Phytochemistry, 1978, Vol 17, pp 19–27 Pergamon Press. Printed in England.

PURIFICATION AND CHARACTERIZATION OF TWO TYROSYL-*t*RNA SYNTHETASE ACTIVITIES FROM SOYBEAN COTYLEDONS*

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(*Revised received* 4 *July* 1977)

Key Word Index—*Glycine max*; Leguminosae; soybean; transfer RNA; aminoacyl *t*RNA synthetase.

Abstract—The tyrosyl-*t*RNA synthetases located in cytoplasm and chloroplasts of soybean cotyledons were purified to near homogeneity by ammonium sulfate precipitation, DEAE-cellulose chromatography, hydroxylapatite chromatography, and DEAE-Sephadex A-25 chromatography. Purified cytoplasmic tyrosyl-*t*RNA synthetase shows only a single band in acrylamide gel electrophoresis which corresponds to a MW of 126000. In SDS-acrylamide gel electrophoresis the enzyme again shows only a single band which corresponds to a MW of 61000. Chloroplast tyrosyl-*t*RNA synthetase shows only one band in both acrylamide and SDS-acrylamide gel electrophoresis with MWs being 98000 and 43000, respectively. For cytoplasmic tyrosyl-*t*RNA synthetase the apparent K_ms determined are 6.8 μM L-tyrosine, 49 μM ATP, and 8.9×10^{-5} M *t*RNA (as total *t*RNA). Apparent K_ms for chloroplast tyrosyl-*t*RNA synthetase are 4.9 μM L-tyrosine, 214 μM ATP and 2.2×10^{-8} M *t*RNA (as BDC-ethanol fraction *t*RNA). Fractionation of soybean cotyledon-*t*RNA on RPC-5 columns gives 4 tyrosyl-*t*RNA species, the first two species ($tRNA^{Tyr}_{1 \text{ and } 2}$) are acylated only by cytoplasmic tyrosyl-*t*RNA synthetase while the last two species ($tRNA^{Tyr}_{3 \text{ and } 4}$) are acylated only by chloroplast tyrosyl-*t*RNA synthetase.

INTRODUCTION

Previous work from our laboratory has shown that enzyme preparations from soybean cotyledons normally acylate 6 isoaccepting leucyl-*t*RNA species, but the same enzyme preparations are incapable of acylating *t*RNA with tyrosine without an associated degradation of the *t*RNA [1]. Subsequent studies have shown that these enzyme preparations are deficient in cytoplasmic tyrosyl-*t*RNA synthetase activity, and that chloroplast tyrosyl-*t*RNAs were being degraded by the enzyme preparations (unpublished data). In order to characterize the tyrosyl-*t*RNA isoaccepting species of soybean cotyledons, and to investigate the properties of soybean tyrosyl-*t*RNA synthetases it was necessary to purify the tyrosyl-*t*RNA synthetase activities of soybean cotyledons more completely. This paper reports the purification (to near homogeneity) and partial characterization of the major tyrosyl-*t*RNA synthetase activities of soybean cotyledons, and the demonstration that one of these activities is localized in chloroplasts.

RESULTS

Purification of cytoplasmic tyrosyl-tRNA synthetase

The steps of purification of cytoplasmic tyrosyl-*t*RNA synthetase from soybean cotyledons are summarized in Table 1. Purification was carried out at 4° as follows: Step 1. 4- or 5-day-old etiolated soybean cotyledons (400 g) were ground for 2 min with 20 g of insoluble PVP and 230 ml of grinding medium consisting of 40 ml KPi, pH 7.8, μM tyrosine, 5 mM 2-mercaptoethanol and 0.1 mM EDTA. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 30000 *g* for 10 min. Step 2. The crude supernatant was spun at 105000 *g* for 2 hr. The protein which could be precipitated from the supernatant between 220 g/l. and 330 g/l. $(NH_4)_2SO_4$ was suspended in 10 ml of 15 mM KPi, pH 7.8, in TDEG (1 μM tyrosine, 0.2 mM dithiothreitol, 0.1 mM EDTA and 10% glycerol). The suspended pellet was passed through a 2.2 × 25 cm G-50 Sephadex column equilibrated with 15 mM KPi, pH 7.8, in TDEG. Step 3. The desalted enzyme from G-50 was applied to a 3.4 × 30 cm DEAE-cellulose column, previously equilibrated with 15 mM KPi, pH 7.8, in TDEG. The column was washed (200 ml/hr, 5 ml fractions) with 200 ml of equilibrating buffer, and an 800 ml linear gradient from 15 mM to 250 mM KPi, pH 7.8, in TEDG was used for elution. The elution profile of the DEAE-cellulose column is shown in Fig. 1. Tyrosyl-*t*RNA synthetase eluted as a single major activity slightly behind the major protein peak eluting from the column. There was a small activity peak eluting at higher salt concentrations (chloroplast synthetase). Fractions constituting the bulk of the activity were pooled, and diluted 2.5 times with TDEG. The pH of the diluted fractions was titrated to 6.7 with 10% H_3PO_4 in TDEG. Step 4. The DEAE-cellulose fractions were applied at a flow rate of 40 ml/hr to a 1.2 × 12 cm hydroxylapatite (HA) column, previously equilibrated with 30 mM KPi, pH 6.7, in TDEG. The column was washed with equilibrating buffer and eluted with a 300 ml linear gradient from 0.03 to 0.3 M KPi, pH 6.7, in TDEG, (3 ml fractions). The tyrosyl-*t*RNA synthetase activity eluted from the column as a single activity peak, well behind the major protein peak at

* Journal paper no. 6132 of the Purdue Agricultural Experiment Station.

