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LEUCINE SPECIFIC TRANSFER RIBONUCLEIC ACIDS AND SYNTHETASES IN THE COTYLEDONS OF MATURE AND GERMINATING PEA SEEDS

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Abstract—Changes in isoaccepting species of $tRNA^{Leu}$ were determined in germinating pea seedlings and in developing pods. Leucine specific transfer ribonucleic acids of pea cotyledons can be fractionated into four isoaccepting species by reversed-phase chromatography (RPC-5) on a Plaskon column. In contrast, only two species of $tRNA^{Leu}$ were observed in developing seed pods. Leucyl-*t*RNA synthetase purified by ammonium sulfate precipitation and DEAE cellulose column chromatography retained the full range of specificity towards all four $tRNA^{Leu}$ species of pea cotyledons. This partially purified pea cotyledon enzyme could be further separated on a hydroxylapatite (HA) column into two peaks of leucyl-*t*RNA synthetase activity. Enzyme 1 is dominant in seed pods while 2 is predominant in cotyledons. Enzymes 1 and 2 from cotyledons were examined for the amino acid acceptor activity of twelve different amino acids. Both these fractions showed less than 3% acceptor activity for eleven other amino acids as compared to leucine-*t*RNA synthetase activity. Preliminary characterization of enzyme 2 from cotyledon, by isoelectric focusing and polyacrylamide gel electrophoresis indicates at least three subspecies.

INTRODUCTION

It is now well established that there are both qualitative and quantitative alterations in chromatographic elution profiles of certain isoaccepting *t*RNAs, when a cell passes from one physiological state to another. Such changes occur in *E. coli* infected with bacterial phage T_2 [1] and in virus infected hamster cells [2]. Similar changes have been observed at different developmental stages of various organisms. Previous studies on *t*RNAs in soybean have revealed that the complement of $tRNA^{Leu}$ isoaccepting species change during cotyledon senescence [3]. Aminoacyl-*t*RNA synthetases and *t*RNAs may be involved in developmental and regulatory functions of cellular systems.

Previous work in our labotatory [4] shows that the fractionation of leucyl-*t*RNA synthetases from soybean cotyledons and seed pods on hydroxylapatite (HA) column results in three peaks of activity. Bick and Strehler [5] demonstrated changes in these enzymes in respect to their ability to acylate certain transfer RNAs in aging soybean cotyledons. This implies that readout of certain messenger ribonucleic acids (*m*RNAs) is not only controlled by the *t*RNA but also by the acylating enzyme. This study was undertaken to determine the differences in $tRNA^{Leu}$ isoaccepting species and partial purification and characterization of leucyl *t*RNA synthetases in two developmental stages, namely the germinating cotyledons and seed pods of peas.

RESULTS AND DISCUSSION

Differences in $tRNA^{Leu}$ species and leucyl-*t*RNA synthetase activities in cotyledons of germinating pea seedlings and developing pea seed pods are discussed here. Leucyl-*t*RNA synthetase activity was investigated in these two systems, after the synthetase preparations were separated on HA columns. Transfer RNA preparations from these samples were acylated with enzyme fractions from HA column and finally the $tRNA^{Leu}$ species separated on Plaskon (RPC-5) columns. Polyclar AT and 2-mercaptoethanol were routinely added to avoid inactivation by phenolics [6], especially since their protective action has been specifically established in the case of leucyl-*t*RNA synthetase from soybean tissue [7]. After extraction, the total leucyl-*t*RNA synthetase was partially purified by ammonium sulfate precipitation, DEAE chromatography and HA column chromatography. Table 1 summarises the purification procedure of the leucyl-*t*RNA synthetase from 10 g of five day old pea cotyledons. A 358-fold purification and 379% recovery at the end of HA column chromatography was obtained.

