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ISOLATION AND PROPERTIES OF THE MAIN ISOLEUCINE tRNAs FROM LUPINUS LUTEUS SEEDS*

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RPC-5 chromatography of [\textsuperscript{14}C]isoleucyl-tRNA shows the presence of 5 tRNA\textsuperscript{Ile} species in lupin seeds. The two major species have been isolated using RPC-5 and Sepharose 4B chromatography. The acceptor activity of the purified tRNAs ranges from 1350 pmoles (tRNA\textsuperscript{Ile}_A) to 1510 pmoles (tRNA\textsuperscript{Ile}_B) of isoleucine per one A\textsubscript{260} unit of tRNA. The two tRNAs have similar thermal denaturation profiles but differ in the composition of the major and minor bases, 3'-terminal nucleotide sequences, and the titration curves with MgCl\textsubscript{2}. It is concluded that the two major tRNA\textsuperscript{Ile} species are coded for by different genes.

During the last years multiplicity of isoaccepting tRNA species was demonstrated in plants (Sueoka & Kano-Sueoka, 1970; Merrick & Dure, 1972; Hiatt & Snyder, 1973; Cornelis et al., 1975). Their number and relative ratios depend upon tissue, age and growth conditions of plants. Variations in the chromatographic profiles of the isoaccepting tRNA species could indicate either changes in the transcription rate of various tRNA genes, or post-transcriptional changes during tRNA maturation, or both. It is not known which of these processes dominates in plants. More information about the structure of different isoaccepting tRNAs should be accumulated to answer these questions.

Lupin seeds contain a considerable amount of tRNA\textsuperscript{Ile} (Legocki et al., 1968a) which can be fractionated into 3 - 4 species by the methods described previously (Legocki et al., 1968b; 1970) or into 5 species by RPC-5 chromatography as shown in the present work. This investigation was undertaken to characterize more fully the main isoleucine tRNAs from lupin seeds and to elucidate whether they were coded for by different genes.

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MATERIALS AND METHODS

General. RPC-5 was obtained from Miles Laboratories Inc. Elkhart (Ind., U.S.A.); Sepharose 4B and Sephadex G-100 were from Pharmacia (Uppsala, Sweden). DEAE-cellulose was purchased from Serva (Heidelberg, G.F.R.), L-[14C]isoleucine (spec. act. 342 mCi/m mole or 210 mCi/mmole) was from the Radiochemical Centre (Amersham, England) or UVVVR (Prague, Czechoslovakia). Pancreatic RNase, snake venom phosphodiesterase and E. coli alkaline phosphatase (BAPF) were obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). T1 RNase and T2 RNase were purchased from Calbiochem (San Diego, Calif., U.S.A.). T.l.c. supports (Prod. No. 5563/0001) and t.l.c. - cellulose precoated plates were purchased from Merck (Darmstadt, G.F.R.). Other chemicals were reagent grade products from P.O.Ch. (Gliwice, Poland). Lupinus luteus seeds var. Express were obtained from seed companies in Poland.

Preparation of tRNA. Crude tRNA from lupin seeds was prepared essentially as described by Augustyniak et al. (1974) and purified on Sephadex G-100 column (2 x 140 cm) equilibrated with 0.5 M-NaCl containing 10 mM-MgCl2 and 2 mM-EDTA. Transfer RNA collected from this column was used for preparation of aminoacyl-tRNA to be used for chromatography on RPC-5 column (Pearson et al., 1971) and for purification of isoleucine tRNA16 species.