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Table 1. Purification of cytoplasmic soybean tyrosyl-*t*RNA synthetase

Step	Total protein (mg)	Total enzyme activity (μ)*	Yield (%)	Specific activity (μ/mg)	Fold purification
Crude homogenate	36 300	1550	100	0.043	1
Ammonium sulfate G-50	2890	1090	71	0.380	9
DEAE-cellulose	388	840	54	2.50	58
Hydroxylapatite	24.8	540	35	22	512
DEAE-Sephadex	1.82	290	19	161	374

*1 unit of tyrosyl-*t*RNA synthetase activity is defined as the amount of enzyme which acylates 1 nmol of tyrosyl-*t*RNA in 10 min.

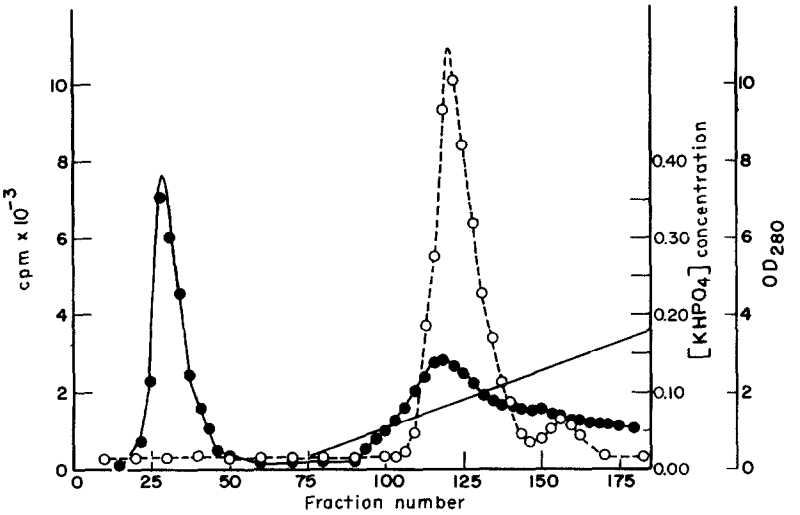

Fig. 1. DEAE-cellulose column chromatography of cytoplasmic tyrosyl-*t*RNA synthetase. An enzyme preparation from 400 g of tissue, after $(NH_4)_2SO_4$ precipitation and G-50 desalting, was chromatographed on a DEAE-cellulose column (see text). The straight solid line denotes KPi concentration. Closed circles denote A_{280} and open circles enzyme activity as cpm tyrosine [^{3}H] acylated onto *t*RNA.

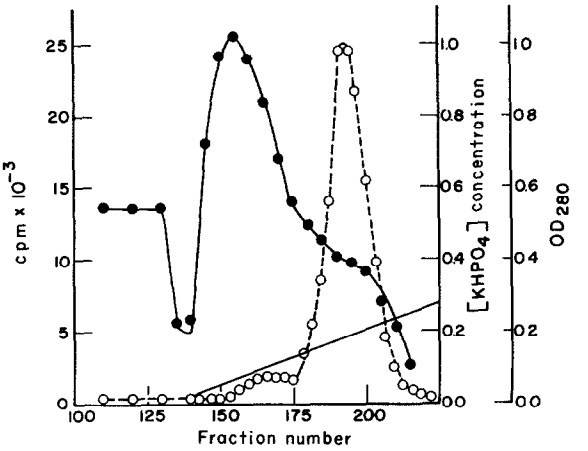

Fig. 2. Hydroxylapatite column chromatography of cytoplasmic tyrosyl-*t*RNA synthetase. The pooled, diluted, pH adjusted fractions from DEAE-cellulose were chromatographed on a HA column as outlined in the text. The straight solid line denotes KPi concentration. Closed circles are A_{280} and open circles are enzyme activity (see Fig. 1).

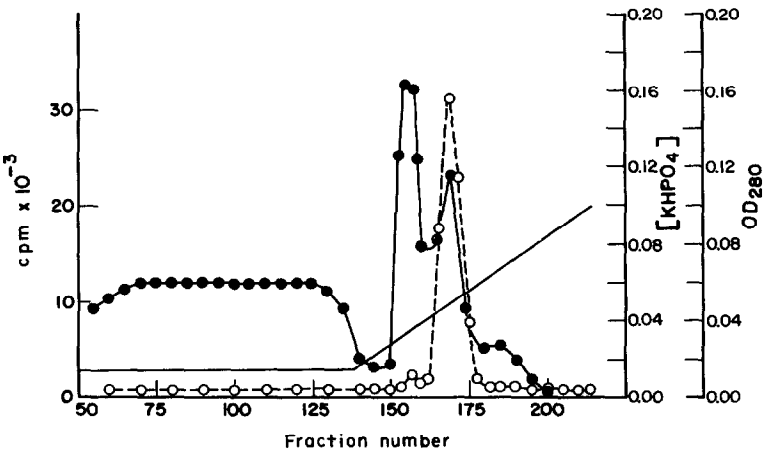

Fig. 3. DEAE-Sephadex A-25 column chromatography of cytoplasmic tyrosyl-*t*RNA synthetase. A DEAE-Sephadex A-25 column was loaded with the pooled diluted fractions from HA, washed and eluted with a linear gradient. The straight solid line represent A_{280} and closed circles represent enzyme activity (see Fig. 1).

about 0.21 M KPi (Fig. 2). Fractions containing the bulk of the activity eluting from HA were pooled and diluted 10 times with TDEG. Step 5. The HA fractions were applied to a 0.9 × 20 cm A-25 Sephadex column equilibrated with 15 mM KPi, pH 6.7, in TDEG. The column was washed with 20 ml of equilibrating buffer at a flow rate of 30 ml/hr. Fractions (2 ml) were collected, and the column was eluted with a 200 ml linear gradient from 0.15 to 0.1 M KPi, pH 6.7, in TDEG.