DEAE-cellulose chromatography

The protein precipitated with ammonium sulfate (30–60% fraction) was dissolved in 5 ml of buffer C (25 mM KPi pH 7·8, 10^{-2} M 2-Mercaptoethanol, 10^{-5} M phenyl methyl sulfonyl chloride; 10^{-6} M L-Leucine), dialysed to remove the ammonium sulfate and chromatographed on a DEAE column. DEAE chromatography was routinely performed using a stepwise elution technique and the enzyme recovered in a single protein fraction by eluting in the cold with 0·1 M Pi buffer. The enzyme recovery after DEAE chromatography was 269% and the specific activity increased about 2–3 fold as compared to the enzyme from the ammonium sulfate fraction (Table 1).

Table 1. Purification of leucyl-*t*RNA synthetase

Enzyme fraction	Volume (ml)	Protein mg/ml	Specific activity (units/mg)	Total activity	Purification fold	% Recovery
Crude extract	38·0	32·38	0·06	73·8	1·0	100
$(NH_4)_2SO_4$ fraction	13·0	22·47	0·46	134·3	7·6	181
DEAE cellulose fraction	30·0	5·58	1·19	199·2	19·8	269
Hydroxylapatite fraction	3·5	3·72	21·50	280·0	358·0	379

Hydroxylapatite column chromatography

Preliminary studies with HA column using buffers without glycerol resulted in poor separation and low enzyme activity (less than 3000 cpm in peak 2). Addition of 10% glycerol to the buffers increased activity up to 40 000 cpm. HA column chromatography gives an 18-fold enrichment of the DEAE fraction. Enzyme recovery after this stage of purification was high. Leucyl-*t*RNA synthetase from pea cotyledons and pea seed pods fractionate into two discrete peaks of activity on an HA column (enzymes 1 and 2).

For further purification and characterization, it was necessary to have an enzyme preparation with little contamination. Therefore, the purity of 1 and 2 from cotyledons following HA column chromatography was checked by testing their amino acid acceptor activity for 12 different amino acids. Results in Table 2 indicate that both are essentially pure for leucyl-*t*RNA synthetase with less than 3% contamination from other synthetases. Cotyledon enzyme 2 from HA column chromatography was concentrated by Diaflo ultrafiltration for use in isoelectric focusing, polyacrylamide gel electrophoresis and analytical ultracentrifugation studies. However, this purification of leucyl-*t*RNA synthetase, from the foregoing observation does not preclude the possibility of cross contamination between enzymes 1 and 2 from HA column. It should also be emphasized that only enzyme 2, being the major enzyme in the cotyledons, was used in these studies.

Isolectric focusing and electrophoresis

Isoelectric focusing of cotyledon enzyme 2 was performed over the pH range 3–10 and acceptor activity for leucyl-*t*RNA synthetase measured for each fraction. Three peaks of activity were observed which indicates the possibility of three subspecies with pI values 6·9, 5·3 and 4·14 respectively. Samples of 50–100 mg concentrated cotyledon enzyme 2 applied to polyacrylamide columns, resulted in three protein bands also indicating that it is heterogeneous. Sedimentation velocity analysis of enzyme 2 resulted in a S_{abs} value of 6·63, which indicates that the subspecies obtained by isoelectric focusing or disc gel electrophoresis are approximately equal in their MW's.

