Plant Physiol. (1976) 58, 140-142

Ribosyl-*cis*-zeatin in a Leucyl Transfer RNA Species from Peas[1]

Received for publication December 5, 1975 and in revised form March 8, 1976

JOHN W. EINSET, SANTHANAM SWAMINATHAN,[2] AND FOLKE SKOOG
Institute of Plant Development, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

$tRNA_6^{Leu}$ in *Pisum sativum* seed has been purified. This tRNA species contains a cytokinin-active nucleoside and accounts for approximately 7% of the total cytokinin activity in acid hydrolysates of pea tRNA. The cytokinin has been identified as ribosyl-*cis*-zeatin, 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine.

The modified nucleoside *c*-io^{6}A[3] was first tentatively identified by Hall *et al.* (7) in tRNA preparations from peas, corn, and spinach and has since been isolated from tRNAs of several other plants (6, 12). It has also been reported, on the basis of bioassays, to occur in tRNA preparations of certain bacterial species; *i.e. Corynebacterium fascians* (8), a plant pathogen, and in species of *Rhizobium* (10), the bacterium associated with nitrogen-fixing nodules in legumes. The 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine, *t*-io^{6}A, the isomer generally found in free form in plants, has also been isolated from *Pisum* tRNA (14).

Gas chromatographic evidence for the presence of *c*-io^{6}A in $tRNA_6^{Leu}$ from peas has been reported (2). This paper reports the isolation of this tRNA species and the definitive identification of *c*-io^{6}A as its cytokinin-active nucleoside constituent.

MATERIALS AND METHODS

Purification of Leucyl tRNA. Pea seeds (*Pisum sativum* cv. Alaska) that had been surface-sterilized and soaked for 48 hr in aerated, sterile distilled H_2O were homogenized with 1 ml/g fresh weight of 0.1 M tris-HCl (pH 7.8), 0.1 mM EDTA, 0.2% SDS, and then this homogenate was extracted twice with equal volumes of phenol saturated with the same buffer. The final aqueous phase was adjusted to 0.1 M NaCl and the RNA was precipitated by addition of 2 volumes of cold 95% (v/v) ethanol. The RNA was then suspended in 0.02 M sodium acetate (pH 4.5), 1 mM EDTA, and applied to a column of DEAE-cellulose previously equilibrated with the same buffer. After washing the column with 5 bed volumes of buffer containing 0.3 M NaCl, the tRNA was eluted with 2.5 bed volumes of the same buffer containing 1 M NaCl.

The tRNA was applied to a column of BD-cellulose (5) previously equilibrated with 0.02 M sodium acetate (pH 4.5), 0.01 M $MgCl_2$, 0.3 M NaCl, and then the column was eluted with 20 bed volumes of the same buffer containing 1 M NaCl. This removed approximately 90% of the tRNA (BD-salt fraction). The remaining tRNA was eluted with 6 bed volumes of buffer containing 1 M NaCl plus 10% ethanol (BD-ethanol fraction). The BD-ethanol tRNA was further fractionated by chromatography on an RPC-2 column (tricaprylylmethylammonium chloride as stationary phase on diatomaceous earth, 15) equilibrated with 0.02 M sodium acetate (pH 4.5), 0.01 M $MgCl_2$, 0.3 M NaCl, and developed with a linear gradient from 0.3 M to 0.7 M NaCl.

Bioassay of Cytokinin Activity. The fractionation of plant tRNA species containing *c*-io^{6}A was monitored with the tobacco callus bioassay, as this compound is active as a cytokinin in the free form (11). The tRNA fractions were dissolved in 10 ml of distilled H_2O, acid-hydrolyzed, and incorporated in serial dilutions into nutrient medium (12). Cytokinin content is expressed either as kinetin equivalents (KE, where 1 KE is the growth response obtained on medium containing 1 μg/l kinetin) or as μmol of *c*-io^{6}A calculated by comparison of the growth response with the response given by acid-hydrolyzed samples of this nucleoside tested in serial dilutions.