Once again the tyrosyl-*t*RNA synthetase activity eluted as a single peak of activity, which was coincident with the second of two major protein peaks eluted from the column (Fig. 3). Fractions containing enzyme of highest sp. act. were pooled, made to 50% in glycerol and stored at −22° for further use. Enzyme stored in this manner showed less than 10% loss of activity during 6 months of storage. The enzyme produced as described above was used for all subsequent experiments on cytoplasmic tyrosyl-*t*RNA synthetase.

Purification of chloroplast tyrosyl-tRNA synthetase

Chloroplast tyrosyl-*t*RNA synthetase was purified from a 3000 *g* pelleted fraction from green soybean cotyledons by essentially the same procedure as outlined for cytoplasmic tyrosyl-*t*RNA synthetase. A table of purification for chloroplast synthetase is not presented because it was not possible during initial purification steps to show activity for chloroplast tyrosyl-*t*RNA synthetase in the standard chloroplast synthetase reaction mixture (see Experimental). This was probably due to the high levels of RNase and ATPase found in chloroplast preparations. To qualitatively detect activity in the early steps of purifications it was necessary to use tyrosine-[^{3}H] of a higher activity. In later purification steps where the lower sp. act. radioisotope could be used, the yield in any given step was about the same as that for cytoplasmic enzyme.

Step 1. A crude chloroplast pellet was used as starting material for the preparation of chloroplast tyrosyl-*t*RNA synthetase. 10 kg of 6-day green soybean cotyledons were ground in 500 g batches with 7 l. of grinding medium consisting of 0.5 M sucrose, 40 mM KPi, pH 7.8, 1 μM tyrosine, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol. The homogenate was filtered through 4 layers of cheesecloth and through 2 layers of Miracloth, and centrifuged at 3000 *g* for 10 min to produce a crude chloroplast pellet. Step 2. The 3000 *g* pellet was suspended in a minimum volume of 20 mM KPi, pH 6.7. in 1 μM tyrosine, 0.2 mM dithiothreitol (DTT), and 0.1 mM EDTA; and homogenized. The lysate was then centrifuged at 30 000*g* for 10 min, and then for 2 hr at 105 000 *g*. The protein which could be precipitated from the high-speed supernatant between 180 g/l. and 360 g/l. $(NH_4)_2SO_4$ was suspended in 10 ml of 15 mM KPi, pH 7.8, in TDEG, and passed through a 2.2 × 25 cm G-50 Sephadex column equilibrated with the same buffer. Step 3. The G-50 desalted protein was applied to a 3.4 × 30 cm DEAE-cellulose column, and chromatographed as in Step 3 of the purification for cytoplasmic enzyme. Fig 4 shows the elution of chloroplast tyrosyl-*t*RNA synthetase from a DEAE-cellulose column. There is one major protein peak in the profile, and one peak of tyrosyl-*t*RNA synthetase activity slightly trailing the protein peak. Fractions containing tyrosyl-*t*RNA synthetase activity were pooled and diluted × 6 with TDEG. The pH of the diluted fractions was then adjusted to 6.7 with 10% H_3PO_4 in TDEG. Step 4. DEAE-cellulose fractions constitute *ca* 1.5 l. and the maximum flow rate for the HA column was about 40 ml/hr. The loss of activity during the lengthy application of the large amount of soln to the column would cause serious losses in final yield and purification. To avoid this a 1.2 × 12 cm HA column was packed and equilibrated with 30 mM KPi, pH 6.7, in TDEG, and *ca* 70% of the adsorbent was removed from the column and stirred into the DEAE-cellulose pooled fractions. After stirring for 1 hr the slurry was centrifuged at 10 000 *g* for 10 min. The ppt. was suspended in 30 mM KPi, pH 6.7 in TDEG, and the column was repacked over the 30% of the gel which was not stirred into the DEAE fractions. This batchwise loading procedure saved about 45 hr, and the loss of activity employing this step was much less than

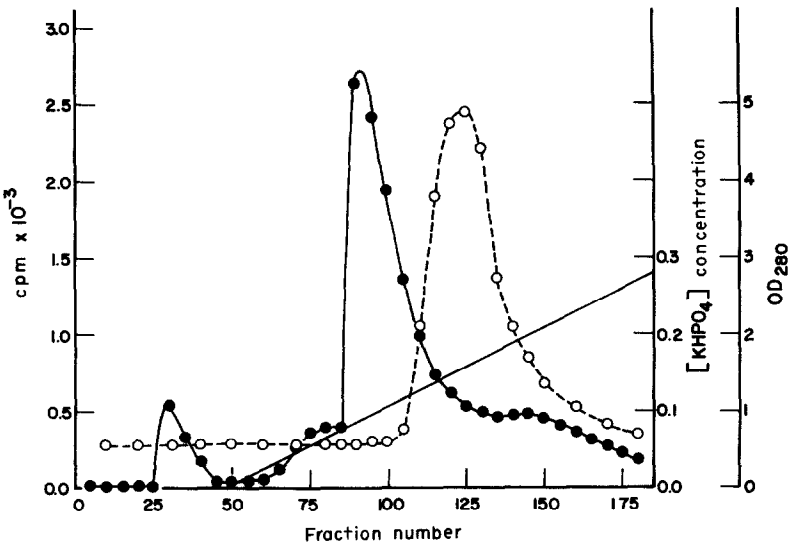

Fig. 4. DEAE-cellulose column chromatography of chloroplast tyrosyl-*t*RNA synthetase. Enzyme prepared by lysis of the 3000 *g* pellet and $(NH_4)_2SO_4$ precipitation was desalted on Sephadex G-50 and chromatographed on a DEAE-cellulose column. The straight solid line indicates KPi concentration. Closed circles indicate A_{280} eluting from the column and open circles denote cpm enzyme activity as cpm tyrosine-[^{3}H] acylated to *t*RNA.