Transfer RNAs in pea cotyledons and seed pods

For routine enzyme assays or acylation of *t*RNAs for fractionation on RPC-5 (Plaskon) Columns, a synthetase preparation, partially purified on DEAE cellulose columns was used. $tRNA^{Leu}$ from cotyledons after acylation with ^{3}H-Leucine, using five day old cotyledon enzyme, separated into 4 peaks on an RPC-5 column. The relative amounts of *t*RNA in peaks 1–4 are 32, 57, 5 and 6% respectively. Acylation of pod *t*RNA with ^{3}H-Leucine, using seed pod enzyme and final separation on Plaskon columns results in only *t*RNA species 1 and 2, the relative amounts being 79·5 and 20·5% respectively. If one compares the relative amounts of $tRNA_1^{Leu}$ in cotyledons and pods, it is clear that in pods $tRNA_1^{Leu}$ is 47·5% more than in cotyledons. The complete absence of $tRNA^{Leu}_{3\text{ and }4}$ and the high amounts of $tRNA_1^{Leu}$ in seed pods indicate differences that could be based on one of the two rate limiting factors, i.e. synthetases or *t*RNAs. Therefore, acylation of pod *t*RNA with cotyledon enzyme (heterologous system) and acylation of cotyledon-*t*RNA with pod enzyme was performed. The charged *t*RNAs were then separated on RPC-5 columns. The elution profile of $tRNA^{Leu}$ species in seed pods charged with synthetase from 5 day old cotyledons shows only $tRNA^{Leu}$ species 1 and 2 with 87 and 13% activity

Table 2. Aminoacyl-*t*RNA synthetase activities of pea cotyledons after HA column chromatography

Amino acid	pmol aminoacyl-*t*RNA formed per mg enz/min		% Activity of aminoacyl-*t*RNA synthetases relative to leu-*t*RNA synthetase	
	Enzyme 1	Enzyme 2	Enzyme 1	Enzyme 2
Alanine	10	5	0·20	0·04
Arginine	50	70	1·10	0·60
Aspartic acid	30	160	0·70	1·50
Glutamic acid	50	40	1·10	0·40
Glycine	7	7	0·15	0·06
Histidine	90	170	2·00	1·60
Isoleucine	60	60	1·30	0·60
Leucine	4410	10160	100·00	100·00
Methionine	20	32	0·40	0·30
Phenylalanine	10	20	0·20	0·20
Tyrosine	15	50	0·34	0·50
Valine	100	100	2·10	1·00

Table 3. Variation of $tRNA^{Leu}$ isoaccepting species in germinating pea cotyledons and seed pods

Source of enzyme	Source of tRNA	Relative amount of each $tRNA^{Leu}$ species (% total) 1	2	3	4
Cotyledons 5 days	Cotyledons 5 days	32·0	57·0	5·0	6·0
	Seed pods	87·0	13·0	0·0	0·0
Seed pods	Cotyledons 5 days	30·0	62·0	3·2	4·8
	Seed pods	79·5	20·5	0·0	0·0

tRNA was acylated in a 2 ml reaction mixture with ^{3}H-leucine and enzyme from DEAE-cellulose column, and fractionated on a Plaskon column. The amount of radioactivity in peaks 1–4 was summed and expressed as % of total counts.

respectively. This suggests that pods are deficient in $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$ indicating tRNAs as the limiting factor. Cross charging cotyledon tRNA with synthetase from seed pods, and separation on Plaskon columns results in four tRNA species with 30, 62, 3·2 and 4·8% activity respectively. This clearly shows that seed pod enzyme is capable of charging all four $tRNA^{Leu}$ species including species 3 and 4 in cotyledons. These data are summarized in Table 3. We can conclude, therefore, that pea pods contain only two species of $tRNA^{Leu}$ with a higher proportion of $tRNA_1^{Leu}$.

Transfer RNA specificity of individual enzyme fraction

Previous reports [8] have shown that RPC-2 (Freon) column chromatography of tRNAs amino acylated by individual leucyl-tRNA synthetase from HA column indicates that enzyme 1 was specific for $tRNAs_{3\text{ and }4}^{Leu}$ and enzyme 2 was specific for $tRNAs_{1\text{ and }2}^{Leu}$. Contrary to the above results, enzyme 1 from cotyledons acylates all four isoaccepting species of tRNAs from cotyledons, with increases in acylation of $tRNA_{3\text{ and }4}^{Leu}$ and decrease in acylation of $tRNA_{1\text{ and }2}^{Leu}$. The percent acylation of tRNAs 1–4 is 28, 45, 15 and 12% respectively. The elution profile of ^{3}H-$tRNA^{Leu}$ species of cotyledons charged with synthetase 2 of cotyledons, which acylates mainly tRNAs 1 and 2 with minor acylation of tRNAs 3 and 4, showed that acylation of tRNAs 1–4 is 32·6, 59, 3·5 and 4·9% respectively.