Determination of Leucine Acceptor Activity. The leucyl tRNA synthetase was prepared from soaked pea seeds by the procedure of Scott and Morris (10) except that the chromatography step on Bio-Gel P-150 was omitted. Reaction mixtures for aminoacylation were incubated at 30 C and contained tRNA, 0.1 M tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 1 mM ATP, 0.5 μCi/ml of [^{14}C]leucine, and 150 μg/ml of protein. At various times after initiating the reaction, 20-μl volumes were removed and mixed with 0.1 ml of cold 10% trichloroacetic acid. After 15 min, the precipitated tRNA was collected on glass fiber filters which were then dried and analyzed for radioactivity in a liquid scintillation counter. To calculate leucine acceptance, the plateau value of incorporated radioactivity was transformed to pmol of leucine and converted to pmol incorporated/A_{260} unit of tRNA.

Purification and Identification of *c*-io^{6}A. The purified leucyl tRNA sample was enzymically hydrolyzed to nucleosides as described (3). The dried hydrolysate was then extracted six times with 5-ml volumes of water-saturated ethyl acetate and the dissolved nucleosides were separated on a Sephadex LH-20 column (1) equilibrated and eluted with distilled H_2O. Fractions corresponding to the elution volume of *c*-io^{6}A were pooled, evaporated to dryness under N_2, and then the solid obtained was derivatized by reaction with *N,O-bis*(trimethylsilyl)trifluoroacetamide in pyridine (13). This silylated sample was fractionated in a Varian 2700 gas chromatograph that was interfaced with a DuPont 21-491B mass spectrometer. The gas chromatograph was equipped with a glass column (180 cm long, 2 mm i.d.) containing 3% OV-1 on 100- to 200-mesh Aeropak and was developed isothermally at 270 C with helium gas (30 ml/min).

RESULTS AND DISCUSSION

Chromatography of pea seed tRNA on BD-cellulose separated cytokinin-containing species into two fractions. The majority (90%) of the cytokinin activity eluted in the BD-salt fraction and the rest eluted in the BD-ethanol fraction (Table I). Further

[1] This work was supported by National Science Foundation Research Grant BMS 72-02226-A03.

[2] Present address: Division of Clinical Oncology, Medical School, University of Wisconsin, Madison, Wisconsin 53706.

[3] Abbreviations: *c*-io^{6}A: 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine; *t*-io^{6}A: 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine; BD-cellulose: benzoylated-diethylaminoethyl cellulose; KE: kinetin equivalents = μg of kinetin required to produce the same growth response as the tested preparation.

chromatography on RPC-2 resolved the BD-ethanol material into two peaks of cytokinin activity with the major peak eluting in fraction V (Fig. 1). This fraction had a relative cytokinin activity of 2.2 KE/20 A_{260} in the experiment shown in Figure 1 and 4.2 KE/20 A_{260} in a repeat experiment. The average value corresponds to 0.8 μmol of *c*-io^{6}A/μmol of tRNA.

Leucine acceptor activity in the RPC-2 profile coincided with the major peak of cytokinin activity (Fig. 2). For more exact determination of the acceptor activity, leucyl tRNA labeled with ^{32}P was used. This gave a value of 330 pmol/A_{260} unit which is in the range expected of purified species charged with this pea synthetase. This value represents a 15-fold enrichment in leucine acceptance over total tRNA and correlates well with the degree of enrichment in cytokinin activity in the same fraction.