normal loading. The resolution of activity was unaffected by the loading procedure used. After batchwise loading of the HA column, the column was washed, and eluted (see Step 4, cytoplasmic enzyme preparation). Fig. 5 shows the elution profile from the HA column. One single, symmetrical peak of tyrosyl-*t*RNA synthetase activity was eluted from the column between 0.07 and 0.12 M KPi. Typically, more activity appeared to be recovered from the HA coumn than was loaded onto it. This was probably because the enzyme was purified from inhibitory activites during this step. Fractions containing the bulk of the tyrosyl-*t*RNA synthetase activity were pooled and diluted 3 fold with TDEG. Step 5. the HA fractions were applied to a 0.9 × 20 cm DEAE-Sephadex A-25 column which had been equilibrated with 20 mM KPi, pH 6.7, in TDEG. The column was eluted with equilibrating buffer and then with a linear gradient from 0.02 to 0.2 M KPi, pH 6.7, in TDEG. Fractions (2 ml) were collected at a flow rate of 39 ml/hr. The elution profile of chloroplast tyrosyl-*t*RNA synthetase from Sephadex A-25 is shown in Fig. 6. The activity eluted with a smaller protein peak ahead of the bulk of the protein eluting from the column. Fractions having highest sp. act. were pooled. The pooled fractions were made to 50 % in glycerol and stored at −20° for use in

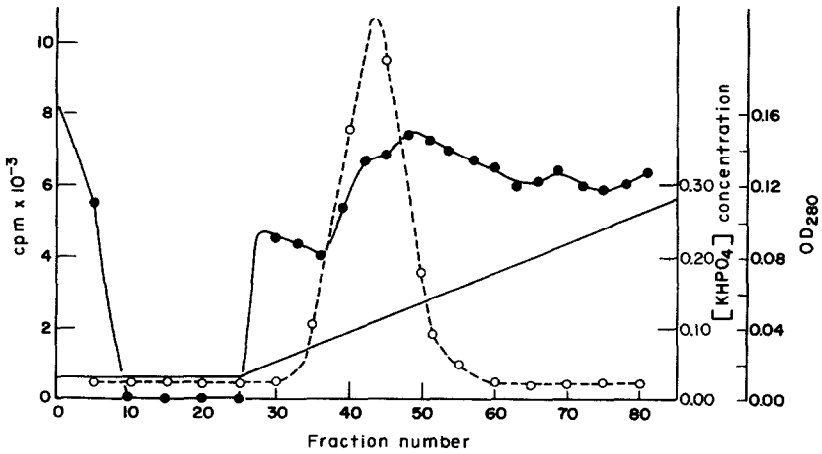

Fig. 5. Hydroxylapatite column chromatography of chloroplast tyrosyl-*t*RNA synthetase The pooled, diluted, pH adjusted fractions were bulk-loaded onto a HA column and chromatographed as described in the text. The straight solid line denotes potassium phosphate concentration. The closed circles are A_{280} eluting from the column, and open circles denote enzyme activity (see Fig. 4).

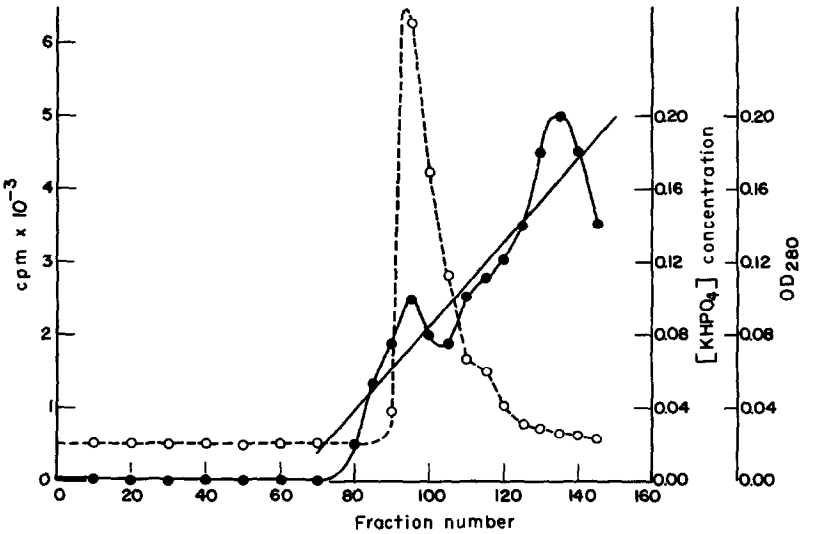

Fig. 6. DEAE-Sephadex A-25 column chromatography of chloroplast tyrosyl-*t*RNA synthetase. The pooled diluted fractions from hydroxylapatite were applied to a DEAE-Sephadex A-25 column, and a linear gradient was used to elute the column. The straight solid line denotes KPi concentration. Closed circles are A_{280} and open circles are enzyme activity.

kinetic studies, or diluted X4 with TDEG and the pH adjusted to 7.8 by the addition of 0.1 M KOH in TDEG for use in the next purification step.