Assay for amino acid acceptor activity. Crude or partially purified lupin seed aminoacyl-tRNA synthetase was prepared as described by Jakubowski & Pawelkiewicz (1975). The incubation mixture contained in a final volume of 100 μl: 10 μmoles of Tris/HCl buffer, pH 7.8, 1 μmole of MgCl2, 0.6 μmole of ATP, 0.3 μmole of 2-mercaptoethanol, 0.2 μmole of KCl, 2.5 nmoles of [14C]isoleucine, 0.05 - 1.00 A260 units of tRNA, and an appropriate amount of the enzyme preparation (25 - 100 μg of protein). To the incubation mixture CTP and the 19 remaining non-radioactive amino acids were added in quantities equal to 1/10th of ATP and 10 times of [14C]isoleucine, respectively. The mixture was incubated for 20 min at 30°C. The amount of aminoacyl-tRNA synthesized was estimated by the filter paper disc technique of Mans & Novelli (1961). Radioactivity was measured in the toluene-based scintillation solution with a Packard Tri Carb, Model 2425, scintillation spectrometer. The samples of [14C]isoleucyl-tRNA were prepared for RPC-5 chromatography by the scaled up aminoacylation procedure. Radioactive aminoacyl-tRNA was recovered from the incubation mixture on a small DEAE-cellulose column as described by Waters & Novelli (1971).

RPC-5 chromatography of aminoacyl-tRNA. A sample of [14C]isoleucyl-tRNA (55 A260 units of unfraccionated tRNA, 150 000 c.p.m.) was applied on a 1 x 50 cm RPC-5 column and eluted at room temperature with 720 ml of a linear NaCl gradient (0.4 to 0.65 M) in 10 mM-sodium acetate buffer, pH 4.5, containing 10 mM-MgCl2 and 1 mM-EDTA. Fractions (2.6 ml) were collected every 2 min. From every second fraction 0.1 ml was taken for radioactivity counting in 10 ml of Bray's solution (Bray, 1960).
Purification of tRNA^{Ile} species. Purification of isoleucine tRNAs was achieved by RPC-5 chromatography followed by chromatography on Sepharose 4B in the reversed ammonium sulphate gradient as described by Holmes et al. (1975), and rechromatography on RPC-5 column.

For the first RPC-5 chromatography, a column of 1.5 x 94 cm was used; bulk tRNA^{Ile} (2250 A_{260} units) was applied to the column, which was eluted with a linear gradient (0.4 to 0.65 M, total volume 2.2 litres) of NaCl in 10 mm-sodium acetate buffer, pH 4.5, containing 10 mm-MgCl\(_2\) and 1 mm-EDTA. Fractions (8 ml) were collected every 5 min. Fractions 77 - 80 and 95 - 98 were pooled for tRNA\(_{1}\)^{Ile} (35 A_{260} units) and tRNA\(_{2}\)^{Ile} (32 A_{260} units), respectively.

For chromatography on Sepharose 4B, tRNA\(_{3}\)^{Ile} collected from several RPC-5 runs (320 A_{260} units) was applied to a 1 x 20 cm column and eluted with a linear (1.6 to 0.0 M, total volume 315 ml) (NH\(_4\))\(_2\)SO\(_4\) gradient in sodium acetate buffer, pH 4.5. Fractions of 2.8 ml were collected every 5 min. Fractions 37 - 42 were pooled for tRNA\(_{1}\)^{Ile} and yielded 81 A_{260} units. tRNA\(_{2}\)^{Ile}, 280 A_{260} units, collected from several RPC-5 columns, chromatographed under the same conditions on Sepharose 4B yielded 75 A_{260} units (fractions 45 - 51). All the chromatographic runs on Sepharose 4B were done in a cold room (at 4°C).

Rechromatography on a small RPC-5 column (0.6 x 20 cm) at pH 7.5 was used for further purification of tRNA\(_{1}\)^{Ile} and tRNA\(_{2}\)^{Ile}. The pooled fractions from the Sepharose 4B column were applied in several portions (usually of 25 A_{260} units each) to the RPC-5 column. The column was eluted with 150 ml of a linear NaCl gradient (0.4 to 0.65 M) in 10 mm-Tris/HCl buffer, pH 7.5, containing 10 mm-MgCl\(_2\) and 1 mm-EDTA. Fractions of 1.8 ml were collected every 2 min. This purification step resulted in symmetrical absorbance peaks which coincided with acceptor activities (data not shown), and gave 20 and 16 A_{260} units of tRNA\(_{1}\)^{Ile} and tRNA\(_{2}\)^{Ile}, respectively. All the RPC-5 chromatography runs were carried out at room temperature.