In order to identify the cytokinin in the leucyl tRNA species, the purified fraction was enzymically hydrolyzed and the nucleosides obtained were separated on a Sephadex LH-20 column. This chromatography gave a distinct fraction having cytokinin activity and eluting in the volume corresponding to *c*-io^{6}A. The UV spectrum of this fraction in water had maximum absorption at 269 nm. After reaction with silylating reagent, the derivatized sample was fractionated in a gas chromatograph that was interfaced with a mass spectrometer. The sample gave a peak corresponding to the retention time of silylated authentic *c*-io^{6}A which was clearly separated from silylated *t*-io^{6}A under these conditions (Fig. 3). The mass spectrum had prominent peaks at m/e 639 (parent ion of silylated *c*-io^{6}A), at m/e 624, 550, 536, 508, and 320, representing characteristic fragments of the silylated nucleoside, at m/e 290 (silylated base peak) and at m/e 349 and 277 due, respectively, to silylated ribose and a characteristic fragment (4, 14). The distinctive ions are listed in Table II and indicated in Figure 4.

The mass spectral data together with the UV spectrum and chromatographic data unequivocally identify *c*-io^{6}A as a constituent of the leucyl tRNA species. This tRNA is the same as the species designated as tRNA$_6^{Leu}$, one of six isoaccepting forms isolated from peas and separated by chromatography on RPC-2 by Babcock and Morris (2). They also reported a nuclease from pea roots which cleaves tRNA$_6^{Leu}$ to a fragment and have presented gas chromatographic evidence for *c*-io^{6}A in purified preparations of this fragment (2).

Table I. *Leucine Acceptor Activity and Cytokinin Activity of Pea Seed tRNA Fractions*

Fraction	Leucine Acceptor Activity		Cytokinin Activity	
	pmol/A_{260}	% of total	KE/20 A_{260}	% of total
Total tRNA	22	100	0.16 0.15 – } 0.16	100
BD-salt tRNA	24	92	0.13 0.16 0.16 } 0.15	90
BD-ethanol tRNA	19	8	0.15 0.17 0.16 } 0.16	10

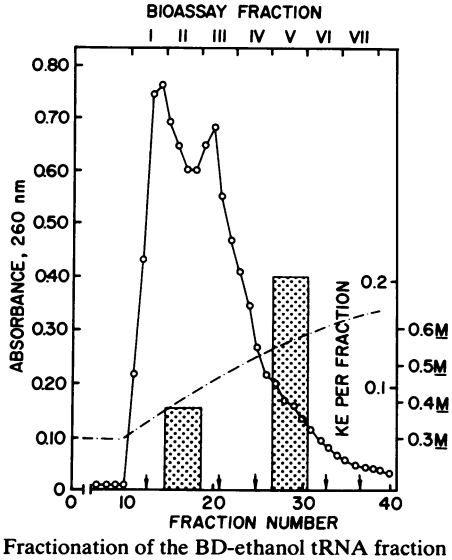

FIG. 1. Fractionation of the BD-ethanol tRNA fraction from peas on a RPC-2 column. After applying the sample which contained 3.75 mg of tRNA, the column (0.5 cm × 40.3 cm) was eluted with 15 ml of 0.02 M sodium acetate (pH 4.5), 0.01 M $MgCl_2$, 0.3 M NaCl, and then with a linear gradient of increasing salt concentration formed by 125 ml of buffer with 0.3 M NaCl and 125 ml of buffer with 0.8 M NaCl. The eluate was collected in 40 3.0-ml fractions from each of which 2.7 ml was incorporated into 100 ml of nutrient medium for bioassays. Bars: cytokinin activity (KE); arrows: absence of cytokinin activity in the tobacco bioassay; solid line: absorbance per fraction; dashed line: salt concentration determined with a conductivity meter.

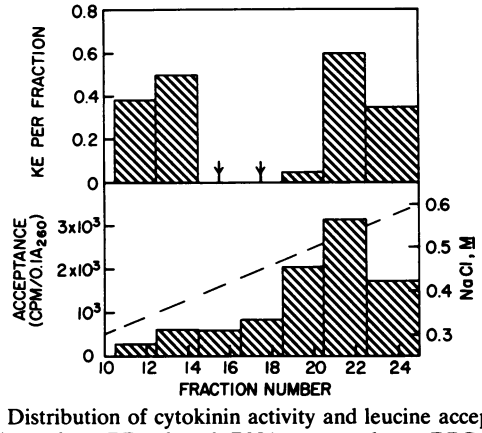

FIG. 2. Distribution of cytokinin activity and leucine acceptor activity in fractions of pea BD-ethanol tRNA separated on a RPC-2 column. The sample which contained 2.6 mg of tRNA was fractionated as described in the legend to Figure 1. CPM > 800: significant acceptor activity.

