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# PHENYLALANINE *t*RNA OF *LUPINUS LUTEUS* SEEDS

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**Key Word Index**—*Lupinus luteus*, Legminosae, lupin, isolation and purification, phenylalanine, $tRNA^{Phe}$

**Abstract**—Two isoaccepting $tRNA^{Phe}$ were isolated from yellow lupin seeds by DEAE-cellulose, BD-cellulose and reversed phase chromatography The products obtained were characterized by aminoacylation and fluorescence The chromatographic behaviour and some properties of the isolated *t*RNAs are discussed and compared with the known $tRNA^{Phe}$ from other sources

## INTRODUCTION

TRANSFER RNA from higher plants are less well studied than those isolated from bacteria, yeast, or animal tissues. This is due mainly to the difficulties in purification of the plant *t*RNA: the methods of *t*RNA isolation developed for bacteria or yeast[1,2] had to be modified to give satisfactory results with plant material[3–7] As a result, the only plant *t*RNA of known primary structure is $tRNA^{Phe}$ from wheat germ,[3] in spite of the great interest in the possible participation of *t*RNA in cellular regulation.[8]

The known $tRNA^{Phe}$ from wheat germ is one of the two phenylalanine isoacceptors shown by Yoshikami *et al.*[9,10] The two isoacceptors differ only in the fluorescing base in the position adjacent to the anticodon The presence of multiple $tRNA^{Phe}$ is rather a common feature for eucaryotes[11–15] and recently Hiatt and Snyder[16] demonstrated nine $tRNA^{Phe}$ species in barley seedlings. This report describes the isolation and purification of two $tRNA^{Phe}$ from *Lupinus luteus* seeds.

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## RESULTS AND DISCUSSION

The procedure used for the preparation of crude *t*RNA from lupin seeds involved successively phenol extraction, isopropanol fractionation, deproteinisation with the chloroform–isoamyl alcohol mixture and finally DEAE-cellulose chromatography as described by Hancher *et al*[17] The yield of crude *t*RNA was about 350 mg/kg of lupin seeds The preparations showed 18–19 $A_{260}$ units/mg and contained small amount of high M W. RNA (as judged by 5% polyacrylamide gel electrophoresis) and less than 2% of protein. The average phenylalanyl acceptance of the selected fractions from DEAE-cellulose column was 90 pmol per $A_{260}$ unit (Table 1).

The procedure described gave a similar yield of crude *t*RNA as the procedure of Vanderhoef and Key[5] in which advantage is taken of different solubility of high and low-MW RNA in 3 M NaOAc Crude *t*RNA from lupin seeds obtained by this method contained, however, more contamination (mostly carbohydrates) than *t*RNA prepared as indicated above

Among diverse methods reported for purification of $tRNA^{Phe}$ the BD-cellulose chromatography[18,19] proved to be the most effective. This chromatographic step was used for isolation of $tRNA^{Phe}$ also from plant material [3,10] As shown in Fig 1 the crude *t*RNA from lupin seeds separated on BD-cellulose into two peaks of phenylalanine acceptor activities. The main one is eluted at about 1 M NaCl and the second one in the EtOH fraction The proportions of the two peaks varied for different crude *t*RNA preparations, but the average ratio was about 7 : 1

The presence of two $tRNA_s^{Phe}$ was checked by BD-cellulose chromatography of the crude *t*RNA aminoacylated with [$^{14}$C]-phenylalanine: the charged *t*RNA was applied on BD-cellulose and the column was successively washed with 0·3, 1 M NaCl and 1 M NaCl containing 20% of EtOH buffered to pH 4·5 About 70% of the total [$^{14}$C]-Phe-*t*RNA ($tRNA_1^{Phe}$) was eluted with 1 M NaCl and about 30% ($tRNA_2^{Phe}$) with the ethanolic solvent The two $tRNA_s^{Phe}$ also eluted separately when a sample of crude *t*RNA, heated at 80° and then slowly cooled, was similarly aminoacylated and run on the same BD-cellulose column This indicates that $tRNA_1^{Phe}$ is not a conformer of $tRNA_2^{Phe}$ or vice versa Lack of the expected shift of Phe-$tRNA_1^{Phe}$ from salt into ethanol fraction[18] indicates that the overall structure-dependent ionic interactions and not the hydrophobic ones are the decisive factor of *t*RNA behaviour during BD-cellulose chromatography Friedman[20] who investigated, the chromatographic properties of *t*RNA acylated with *N*-hydroxysuccinimide ester of phenoxyacetic acid come to a similar conclusion