When acrylamide gel electrophoresis was carried out on the DEAE-Sephadex chloroplast enzyme, 3 bands were observed. For kinetic studies and other enzymatic uses, the A-25 Sephadex purified enzyme was used because further purification resulted in low yield. However, it was necessary to further purify the enzyme for electrophoretic study. Therefore, the pooled, diluted, pH adjusted DEAE-Sephadex fractions were applied to a 0.5 × 4 cm HA column which had been previously equilibrated with 30 mM KPi, pH 7.8 in TDEG. After washing the column with equilibrating buffer, the column was eluted with a 0.03 to 0.2 M KPi, pH 7.8, linear gradient in TDEG. Fractions (1 ml) were collected (flow rate 10 ml/hr). Fig. 7 shows the elution profile of the second HA column. Four protein peaks were observable in the profile. Chloroplast tyrosyl-*t*RNA synthetase was coincident with the first of the four peaks. The bulk of the activity was pooled, concentrated and stored at −20° for electrophoretic studies.

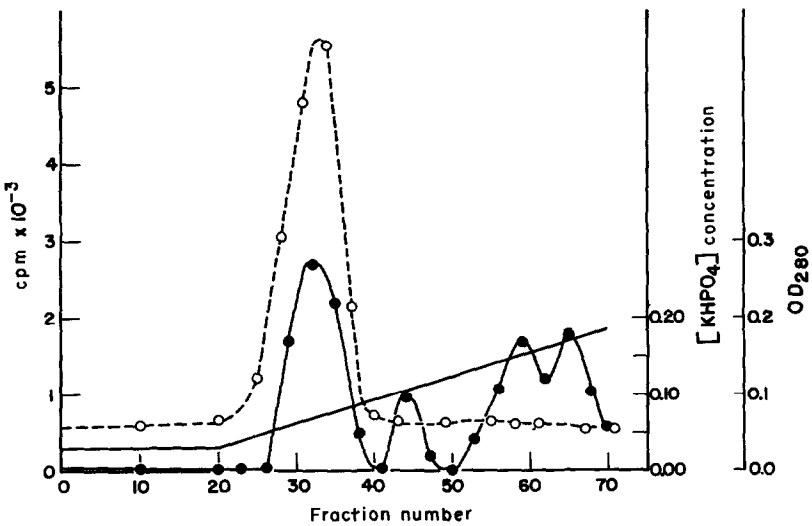

Fig. 7. Second hydroxylapatite column chromatography of chloroplast tyrosyl-*t*RNA synthetase. The pooled, diluted, pH adjusted DEAE–Sephadex fractions were chromatographed on a HA column. The straight solid line denotes KPi concentration. Closed circles are A_{280}, and open circles are enzyme activity.

Table 2. Tyrosyl-*t*RNA synthetase activity in the sucrose gradient used to fractionate the 3000*g* pellet

Pooled fractions from gradient		Tyrosyl-*t*RNA synthetase (cpm/min)	Activity % of total
Top	I	200	11
	II	100	5
	III	1500	82
	IV	50	3
Bottom pellet		—	

Chloroplast specificity if the tyrosyl-tRNA synthetase activity in the 3000 g *pellet*

To demonstrate that the tyrosyl-*t*RNA synthetase activity obtained by lysis of the 3000 *g* pellet was localized in the chloroplast, an aliquot of the 3000 *g* pellet was suspended in 35% sucrose, containing 20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, and 0.2 mM DTT; and 40–60 A_{280} units of protein were layered onto a 35 to 65% sucrose gradient in 20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, and 0.2 mM DTT. The gradient centrifuged for 4 hr 105 000 *g* and fractionated. The A_{280} profile (protein) and A_{650} profile (chlorophyll) show only one major component. To assay tyrosyl-*t*RNA synthetase in various parts of the gradient, fractions were pooled into 4 larger fractions, and lysed by dilution with 1 vol. of cold H_2O. Since the protein and chlorophyll were coincident and since the position of the single peak in the gradient was equivalent to the reported position of chloroplasts under these conditions [2], the fractions pooled into Fraction III (Table 2) of the gradient were considered to be chloroplastic. 80% of the tyrosyl-*t*RNA synthetase activity was found in Fraction III. Fraction II, which would contain any mitochondria present, and the fraction pelleting through the gradient which would contain nuclei and glyoxysomes contained no measurable activity.

That the activity in the 3000 *g* pellet is distinct from the activity in the whole cell is shown in Fig. 8. Soybean cotyledons (200 g) were taken through Steps 1 and 2 of the cytoplasmic enzyme purification procedure. After Step 2, however, the activity was adsorbed onto a 2.5 × 30 cm DEAE-cellulose column which was washed with several column vols of 50 mM KPi pH 7.8 in TDEG. The synthetase activities were then eluted from the DEAE column with 0.3 M KPi, pH 7.8, in TDEG. The 0.3 M KPi DEAE-cellulose fraction was diluted 10 times with TDEG and the pH adjusted to 6.5. The DEAE-cellulose fractions were applied to a HA column and chromatographed as in Step 4 for cytoplasmic enzyme. The elution profile of this column which was loaded with the lysate of 3000 *g* pellet in Fig. 8. It is clear that the 3000 *g* pellet contains almost no tyrosyl-*t*RNA synthetase activity eluting at high Pi concentration. This fact combined with the sucrose gradient data suggests that the tyrosyl-*t*RNA synthetase activity of the 3000 *g* pellet is a distinct tyrosyl-*t*RNA synthetase associated with the chloroplast.