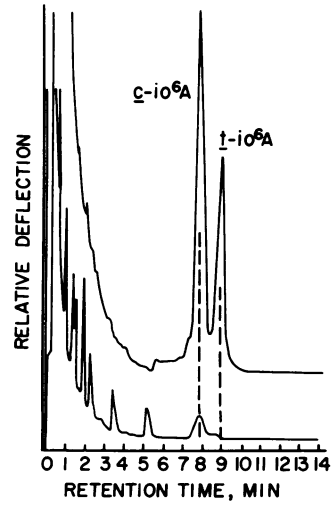

FIG. 3. Gas chromatographic separation of the trimethylsilylated derivatives of *cis* and *trans* isomers of ribosylzeatin and the cytokinin from pea leucyl tRNA. Upper curve is a trace obtained with a sample containing derivatives of both authentic *c*-io^{6}A and authentic *t*-io^{6}A. The retention times for silylated *c*-io^{6}A and silylated *t*-io^{6}A were 7.9 min and 9 min, respectively. Lower curve is a trace obtained with a derivatized sample of the cytokinin from the leucyl tRNA and shows a peak corresponding to the retention time of silylated *c*-io^{6}A.

Table II. *Mass Spectrum of Trimethylsilylated Derivatives of Synthetic c-io⁶A and of the Purified Cytokinin from Pea Leucyl tRNA*

c-io⁶A		Sample			Composition
m/e	Relative abundance	m/e	Relative abundance		
639	0.33	639	0.54	a	M^+
624	0.27	624	0.59		M^+-CH_3
550	0.70	550	1.13	b	M^+-OTMS
536	1.00	536	1.00	c	M^+-CH_2OTMS
508	0.08	508	0.33	d	M^+-$C(CH_3)CH_2$OTMS
483	0.02			e	M^+-$CH_2CH_2CH(CH_3)CH_2$OTMS
406	0.08	406	0.33	f	
349	0.12	349	0.66	g	$(TMS)_3$Ribose$^+$
320	0.80	320	0.77	h	M^+-$C_4H_4(OTMS)_3$
290	0.20	290	2.27	i	M^+-$(TMS)_3$Ribose
277	0.37	277	3.18	j	$(TMS)_2$Ribose$^+$

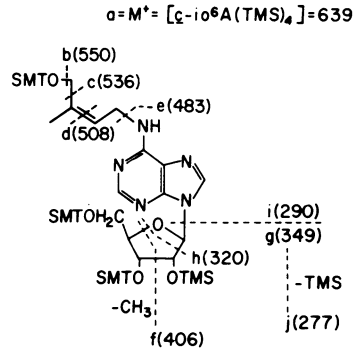

FIG. 4. Major fragment ions of trimethylsilylated *c*-io⁶A.

In addition to leucyl tRNA, we can distinguish at least two other cytokinin-containing tRNAs in pea seeds. On the basis of bioassay evidence, there are at least three tRNA species in pea seeds that contain *c*-io⁶A (unpublished). Although the amino acid specificity has not been determined, it is expected by analogy with other isoprenoid nucleosides that *c*-io⁶A-containing tRNAs will correspond to codons with uridine as the first base (12). Two of the six codons for leucine begin with uridine. The codon specificity of $tRNA_6^{Leu}$ is being determined.

Acknowledgments – Synthetic io⁶A was kindly furnished by N. J. Leonard, University of Illinois, Urbana. We thank H. Schnoes, University of Wisconsin for advice and the use of gas chromatographic and mass spectrometric apparatus.

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