaOAc (pH 7) using a glass homogenizer. The soln was then quickly brought to room temp, 0·15 vol of *iso*-PrOH added with mixing, centrifuged (5 min at 8000 *g*) at room temp., and the ppt discarded. Additional cold *iso*-PrOH (1·5 vol) was then added to the supernatant fraction and the partially purified *t*RNA was allowed to ppt for 18 hr at −20°. Final purification involved the use of a 13 mm diam. column packed with 2 g of DEAE cellulose (BioRad. cellex D, 0·92 meq/g) which had been dispersed in 1 M NaOH, rinsed with H_2O to neutrality, and titrated to pH 3 with HCl in the presence of added NaCl (*ca* 0·25 M). The column was equilibrated at 0° with a soln of 50 mM NaCl and 1 mM –SH. A similar soln was used to dissolve the crude RNA ppt. which was then loaded onto the column and eluted with 0·2 M NaCl + 1 mM –SH until the effluent no longer absorbed at 254 nm. The *t*RNA was then eluted with 0·65 M NaCl + 1 mM –SH. H_2O (0·5 vol) was added to reduce the NaCl concn. followed by 2 vol of cold EtOH to ppt. the *t*RNA at −20° for 18 hr. The final ppt. was taken up in a small amount of 10 mM Tris (pH 7·4), 10 mM $MgCl_2$, 1 mM –SH, and either charged with an amino acid or held at −78°. As a result of the extraction at pH 8·8 additional "stripping" was not necessary. Yields were estimated on the assumption that A254 nm for 1 mg *t*RNA = 20.

Preparation of synthetase. The procedure followed closely that described in ref [24] with the exception that 20 mM –SH was added to all solns and the tissue was macerated in the frozen state. 100 g of frozen fruit tissue, enough M Tris to give a final pH of 8 (based on prior titration), and 100 ml of extraction buffer (10 mM Tris–pH 8, 10 mM $MgCl_2$, 20 mM –SH, and 10% glycerol) were placed in a mortar and kept frozen with liquid N_2 as the mixture was ground to a fine powder. After thawing, but while a few ice crystals still remained, the homogenate was transferred to tubes and centrifuged at 30000 *g* for 10 min. All subsequent steps were done at 0°.

The resultant supernatant fraction was filtered through glass wool and centrifuged at 105000 *g* for 3 hr. The final supernatant fraction was loaded onto a 12 mm diam. column containing 3 g of DEAE cellulose (BioRad. cellex D washed according to ref [25]) and equilibrated at 0° with buffered soln (20 mM buffer—pH 7·5, 1 mM $MgCl_2$, 20 mM –SH and 10% glycerol). The same soln was used to elute all material absorbing at 280 nm. Synthetases were then eluted from the DEAE cellulose with a soln containing 0·25 M KPi buffer (pH 6·5), 1 mM $MgCl_2$, 20 mM –SH and 10% glycerol. The resultant enzyme soln was dialyzed for 18 hr in 1 l. of 1 mM KPi buffer (pH 6·8), 20 mM –SH, 10% glycerol and 15% polyethylene glycol (MW 6000), utilizing dialysis tubing previously boiled in 1 mM EDTA. Additional glycerol, to 20% (v/v), was added to the final enzyme soln prior to storate at −20° where the leucyl-, tyrosyl-, lysyl-, methionyl-, and phenylalanyl-*t*RNA synthetase components were stable for several weeks. Enzyme protein

was determined by a modified Lowry procedure [26] with 5× crystallized egg albumen as a standard

Aminoacylation of tRNA. The acylation procedure is essentially that described in ref [19], with minor modifications The 0 25 ml reaction mixture contained 20 μmol Tris (pH 7 5), 10 μmol -SH, 3 5 μmol KCl, 5 μmol $Mg(OAc)_2$, 0 5 μmol ATP, 0 2–0 5 μμmol amino acid labelled with either ^{14}C (250–450 μCi/μmol) or ^{3}H (2–20 mCi/μmol) and *ca* 3 μg *t*RNA Optimum Mg, ATP, and enzyme concentrations were determined for each amino acid The reaction was initiated with the addition of 20–40 μg synthetase and the mixture incubated at 30° for 30 min A 200 μl portion was then transferred onto 2 3 cm filter paper (Whatman 3) discs (numbered and affixed on pins as described in ref [27]) and exposed to an IR lamp Before reaching absolute dryness, which results in high background cpm, the discs were placed in 10% TCA at 0° and rinsed for additional 10 min periods, twice in 5% TCA, once in 70% EtOH, and once in Et_2O The discs were dried under IR and then counted in a liquid scintillation spectrophotometer