Phenylalanine accepting portions of effluents from a preparative BD-cellulose column were pooled as indicated in Fig. 1 The amino acid acceptor activities of the pooled fractions were not high when compared with appropriate values reported for $tRNA^{Phe}$ from other sources. The average (four runs) amino acid acceptance was 245 and 180 pmol per $A_{260}$ unit for $tRNA_1^{Phe}$ and $tRNA_2^{Phe}$ respectively Gel electrophoresis of both the $tRNA^{Phe}$ fractions showed besides the main 4S band also a small band of high MW RNA and sometimes some hydrolysis products due to incidentally introduced ribonucleases The EtOH fraction contained more high MW contamination than the 1 M NaCl fraction of $tRNA^{Phe}$

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The presence of similar inactive material in the EtOH fraction was observed also by other authors.[19] We think that the relatively low acceptor activity of $tRNA^{Phe}$ obtained after BD-cellulose chromatography was due mainly to an aggregation of lupin tRNA. The possible aggregates could not be resolved by heating the tRNA solution to 75° followed by slow cooling.

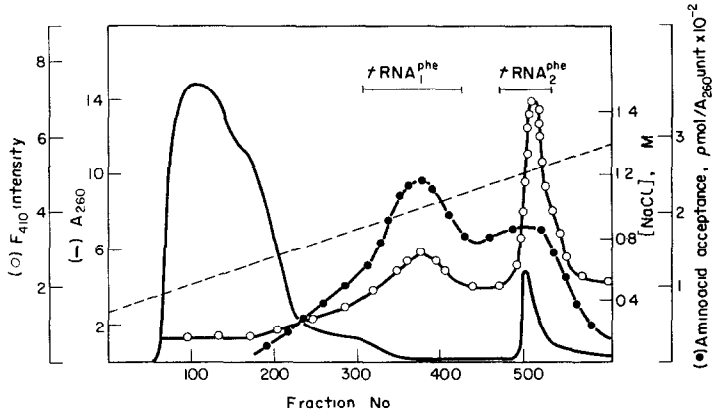


FIG 1 BD-CELLULOSE CHROMATOGRAPHY OF CRUDE LUPIN SEEDS tRNA

6000 $A_{260}$ units were applied (in 60 ml of starting buffer) on a 2 5 × 80 cm BD-cellulose column equilibrated with 0 05 M NaOAc buffer pH 5 containing 0 3 M NaCl and 0 01 M $MgCl_2$ The column was eluted with a linear gradient (5 5 l tot vol) of 0 3–1 2 M NaCl in the same buffer followed by 1 3 M NaCl in the above buffer and 1 3 M NaCl with 20% of ethanol v/v Fractions of 10 ml were collected at a flow rate 1 2 ml/min and assayed for $A_{260}$ absorbance, [$^{14}$C]-phenylalanine acceptance and fluorescence intensity at 410 nm

Although there was little doubt about the presence of two $tRNA_s^{Phe}$ in the crude lupin seed tRNA, the possibility still existed that the EtOH fraction contained much of the salt $tRNA^{Phe}$ fraction due to the tailing of the previous peak. The observed tailing of this fraction resulted in an incomplete separation of the two $tRNA_s$ and therefore for further purification both the salt and the EtOH fractions were combined except for small parts of the fractions left for rechromatography experiments.