Purity of the final tRNA preparations was checked by electrophoresis in 12% polyacrylamide gel in the presence of urea as described by Phillipsen & Zachau (1972), and by estimation of amino acid acceptor activity of the tRNA species isolated.

Comparison of the 3'-terminal nucleotides. Crude tRNA aminoacylated with [\(^{14}\)C]isoleucine or the purified [\(^{14}\)C]isoleucyl-tRNA\(_{1}\)^{Ile} were hydrolysed with RNase T\(_1\) at pH 5.5 and the products were chromatographed on a DEAE-cellulose column (1 x 22 cm) using a 0.0 - 0.4 m-sodium acetate linear gradient (total volume 600 ml) as described by Merrick & Dure (1972).

Thermal denaturation of tRNA\(_{1}\)^{Ile} and tRNA\(_{2}\)^{Ile}. The thermal denaturation profiles of tRNA were recorded on a Specord UV-Vis spectrophotometer (Carl Zeiss, Jena, G.D.R.), equipped with thermostatically controlled cuvette blocks and a thermostir. Samples to be measured were dialysed for 30 h against 5 mm-Tris/HCl buffer, pH 7.5, containing 5 mm-MgCl\(_2\). Salt-free tRNA\(_{1}\)^{Ile} and tRNA\(_{2}\)^{Ile} were prepared as described by Augustyniak et al. (1976). The heating rate was 1°/min. Readings
taken from the spectrophotometer were corrected for the thermal expansion of water.

*Titration of tRNA*$_{1}^{16}$ and tRNA*$_{2}^{16}$ with *Mg*$_{2}^{2+}$. Titration of the desalted tRNA with MgCl$_{2}$ was performed by measuring the decrease in absorbance of tRNA on addition of MgCl$_{2}$. The readings were taken at 260, 265, 270 and 285 nm. The results were normalized to the absorbance of the initial tRNA solution, taken as unity.

*Nucleoside composition.* The composition of tRNA*$_{1}^{16}$ and tRNA*$_{2}^{16}$ was determined by high-pressure liquid chromatography of nucleosides on Aminex A-7 column as described by Sen & Ghosh (1974), using a DuPont Model 830 Liquid Chromatograph, and the t.l.c. method of Rogg *et al.* (1976). The nucleosides were identified by their positions on the chromatograms and by u.v. absorption spectra taken at pH 1, 6 and 12 (Hall, 1971). tRNA was degraded to nucleosides by phosphodiesterase and alkaline phosphatase (Sen & Ghosh, 1974). Alternatively, the nucleotides obtained after T$_{2}$ RNase digestion of tRNA were analysed by the t.l.c. method recommended by Kimura-Harada *et al.* (1972).

**RESULTS AND DISCUSSION**

 tRNA*$_{1}^{16}$ after acylation with [*1*^4*C]*isoleucine was resolved into five species by RPC-5 chromatography (Fig. 1). Two species were always predominant, irrespective of the purification degree of the enzyme used for aminoacylation. On DEAE-cellulose chromatography, the crude tRNA esterified with isoleucine and digested with T$_{1}$ RNase showed also the presence of two major [*1*^4*C]*aminoacyl-oligonucleotides (cf Fig. 3). The two main isoleucine tRNA species were purified by three chromatographic steps: RPC-5 chromatography at pH 4.5, Sepharose 4B chromatography in the reversed gradient of ammonium sulphate, and rechromatography on RPC-5 at pH 7.5 (Figs. 1 and 2).

After the first purification step the pooled fractions corresponding to tRNA*$_{1}^{16}$ and tRNA*$_{2}^{16}$ (Fig. 1) accepted about 530 and 350 pmoles of isoleucine per one A$_{260}$ unit, respectively. Further purification of the pooled fractions on Sepharose 4B column by the reversed ammonium sulphate gradient chromatography (Fig. 2) removed a considerable amount of other tRNAs from the isoleucine tRNA species, the two major tRNA*$_{16}^{16}$ were, however, still incompletely separated.