Polyacrylamide gel electrophores. Comparison of cytoplasmic and chloroplast tyrosyl-tRNA synthetases

MWs were determined for chloroplast and cytoplasmic tyrosyl-*t*RNA synthetase by electrophoresis on 7% acrylamide gels. Standards used to estimate MWs

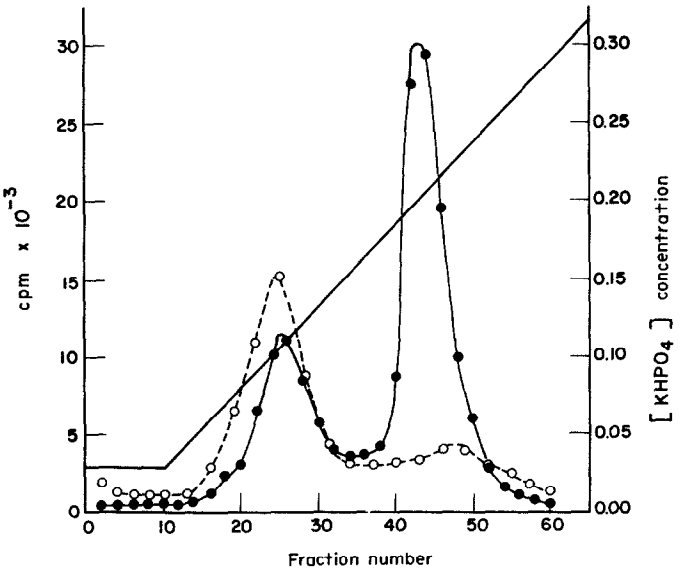

Fig. 8. Comparison of tyrosyl-*t*RNA synthetase activity in a lysed 3000 *g* pellet with whole cell tyrosyl-*t*RNA synthetase on hydroxylapatite. A tyrosyl-*t*RNA synthetase preparation partially purified from 200 g of cotyledons (see text) and the lysate from a 3000 *g* pellet were loaded onto identical hydroxylapatite columns and the columns were eluted with a linear gradient of potassium phosphate (solid line). Open circles are the elution of the 3000 *g* lysate and closed circles are the elution profile of the partially purified whole cell tyrosyl-*t*RNA synthetase.

Table 3. Apparent K_ms determined for cytoplasmic and chloroplast tyrosyl-*t*RNA synthetases

Substrate	Cytoplasmic tyrosyl–*t*RNA synthetase	Chloroplast tyrosyl–*t*RNA synthetase
L-tyrosine	6.8×10^{-6}	4.9×10^{-6}
ATP	4.9×10^{-5}	2.1×10^{-4}
*t*RNA	8.9×10^{-8}	2.2×10^{-8}

included: catalase, bovine serum albumin, intestinal alkaline phosphatase, bacterial alkaline phosphatase, peroxidase, and α-chymotrypsin. The relative migration of cytoplasmic tyrosyl-*t*RNA synthetase in this system was 0.43, which corresponded to a MW of 126 000. When samples of up to 50 μg of protein were used, only a single protein band was observed. Chloroplast tyrosyl-*t*RNA synthetase had a relative migration of 0.52 corresponding to an estimated MW of 98 000.

The relative migration of cytoplasmic tyrosyl-*t*RNA synthetase in the SDS gels was 0.49, corresponding to a MW of 60 000. There was only a single band in electrophoresis, suggesting that cytoplasmic synthetase was a dimer of 2 identical subunits.

Chloroplast tyrosyl-*t*RNA synthetase taken from the second HA column showed only one band in SDS electrophoresis. The relative mobility of this band was 0.71, corresponding to a MW of 43 000, suggesting that chloroplast tyrosyl-*t*RNA synthetase was also a dimer of two identical subunits of MW of 43 000.

Optimum ATP concentration and optimum magnesium to ATP ratio

The optimum ATP and Mg^{2+} for cytoplasmic tyrosyl-*t*RNA synthetase was found to be 1 mM and 3 mM respectively. For chloroplast tyrosyl-*t*RNA synthetase the optimum ATP concentration was found to be 2 mM ATP. The optimum Mg^{2+} to ATP ratio was 2. The optimum ATP concentration range for chloroplast enzyme was broader than that for cytoplasmic enzyme, since 10 mM ATP was only slightly inhibitory for chloroplast enzyme. Accordingly, chloroplast tyrosyl-*t*RNA synthetase was assayed at 2 mM ATP and 4 mM $MgCl_2$.

Apparent K_m

Apparent K_ms for chloroplast and cytoplasmic tyrosyl-*t*RNA synthetase were determined using Lineweaver–Burk plots. Values determined for all 3 substrates are given in Table 3.

Transfer RNA specificity

Reversed phase chromatography (RPC-5) was used to demonstrate the *t*RNA isoacceptor specificity of the purified tyrosyl-*t*RNA synthetases. Samples were acylated for 5 or 30 min using either chloroplast or cytoplasmic tyrosyl-*t*RNA synthetase in the standard reaction mixture (see Experimental) except that 1 mg/ml total soluble RNA was used in both reaction mixtures. Since incubation time did not alter the RPC-5 profile, only the 30 min incubation is shown. Four major species of tyrosyl-*t*RNA are acylated by the two enzymes, which will be referred to as $tRNA_1^{Tyr}$, $tRNA_2^{Tyr}$, $tRNA_3^{Tyr}$, and