The above results indicate that there is an increase in acylation of $tRNA_{3\text{ and }4}^{Leu}$ when cotyledon tRNA is acylated with synthetase 1 from cotyledons as compared to the acylation of $tRNA_{3\text{ and }4}^{Leu}$ using synthetase 2. When pod enzymes 1 and 2 from HA column are used for acylation of pod tRNA and subsequent separation on RPC-5 columns, no preferential specificity for tRNAs is observed. Both enzyme peaks are capable of acylating only $tRNA_{1\text{ and }2}^{Leu}$ and on a quantitative basis 75–78% activity expressed as cpm is found associated with $tRNA_1^{Leu}$ and 22–25% with $tRNA_2^{Leu}$.

With the onset of seed germination, numerous biochemical changes occur; some of these changes, like the increase in activity of specific enzymes, are directly related to protein synthesis. Since transfer RNA serves as a functional link between the genetic information encoded in the messenger RNA and protein synthesis, specific changes in the tRNA population have various implications for the regulation of cellular events during differentiation and development.

Data presented here show changes in isoaccepting $tRNA^{Leu}$ species and multiple forms of synthetases between seed pods, i.e. a stage before germination, and cotyledons, a stage after germination. Pea pods contain only two isoaccepting species of $tRNA^{Leu}$ in contrast to pea cotyledons which contain four $tRNA^{Leu}$ species. In pods, $tRNA_1^{Leu}$ constitutes 79·5% and $tRNA_2^{Leu}$ constitutes 20·5% to the total $tRNA^{Leu}$ complement. Pea cotyledons, however, contain four isoaccepting species of $tRNA^{Leu}$. From this, one can conclude that with the onset of germination two new tRNAs appear in cotyledons. Further, if one considers the relative amounts of these four tRNAs in cotyledons, it is observed, that $tRNA_1^{Leu}$ decreases to 32%, while $tRNA_2^{Leu}$ increases up to 57%; the two new species $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$ amount to 5 and 6%.

RPC-5 chromatography of radioactively labelled tyrosyl, aspartyl, asparaginyl, and histidyl tRNAs from developmental stages of wild type *Drosophila melanogaster* has revealed qualitative and quantitative changes in their major isoaccepting forms [9]. These authors attribute such differences to post transcriptional modifications of the same tRNA gene products [10]. They propose a term "homogeneic" to describe tRNAs having the same sequence and presumably products of the same gene but which are chromatographically distinct because of different degrees of post-transcriptional modification. It is well established that there are tRNA modifying enzymes like tRNA methylases which modify tRNAs after the three dimensional structure of tRNA is already formed [10, 11]. Therefore, it is tempting to propose here, that with germination, there is post-transcriptional modification of $tRNA_1^{Leu}$, leading to observed changes (decrease or increase) in $tRNA_{1-4}^{Leu}$, especially with the appearance of $tRNA_{3\text{ and }4}^{Leu}$. Further, all these isoaccepting tRNAs have the possibility of being "homogeneic".

EXPERIMENTAL

Green peas (*Pisum sativum* L. cv. Alaska) were surface sterilized in 10% chlorox, soaked in dist. H_2O overnight and sown in moist vermiculite. Cotyledons were harvested after 5 days of germination in the dark at 27°–29°. Harvested cotyledons were either used immediately or stored at −20° for tRNA and enzyme extractions. Samples of mature seed pods were harvested from field grown Alaska peas. The harvested seed pods were stored at −20° and used for extraction later.