The two species obtained from the Sepharose column showed the isoleucine acceptor activities of 1280 and 965 pmoles/A$_{260}$ unit, respectively. After rechromatography on RPC-5 column at pH 7.5, the fractions accepting 1510 pmoles of isoleucine/A$_{260}$ unit (tRNA*$_{16}^{16}$) and 1350 pmoles/A$_{260}$ unit (tRNA*$_{26}^{16}$) were collected and used for further analysis. These fractions accepted less than 25 pmoles of amino acids when charged with a mixture of 19 amino acids deprived of isoleucine, and showed only one band on acrylamide gel.

To sum up, about 28 000 A$_{260}$ units of crude tRNA yielded 64 A$_{260}$ units of tRNA*$_{16}^{16}$ and 43 units of tRNA*$_{26}^{16}$ with the purity as indicated above. The overall
recovery was about 18% if calculated on the basis of the content of isoleucine tRNA species in the initial quantity of crude tRNA.

**Fig. 1.** Preparative RPC-5 chromatography of crude tRNA from lupin seeds. 2250 A_{260} units of tRNA were applied on a 1.5x94 cm column. The column was eluted with a linear gradient (2.2 litres total volume) of 0.4 - 0.65 M-NaCl in NaOAc buffer, pH 4.5. Fractions of 8 ml were collected at a flow rate of 1.6 ml/min.

**Fig. 2.** Sepharose 4B chromatography of the tRNA^{[1]i} and tRNA^{[2]i} partially purified by RPC-5 (see Fig. 1). 320 A_{260} units of tRNA^{[1]i}, and 280 A_{260} units of tRNA^{[2]i} were applied separately to a 1x20 cm column and eluted with a linear 1.6 - 0.0 M-(NH$_4$)$_2$SO$_4$ gradient in sodium acetate buffer, pH 4.5 (total vol. 315 ml). The flow rate was 0.5 ml/min.
The thermal denaturation curves of the purified isoleucine tRNA species, determined in the presence and absence of Mg\(^{2+}\), are shown in Fig. 4. In the absence of Mg\(^{2+}\) the hyperchromicity values were 26.7% for tRNA\(^{14a}\) and 21.7 for tRNA\(^{14e}\) and the melting profiles obtained resembled the multiphasic transition curves measured in the absence of divalent cations and reported for numerous tRNAs (Cole et al., 1972). In the presence of Mg\(^{2+}\) the hyperchromicity values were very similar, 27.4%, for both tRNAs.

Fig. 3. DEAE-cellulose chromatography of the T\(_1\) digest of crude (●) and purified (○) [\(^{14}\)C]isoleucyl-tRNA\(^{14a}\). 120,000 c.p.m. of crude aminocylated tRNA\(^{14a}\) and 48,000 c.p.m. of tRNA\(^{14e}\) were applied to the column.

Fig. 4. Thermal denaturation profiles of tRNA\(^{14a}\) and tRNA\(^{14e}\) from lupin seeds. (——) tRNA\(^{14a}\) in 5 mm-MgCl\(_2\); (• • •) tRNA\(^{14a}\) in 5 mm-MgCl\(_2\); (——) tRNA\(^{14e}\) salt-free; and (——) tRNA\(^{14e}\) salt-free.
Fig. 5. Spectrophotometric titration of tRNA\textsubscript{1}\textsuperscript{14} at 285 nm (---) and 265 nm (-----); and of tRNA\textsubscript{2}\textsuperscript{14} at 285 nm ( • • • ) and 265 nm (———) in the presence of Mg\textsuperscript{2+}.

The two isolated isoleucine tRNAs show evident differences in their Mg\textsuperscript{2+} titration curves (Fig. 5). During the transition from a salt-free to a Mg\textsuperscript{2+}-rich medium, the absorbance of tRNA\textsubscript{2}\textsuperscript{14} was decreased more than in the case of tRNA\textsubscript{1}\textsuperscript{14}.