Transfer RNA fractions pooled from a BD-cellulose column were precipitated with EtOH, dissolved in and dialysed against 0·01 M Tris–HCl buffer pH 7·5 containing 0·01 M $MgCl_2$ and than applied to a Sephadex G–100 column. As shown in Table 1 this step removed the high MW material and the degradation products and resulted in an increase of amino acid acceptor activity of the tRNA.

tRNA obtained from the Sephadex G-100 column was subjected to reversed phase column chromatography A typical elution profile of tRNA from a RPC-5 column is shown in Fig. 2 Phenylalanine acceptor activity was found in two peaks ($tRNA_1^{Phe}$ and $tRNA_2^{Phe}$). In terms of $A_{260}$ units the recovery of tRNA from this column was nearly quantitative (95%); however, of the 75 nmol of $tRNA^{Phe}$ applied on the column only 45 nmol were recovered. A comparable low yield of specific tRNAs from RPC-5 columns was observed by other authors.[21] In an experiment when [$^{14}$C]-Phe aminoacylated tRNA sample was chromatographed on RPC-5, Phe-$tRNA^{Phe}$ was eluted in two peaks, but measurable radioactivity was found also in the fractions emerging at the void volume of

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the column. This radioactivity, however, was acid soluble and therefore it could not be [$^{14}$C]-Phe *t*RNA. The presence of two peaks of phenylalanine acceptor activities on the elution profiles from RPC-5 column of the uncharged *t*RNA as well as separation of the [$^{14}$C]-Phe aminoacylated samples into two distinct peaks confirmed the BD-cellulose observations that *Lupinus luteus* seeds contain at least two phenylalanine *t*RNA.

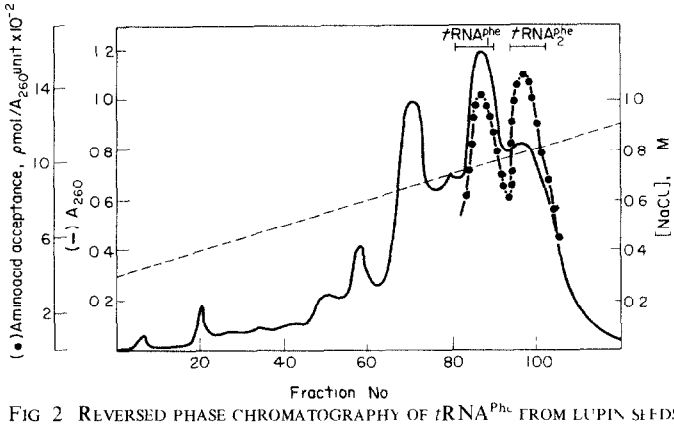


FIG 2 REVERSED PHASE CHROMATOGRAPHY OF *t*RNA$^{Phe}$ FROM LUPIN SEEDS
Transfer RNA$^{Phe}$ rich fractions from BD-cellulose and purified by gel filtration on a Sephadex G-100 column were applied on a 1·2 × 130 cm RPC-5 column. Fractions of 5 ml were collected. For other details see Experimental

To exclude the possibility that the separated *t*RNA$_2^{Phe}$ are mutually transversing conformers, *t*RNA$_1^{Phe}$ was aminoacylated with [$^{14}$C]-Phe and *t*RNA$_2^{Phe}$ with [$^{3}$H]-Phe (or vice versa) and the mixture was co-chromatographed on RPC-5 column. For this experiment the separated fractions both from BD-cellulose and RPC-5 columns were used. The salt *t*RNA$^{Phe}$ from BD-cellulose appeared to be identical with *t*RNA$_1^{Phe}$ from RPC-5, and EtOH *t*RNA$^{Phe}$ from BD-cellulose with *t*RNA$_2^{Phe}$ from RPC-5. Co-chromatography of *t*RNA$_1^{Phe}$ from BD-cellulose with *t*RNA$_2^{Phe}$ from RPC-5 always gave two peaks, one of which was labelled with [$^{14}$C]-Phe the other one with [$^{3}$H]-Phe.