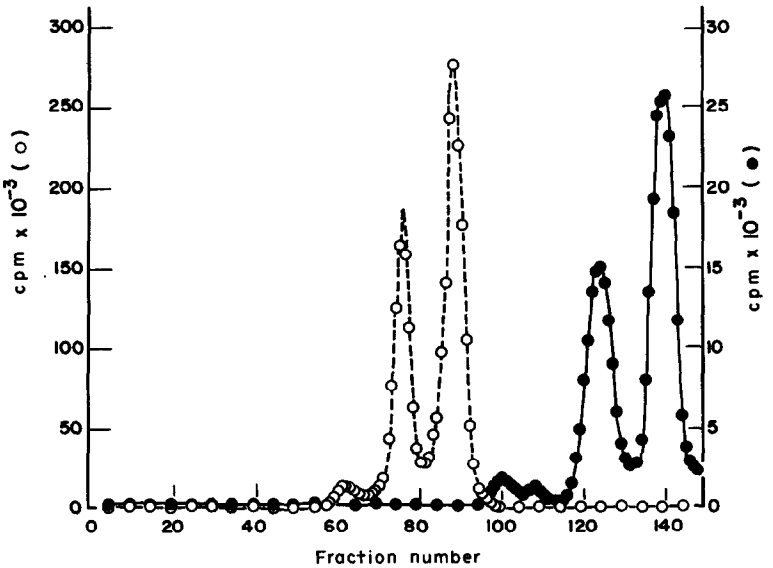

Fig. 9. RPC-5 chromatography of *t*RNA acylated by cytoplasmic and chloroplast tyrosyl-*t*RNA synthetase. Two samples of total soluble RNA were acylated separately with cytoplasmic and chloroplastic tyrosyl-*t*RNA synthetase in the appropriate standard reaction mixture. The samples were incubated at 30° for 30 min. RPC-5 chromatography was carried out separately on each of the two samples. Open circles represent a sample acylated by cytoplasmic tyrosyl-*t*RNA synthetase and closed circles represent a sample acylated with chloroplast tyrosyl-*t*RNA synthetase.

$tRNA_4^{Tyr}$ according to the relative elution position from RPC-5 column (Fig. 9). $tRNA_{1\text{ and }2}^{Tyr}$ are acylated by cytoplasmic tyrosyl-*t*RNA synthetase and $tRNA_{3\text{ and }4}^{Tyr}$ are acylated by chloroplast tyrosyl-*t*RNA synthetase. This absolute *t*RNA specificity holds at higher enzyme or *t*RNA concentrations.

Because the possibility exists that isoaccepting species obtained by reversed phase chromatography may be artifacts of chromatography, cross-checks have been performed to ensure that the 4 isoaccepting species were recognized by their cognate synthetases after chromatography. All 4 species are enzymatically deacylatable in the presence of AMP and PPi after chromatography, and the same profile is observed if the fractions are acylated after chromatography. Also heating and quick cooling the *t*RNA before or after acylation does not cause any changes in the profile. The minor species of *t*RNA shown in Fig. 9 could not meet these criteria, or were not reproducibly found in all *t*RNA preparations, and therefore, were not considered real.

DISCUSSION

A technique routinely used to prepare aminoacyl-*t*RNA synthetases in our laboratory [3] yields an unsatisfactory source of tyrosyl-*t*RNA synthetase; however, enzyme preparations by another technique [4] originally appeared suited for our experimental purposes. Two problems were encountered which ultimately created the need for further modification of the purification procedure. Usually two tyrosyl-*t*RNA synthetase activities were eluted from a HA column, but occasionally 3 peaks of activity were observed and there was present in the enzyme preparations a nuclease which degraded chloroplast *t*RNA species.

During the process of optimizing conditions for purification of the tyrosyl-*t*RNA synthetases, the smaller, earlier eluting activity from HA was lost. The presence of this early eluting tyrosyl-*t*RNA synthetase in chloroplasts had been investigated previously, and a parallel procedure was developed for the purification of this enzyme from chloroplasts. Using the procedures outlined here, it is possible to prepare cytoplasmic and chloroplast tyrosyl-*t*RNA synthetases from soybean cotyledons both of which are essentially free of *t*RNA degrading enzymes and other proteins.

That these cytoplasmic and chloroplast tyrosyl-*t*RNA synthetases are distinct enzyme species is demonstrated in several ways by the data presented here. The two enzymes each acylate distinct *t*RNA species and have different elution properties from 3 different chromatographic media. While both enzymes appear to be made up of two identical subunits, their MWs differ by *ca* 20%.

Since the demonstration of multiple synthetase activities in crude extracts from plant tissues is extremely difficult, the possibility that other tyrosyl-*t*RNA synthetases may exist in soybean has not been completely ruled-out by the data presented here. It is to be expected that soybean mitochondria contain a tyrosyl-*t*RNA synthetase since they contain a mitochondrial specific *t*RNA [6]. However, we were unable to show a unique tyrosyl-*t*RNA synthetase activity in mitochondrial homogenates. This is probably due to the fact that our cotyledon *t*RNA preparations seem to lack the mitochondrial specific *t*RNA reported in seedling *t*RNA preparations. Also the possibility exists that mitochondria contain a synthetase which is not easily distinguishable from cytoplasmic or chloroplast tyrosyl-*t*RNA synthetase.

The data presented here show that chloroplast and cytoplasmic tyrosyl-*t*RNA synthetases from soybean exhibit substrate specificity for their homologous *t*RNAs. It has been shown in other studies that chloroplast and cytoplasmic synthetases exhibit substrate specificity for homologous *t*RNA species [7–10]. Synthetase preparations for other amino acids, however, do not show such rigorous *t*RNA specificity [7,8]. In *Phaseolus vulgaris* the chloroplast and cytoplasmic tyrosyl-*t*RNA synthetases are reported not to show *t*RNA specificity [8]. The difference between the *Phaseolus* results and those presented in this paper could be attributed either to a difference between soybean and *Phaseolus*, or to the less pure enzyme and *t*RNA preparations used in the studies with *Phaseolus*.