Table 1

Nucleoside composition of lupin isoleucine tRNA species

The results are expressed as moles of nucleosides/mole tRNA. It was assumed that these tRNAs are 77 nucleotides long and the values were rounded off to the nearest integer.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>tRNA\textsubscript{1}\textsuperscript{14}</th>
<th>tRNA\textsubscript{2}\textsuperscript{14}</th>
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</thead>
<tbody>
<tr>
<td>A</td>
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<td>11</td>
</tr>
<tr>
<td>U</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>C</td>
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<td>17</td>
</tr>
<tr>
<td>G</td>
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</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ψ</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>hU</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>m\textsuperscript{1}A</td>
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<td>1</td>
</tr>
<tr>
<td>m\textsuperscript{1}C</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>m\textsuperscript{1}G</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>t\textsuperscript{1}A</td>
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<td>1</td>
</tr>
<tr>
<td>m\textsuperscript{1}G</td>
<td>1</td>
<td>* 1</td>
</tr>
<tr>
<td>m\textsuperscript{2}G</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>77</td>
</tr>
</tbody>
</table>
The analysis of the purified tRNA species (Table 1) revealed a difference in the composition of the major and modified bases: tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} contained twice as much \textit{\textasciitilde{7}} residues as tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} and did not contain t\textasciitilde{6}A. The presence of m\textasciitilde{7}G was evidenced only by its t.i.c. position according to Rogg et al. (1976). The absence of t\textasciitilde{6}A in tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} does not exclude the presence of mt\textasciitilde{4}A in that tRNA, however, up to now we did not obtain firm evidence of its content in tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16}. The observed higher content of G in tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} than in tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} agrees well with their melting profiles and the MgCl\textsubscript{2} titration curves. During the melting of tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} in the absence of Mg\textsuperscript{2+} relatively high hypochromicity was observed which suggests considerable ordering of this species. Having a large part of its structure in an ordered state this tRNA underwent smaller changes during MgCl\textsubscript{2} titration than tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16}.

As shown in Fig. 3, the tRNAs investigated have different nucleotide sequences at their 3' ends. The differences in the nucleotide sequence, in the composition of the bases, and in physical properties between tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} and tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} suggest that these species are coded for by separate genes.

As judged by chromatography and amino acid acceptor activity, the purity of the isolated tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} exceeded 80\%, and the purity of tRNA\textsuperscript{\textasciitilde{34}}\textsubscript{16} was slightly lower. This relatively high degree of purification was achieved by an only three-step chromatographic procedure. No simple method of purification of tRNA from plants is yet available. As demonstrated in this paper, the combination of RPC-5 and Sepharose 4B chromatographies proved to be effective in purification of specific tRNAs from plant material.

REFERENCES

IZOLACJA I WŁASNOŚCI GŁÓWNYCH IZOOKCEPTORÓW tRNA™
Z NASION LUBINU ŻÓLTEGO

Streszczenie

[^14C]tRNA™ z nasion lubinu rozdziela się w warunkach chromatografii RPC-5 na 5 izookceptorów. Dwa główne izookceptorowe tRNA wyizolowano stosując chromatografię na RPC-5 i na Sepharose 4B. Aktywność akceptorowa tRNA™ wynosiła 1510 pmoli izoleucyny na jednostkę optyczną A260 tRNA. tRNA™ 1350 pmoli. Obydwa izookceptorowe tRNA wykazują podobne profile denaturacji termicznej, ale różnią się składem podstawowych i modyfikowanych nukleozydów oraz 3'końcową sekwencją cząsteczki. Oczyszczone izoleucynowe tRNA różnią się ponadto przebiegiem krzywych spektrofotometrycznego miareczkowania chłorkiem magnezu. Otrzymane wyniki wskazują, że tRNA™ i tRNA™ są kodowane przez odrębne geny.

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