TABLE 1 RECOVERY OF *t*RNA$^{Phe}$ AT VARIOUS STAGES OF ITS PURIFICATION

| Purification stage | $A_{260}$ units | Phe acceptance* pmol per $A_{260}$ unit | Recovery (%) |
|---|---|---|---|
| Crude *t*RNA | 6000 | 90 | 100 |
| Pool after BD-cellulose | 1200 | 200 | 47 |
| Pool after Sephadex G-100 | 640 | 350 | 40 |
| Product after RPC-5 | 90 | - | 24 |
| *t*RNA$_1^{Phe}$ | 58 | 1370 | 15 |
| *t*RNA$_2^{Phe}$ | 32 | 1500 | 9 |

* The aminoacid acceptance values are an average of three sets of triplicate assays. Occasionally the assay was checked by a purified synthetase [21].

The fluorescence emission spectra of both the isolated *t*RNA$_2$ were similar and had emission maxima at 410 nm upon excitation at 310 nm. This value differs from data published for other *t*RNA$^{Phe}$ and also from the maximum observed for the peroxy Y base isolated

[21] JAKUBOWSKI, H. and PAWIŁKIEWICZ, J. (1973) *FEBS Letters* **34**, 150

from lupin *t*RNA.[22] This may reflect the environmental differences of the Y bases free in solution and bound to their parent $tRNA_s$, or the isomerization as suggested by Hancock *et al.*[23] Recently it was shown that lupin *t*RNA contains two Y-type bases, one of which was identified as peroxy-Y and the other was chromatographically similar to Y.[22] The proportions of $tRNA_1^{Phe}$ to $tRNA_2^{Phe}$ and of peroxy-Y to Y (7 : 1 and 9 : 1 respectively) suggest that $tRNA_1^{Phe}$ contains the peroxy-Y base and $tRNA_2^{Phe}$ Y base. This has not been definitely proved.

## EXPERIMENTAL

*Preparation of crude tRNA* Freshly ground seeds (10 kg) of *Lupinus luteus* (var Express) were mixed with 40 l. of dist 80% *PhOH* and 40 l. of 0·1 M acetate buffer 6·8–7·0 containing 2 mM EDTA and 2 mM $Na_2S_2O_3$ and 0·01 M $MgCl_2$. After 24 hr the aq layer was drawn off and again blended with 10 l. of $H_2O$-satd pHOH After separation of the layers cold isoPrOH (1 vol) was added with continuous stirring to the aq sol made 0·3 M with NaOAc buffer, pH 6·5 (4 vol) The resultant ppt was removed by centrifugation and discarded RNA was precipitated from the supernatant by addition of 2·5 vol EtOH cooled to −20° The ppt was pelleted by centrifugation and dissolved in 0·01 M Tris–HCl buffer pH 7·5 containing 0·01 M $MgCl_2$, 10 mM EDTA and 2 mM $Na_2S_2O_3$ An equal vol of $CHCl_3$–isoamyl alcohol (24 : 1) mixture was added with shaking This step was repeated twice *t*RNA was precipitated from the aq layer (made 0·15 M with NaOAc buffer pH 5·2) by addition of 2·5 vol of cold EtOH, collected by centrifugation and deacylated by incubation of its soln in Tris–HCl buffer 1·8 M pH 8·1 for 30 min at 37°[24] Transfer RNA was further purified on DEAE-cellulose in NaCl gradient 0·3–0·65 M as described by Hancher.[17] Selected fractions defined by amino acid acceptance assays were pooled, precipitated with EtOH and *t*RNA was recovered by centrifugation