Transfer RNA. Transfer RNA was prepared from total RNA of 5-day-old freshly harvested cotyledons or those stored

at −20°. Total RNA was extracted by the phenol technique of Cherry *et al.* [12] with minor modifications. Batches of 100 g of chilled tissue were ground for 3 min in a Waring blender with 100 ml of buffer A (10 mM Tris-HCl pH 7·6, 60 mM KCl and 10 mM $MgCl_2$), 100 ml of buffer washed phenol, 40 ml 11% Dupanol (Sodium lauryl sulfate) and 20 ml Bentonite suspension (40 mg/ml) [13]. The homogenate was strained through four layers of cheese cloth and centrifuged for 10 min at 10 000 *g*. The supernatant was stirred in cold for two hr with an equal vol of organic (phenolic) phase. After centrifugation at 10000 *g* for 15 min, the aq phase was collected. Equal vol of buffer-washed phenol and 1/18 vol of bentonite was added to the aq phase. This mixture was vigorously stirred in the cold for 30 min followed by centrifugation at 10000 *g* for 15 min. The phenol extraction of the aq phase was repeated 2 more times and finally the aq phase was collected and the total nucleic acids precipitated by addition of two vols of cold 95% EtOH and leaving in the freezer overnight. The ppt. was collected by centrifugation and the resulting pellet was extracted 4× with 2·0 M KOAc, pH 6·5 (1 ml/100 g tissue) followed by centrifugation. This crude *t*RNA was further purified on a 2 ml column of DEAE-23 cellulose in buffer B (10 mM NaOAc pH 4·5 containing 10 mM $MgCl_2$) and eluted with 1 M NaCl in buffer B. The *t*RNA soln was dialysed against dist. H_2O in the cold, and the concentration determined by UV absorbancy at 260 nm.

Leucyl-tRNA synthetase. Extraction, purification and fractionation of the enzyme was performed at 0–4°. Freshly harvested or frozen cotyledons were ground for a few minutes with insoluble polyvinylpyrolidone (Polyclar AT; 200 mg/g tissue) using a mortar and pestle. The grinding was continued for the next 15 min with stepwise addition of grinding medium, which consisted of 25 mM KPi pH 7·8, in soln C (10^{-2} M 2-Mercaptoethanol; 10^{-5} M phenylmethylsulfonyl chloride; 10^{-6} M L-leucine) saturated to 30% with respect to $(NH_4)_2SO_4$. The homogenate was strained through cheese cloth and centrifuged for 15 min at 27 000 *g*. Supernatant was filtered through a miracloth filter and the $(NH_4)_2SO_4$ concn increased to 60% saturation (195 mg added per ml) and stirred in the cold for 30 min. The enzyme pellet collected after centrifugation at 10 000 *g* for 10 min was dissolved in 5 ml 25 mM KPi pH 7·8 in soln C, and dialysed against the same buffer for several hours in the cold.

DEAE-cellulose chromatography. DEAE-23 cellulose chromatography was performed using a stepwise elution technique. Dialysed enzyme was adsorbed on a 20 ml column of DEAE cellulose, which was previously equilibrated in 25 mM potassium phosphate, pH 7·8 in soln C. After washing the column with 50 ml of the same buffer, the enzyme was eluted with 100 mM KPi, pH 7·8 in soln C. Five ml fractions were collected and assayed for leucyl-*t*RNA synthetase activity and UV absorption at 280 nm was recorded.

Hydroxylapatite column chromatography. Material from the DEAE-23 cellulose column chromatography was diluted 1:1 with cold distilled H_2O and the pH adjusted to 6·5 with 0·05 M KH_2PO_4. The soln was applied to a hydroxylapatite column (HA) column, made of a mixture of 10 g hydroxylapatite and one g cellulose powder (Whatman CF 11) with small pads of cellulose at the bottom and top of the packing. The column was previously equilibrated with 50 mM KPi buffer pH 6·5 in soln C, containing 10% glycerol. The column was washed with 50 ml of the above buffer and finally eluted by a linear gradient of KPi buffer, pH 6·5 from 50 to 400 mM in soln C, both containing 10% glycerol. Fractions of 6 ml were collected and assayed for leucyl-*t*RNA synthetase activity in a reaction mixture of 0·25 ml containing 0·05 ml of fractionated protein, and all other components as described in amino acylation assay.