For co-chromatography of pre-charged *t*RNA species, the requisite amounts were acylated via a 20 to 50× scale-up of the above procedure Individually charged ^{14}C and ^{3}H *t*RNAs were then combined and precipitated with 2 vol EtOH for 18 hr at −20°

Reverse-phase chromatography Both RPC-2 [28] and RPC-6 [29] procedures with the columns reduced to 5 × 120 mm permitted the separation of small quantities of fruit *t*RNA The chromatographic separation was done at room temp with 500 ml eluting buffer containing 10 mM $Mg(OAc)_2$, 10 mM HOAc (pH 4 5), 2 mM $Na_2S_2O_3$ and 0 02% NaN_3 (to prevent bacterial action—L Waters, personal comm), plus the NaCl gradient as indicated for each amino-acyl-*t*RNA Flow rate was 0 2 ml/min 2 ml fractions were collected in test tubes and held at 0° To determine cpm, 50 μg of 'carrier' yeast RNA and 0 5 ml of 52% TCA were added per tube and left for 2 hr at 0° The RNA was collected on 0 45 μm membrane filters (Schleicher & Schuell, No. B-6, 22 mm diam), washed with 5% TCA then with 70% EtOH, dried, and counted by liquid scintillation In all instances the H_2O was first deionized and then glass dist from 1 mM $KMnO_4$ in 0 5 mM NaOH before use

Acknowledgements—One of us (RJR) benefited from the counsel and encouragement of Dr David Novelli and Dr Larry Waters in preliminary studies conducted at the Oak Ridge National Laboratory during tenure of an NSF Fellowship

REFERENCES

1 Stent, G S (1964) *Science* **144**, 816
2 Neidhardt, F (1966) *Bacteriol. Rev.* **30**, 701
3 Novelli, G (1967) *Ann Rev Biochem* **36**, 449
4 Strehler, B L , Hendley, D D and Hirsch, G P (1967) *Proc. Nat Acad Sci* (U S.) **57**, 1751
5 Andron, L A and Strehler, B L (1973) *Mechan Ageing Develop* **2**, 97
6 Leopold, A C (1961) *Science* **134**, 1727
7 Venkataraman, R and De Leo, P (1972) *Phytochemistry* **11**, 923
8 Bick, M D , Liebke, H , Cherry, J H and Strehler, B L (1970) *Biochim Biophys Acta* **204**, 175
9 Shugart, L (1972) *Exp Geront* **7**, 251
10 Biale, J B (1964) *Science* **146**, 880
11 Sacher, J A (1973) *Ann Rev Plant Physiol* **24**, 197
12 Lea, P J and Norris, R D (1972) *Phytochemistry* **11**, 2897
13 Vanderhoef, L N , Bohannon, R F and Key, J L (1970) *Phytochemistry* **9**, 2291
14 Romani, R J and Sproule, B (1972) *Plant Physiol* **49** (suppl), 12
15 Goren, R (1971) *J Am Soc Hort Sci* **96**, 10
16 Workman, M and Pratt, H K (1957) *Plant Physiol* **32**, 330
17 Zubay, G (1962) *J Mol Biol* **4**, 347
18 Hastings, J R B and Kirby, K S (1966) *Biochem J* **100**, 532
19 Kelmers, A D , Novelli, G D and Stulberg, M P (1965) *J Biol Chem* **240**, 3979
20 Breen, M D , Whitehead, E I and Kenefick, D G (1971) *Plant Physiol* **49**, 733,
21 Mandell, J and Hershey, A (1966) *Anal Biochem* **1**, 66
22 Kirby, K S (1956) *Biochem J* **64**, 405
23 Sein, K T , Becarevic, A and Kanazir, D (1969) *Anal Chem* **28**, 65
24 Muench, K and Berg, P (1966) In *Procedures in Nucleic Acid Research*, (Cantoni, G L and Davis, D R , eds) pp 375–383
25 Peterson, E A and Sober, H A (1962) In *Methods of Enzymology* V (Colowick, S P and Kaplan, N. O , eds) pp 3–26
26 Miller, G L (1959) *Anal Chem* **31**, 946
27 Bollum, F J (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G L and Davies, D R , eds), pp 296–300
28 Weiss, J F and Kelmers, A D (1967) *Biochemistry* **6**, 2507
29 Pearson, R L , Weiss, J F and Kelmers, A D (1971) *Biochim Biophys Acta* **228**, 770
30 Avital, S and Elson, D (1969) *Biochim Biophys Acta* **179**, 297