*Purification of* $tRNA^{Phe}$ $tRNA^{Phe}$-rich fractions from DEAE-cellulose were applied to BD-cellulose BD-cellulose chromatography was carried out at room temp according to Gillam *et al*[18] Fractions from the column were assayed for fluorescence intensity at 410 nm (excitation at 310 nm) and for phenylalanine-acceptor activity Elution was either stepwise or with linear gradient 0·3–1·2 M NaCl followed by 1·3 M NaCl and 1·3 M NaCl containing 20% EtOH The fractions containing $tRNA^{Phe}$ were pooled and mixed with 2·5 vol of cold EtOH The precipitated RNA was collected by centrifugation, dissolved in 0·01 M Tris–HCl buffer pH 7·5 containing 0·01 M $MgCl_2$ and 0·05 M NaCl (1000 $A_{260}$ units) and applied on a 2 × 95 cm Sephadex G-100 column The column was equilibrated with the same buffer and the same buffer was used for elution of RNA The $tRNA^{Phe}$ containing fractions were used for further purification on RPC-5 columns

The packing for RPC-5 chromatography was prepared according to[25] slurried in the equilibration buffer (0·01 M NaOAc, 0·3 M NaCl, 0·01 M $MgCl_2$, pH 4·5) and poured into a 1·2 × 130 cm column The column was run at 25° and a flow rate of 0·2 ml/min was employed. *t*RNA (200 $A_{260}$ units) was dissolved on 10 ml of equilibration buffer (containing 0·18 M NaCl) and pumped onto the column A linear gradient of 0·3–0·9 M NaCl in the same buffer was generated by Ultrograd (LKB, Sweden) with a total vol of 500 ml The gradient elution was completed within 48 hr Alternatively 400 $A_{260}$ units were applied on a 1·5 × 85 cm column with a flow rate 0·5 ml/min and the gradient was completed in 24 hr.

*Analytical co-chromatography* The [$^{14}$C] or [$^3$H]-Phe aminoacylated $tRNA_1^{Phe}$ and $tRNA_2^{Phe}$ was on a 0·7 × 30 cm RPC-5 column equilibrated with 0·5 M NaCl in the same acetate buffer as used for preparative runs A concave gradient of 0·5–0·9 M NaCl was applied, the total gradient vol was 200 ml and 2·5 ml fractions were collected 0·5 ml of each fraction was counted in a Bray's scintillation soln[26]

*The aminoacylation* About 10 $A_{260}$ units of *t*RNA were incubated at 37° in 100 mM Tris–HCl buffer pH 7·5, 2 mM ATP, 2·5 mM 2-mercaptoethanol, 10 mM $MgCl_2$ with 25 nmol of radioactive aminoacid [$^{14}$C]-Phe, 270 mCi/mmol or [$^3$H]-Phe, 1000 mCi/mmol and about 1 mg of enzyme protein prepared according to ref.[27] After 75 min the incubation mixture was extracted 3 × with an equal vol of $H_2O$ satd pHOH followed by 3 extractions with $Et_2O$ *t*RNA was precipitated from the aq phase with 3 vol of cold EtOH The ppt was dissolved in a small vol of 0·01 M NaOAc buffer pH 4·5 containing 0·18 M NaCl, 0·01 M $MgCl_2$ and was dialysed for 6 hr against this buffer and applied on a RPC-5 column

*Assay procedures* The amino acid acceptor activity of *t*RNA was assayed by the filter paper disc method as described[4] One $A_{260}$ unit of unfractionated *t*RNA or 0·05 $A_{260}$ unit of the purified species were incubated for

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30 min in a reaction mixture containing 10 μmol of Tris–HCl buffer pH 7.5, 0.5 μmol of $MgCl_2$, 0.1 μmol of ATP, 0.2 μmol of 2-mercaptoethanol, 3 nmol of [$^{14}$C]-Phe and 0.1–0.15 mg of the crude enzyme protein in a final vol of 100 μl. The filter paper discs containing TCA-insoluble material from the incubation mixture were placed in vials containing 5 ml of toluene scintillation soln and counted in a scintillation counter with an efficiency of 55%.

*Other methods.* Gel (5 or 7%) electrophoresis was performed according to Loening [28]. Protein content was determined by the Lowry method [29]. Fluorescence measurements were conducted at room temp. in a spectrophotofluorimeter fitted with a xenon lamp. Standard quartz 1 × 1 cm light paths cuvets were used. The relative fluorescence readings obtained at 410 nm are called $F_{410}$.

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