While it is not unreasonable that different plant species would have different *t*RNA species and synthetases, it is interesting that pea roots appear to lack chloroplast *t*RNA species analogous to $tRNA_{3\text{ and }4}^{Tyr}$ and/or a synthetase for acylating these species [5,11]. These authors, however, suggest that a third *t*RNA species eluting from reversed phase columns ahead of peaks analogous to $tRNA_{1\text{ and }2}^{Tyr}$ might be chloroplast in origin. Earlier reports on tyrosyl-*t*RNAs in soybean also show a similar pre-$tRNA_1^{Tyr}$ peak [6, 12]. Using crude tyrosyl-*t*RNA synthetase preparations we have also found a similar result, but more purified preparations lack the pre-$tRNA_1^{Tyr}$ peak and contain $tRNA_{3\text{ and }4}^{Tyr}$. Recent results from our laboratory indicated the pre-$tRNA_1^{Tyr}$ peak may be a degradation product of $tRNA_{3\text{ and }4}^{Tyr}$ [13].

EXPERIMENTAL

Chemicals. Acrylamide, *N,N-Bis*-methylene acrylamide, ammonium persulfate, tetramethylethylenediamine, and hydroxylapatite (BioRad), L-tyrosine-[3,5-^{3}H], either 8 Ci/mmol, and benzoylated DEAE-cellulose (BDC) (Schwartz–Mann). RPC-5 chromatographic absorbent (Miles Laboratories) were used.

Plant material. Etiolated cotyledons were obtained from seedlings of Wayne soybean seed (Purdue Ag Alumni Seed Association, Remington, Indiana) as previously reported [4] and used as a source of cytoplasmic tyrosyl-*t*RNA synthetase. 6-day-old green cotyledons, also containing small unexpanded leaves used for the prepn of chloroplast tyrosyl-*t*RNA synthetase were obtained from seedlings grown under constant illumination from 4 × 100 W cool white fluorescent light bulbs.

Preparation of tRNA. *t*RNA from soybean cotyledons was prepared from 4- or 5-day-old etiolated soybean cotyledons by the method of [4]. Typically, *t*RNA prepared in this fashion shows a single peak in gel electrophoresis, and *ca* 3% of the total *t*RNA can be acylated with tyrosine.

BDC-EtOH fraction tRNA. Since total soluble RNA contained extremely low levels of *t*RNA species charged by chloroplastic tyrosyl-*t*RNA synthetase (*ca* 0.12–0.15%) it was necessary to find a source of *t*RNA enriched in these species for use in assaying chloroplast tyrosyl-*t*RNA synthetase. Such enrichment could be obtained by using an EtOH eluted fraction from a benzoylated DEAE-cellulose (BDC) column [14].

Assay procedure Cytoplasmic tyrosyl-*t*RNA synthetase was assayed at 30° in a standard reaction mixture containing: 50 mM HEPES buffer, pH 7.6, 1 mM ATP, 3 mM $MgCl_2$, 0.2 g/ml BSA, 12.5 μM tyrosine [3,5-^{3}H] (8 Ci/mmol), 0.67 μM tyrosyl-*t*RNA (as total soybean *t*RNA). Duplicate 0.2 ml reaction mixtures were prepared, and 50 μl aliquots were removed at 1, 3 and 5 min. The reactions were stopped with a 4 ml of cold 10% TCA

containing 2 M NaCl and 0.01 M tyrosine. The stopped reactions were allowed to stand on ice for 15 min, and the pptd *t*RNA was collected on Whatman GF/A filters. The filters were washed, dried and the radioactivity remaining on each filter determined by liquid scintillation spectrometry at a counting efficiency of 15%. Chloroplast tyrosyl-*t*RNA synthetase was assayed by the same procedure as cytoplasmic enzyme except that the standard reaction mixture contained: 50 mM HEPES buffer, pH 7.6, 2 mM ATP, 4 mM $MgCl_2$, 0.2 g/ml BSA, 9 μM tyrosine [3,5-^{3}H] (8 Ci/mmol), and amount of soybean EtOH fraction *t*RNA to give 0.15 μm tyrosyl-*t*RNA in the assay. For both chloroplast and cytoplasmic tryosyl-*t*RNA synthetase a unit of activity was defined as the amount of enzyme which acylated 1 mmol of tyrosyl-*t*RNA in 10 min.

Acrylamide gel electrophoresis was performed at pH 8.7 on 7% acrylamide gels [15]. Gels (12 × 0.8 cm) were run without stacking gels at 2 mA per tube using Bromophenol Blue as a tracking dye. Following electrophoresis, gels were stained in Coomassie Brilliant Blue [16].

SDS gel electrophoresis. Acrylamide gels (10%, 0.8 cm) were run without stacking gels [17]. The samples were denatured prior to electrophoresis by incubating them for 2 min at 100° in 50 mM Tris–HCl, pH 8.3 containing 5% 2-mercaptoethanol and 2% SDS. Gels containing 0·1% SDS were run at 1.5 mA per tube using Bromophenol Blue as a tracking dye, and stained as above.

RPC-5 chromatography. Reversed phase chromatography was performed at room temp. using the RPC-5 system [18]. Samples containing up to 2 mg of soybean soluble RNA were acylated in a standard reaction mixture (see Assay procedure) subject to modifications in enzyme concn or *t*RNA concn given in the legends to the Fig. The sample was applied to the 0.7 × 80 cm RPC-5 column, and the column was then eluted with a 300 ml linear salt gradient from 0.4 M NaCl to 0.8 M NaCl in 10 mM NaBAc, pH 4.5, and 10 mM $MgCl_2$, at a flow rate of 15 ml/hr. Fractions (2 ml) were collected, chilled to 4°, and the RNA in each fraction was prepared by the addition of 0 1 vol. of 55% TCA. The radioactivity in the ppt. was determined by scintillation spectroscopy as above (see Assay procedures).

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