Transfer RNA aminoacylation assay. The reaction was carried out at 30°. Unless otherwise stated, a 1 ml reaction mixture contained: 10 μmol Tris–HCl, pH 7·8; 5 μmol $MgCl_2$; 0·5 μmol ATP; 0·2% soluble polyvinylpyrollidone; 0·2 mg *t*RNA; 0·2–0·5 mg of enzyme and 20 μl unneutralized solution of L-(4,5-^{3}H) Leucine solution (60 Ci/μmol). Reaction was terminated after 20 min by the addition of 5 ml of ice cold 5% trichloracetic acid. The samples were filtered through glass fiber filters (Whatman GF/A) and counted in a liquid scintillation counter.

Assay for aminoacyl-tRNA synthetase activity. The reaction was carried out at 30°C. The amino acids studied were arginine, aspartic acid, alanine, glycine, histidine, glutamic acid, isoleucine, leucine, methionine, phenylalanine, tyrosine, and valine. The reaction mixture consisted of 250 μl which included: 4 nmol *t*RNA, 100 μg protein, 200 pmol of the amino acid under study and the concentration of other components as used for *t*RNA-aminoacylation assay. Reaction tubes were incubated at 30° and 50 ml aliquots were withdrawn in duplicate after 2 and 4 min. The aliquots were spotted onto Whatman 3 mm paper discs and placed immediately into cold 5% TCA. Filters were washed a second time with cold 5% TCA, once with EtOH–Et_2O (1:1), dried under a hot lamp, and put into vials containing a toluene scintillation fluid (toluene 1000 ml, PPO (2, 5-diphenyloxalate) 4g, POPOP (P-bis-2,5-phenyloxazolyl) C_6H_6 50 mg) and the radioactivity determined in a liquid scintillation counter.

Reversed phase chromatography-(RPC-5) on Plaskon. A mixture of 4 ml of Adogen 464 in 200 ml $CHCl_3$ was coated on to 100 g of polychlorotrifluoroethylene (Plaskon) support. For Plaskon column fractionation, *t*RNA was aminoacylated as described above in a 2 ml reaction mixture containing larger quantities of *t*RNA and enzyme. The concentration of other components were the same. Reactions were run for 20 min at 30° followed by chromatography on a DEAE-23 cellulose column (1 × 3 cm) as described by Anderson and Cherry [14]. Approximately 50 000 cpm of the acylated *t*RNA sample were applied to the RPC-5 column and eluted with a linear gradient of 0·5–0·9 M NaCl in buffer B. Fractions of 10 ml each were collected, made 5% with respect to trichloroacetic acid and filtered through glass fiber filters (Whatman GF/A). The amount of radioactivity on the filter was determined in Mark II Nuclear Chicago Liquid scintillation counter.

Isoelectric focusing. Isoelectric focusing was performed using the LKB 8100 Ampholine Electrofocusing equipment (LKB 8101, Column Capacity 110 mls).

Polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed as described by Davis [15] and Ornstein [16]. A 7·5% gel, alkaline system with separation at pH 8·3 was used. After extrusion, the gels were stained with 1% Amido Schwartz in 7% acetic acid for several hours and then electrophoretically destained.

Analytical ultracentrifugation. The sedimentation coefficient for the concentrated peak II synthetase from HA column chromatography (6 mg/ml) was measured from the schleiren patterns obtained using a Beckman Model E Analytical Ultracentrifuge equipped with schleiren optics and electronic speed control. The sample was analysed at 56 000 rpm and 4–8°C in an An-H rotor.

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