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Isolation of an Organ-Specific Leucyl-tRNA Synthetase from Soybean Seedling

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ABSTRACT **The leucyl-tRNA synthetase activity from cotyledons of 4-day-old soybean seedlings has been fractionated into three components. One of these exclusively acylates two of the six $tRNA^{Leu}$ species present in this tissue. The remaining two enzyme fractions charge the other four $tRNA^{Leu}$ species equally well. Soybean hypocotyls appear to contain only the last two enzyme fractions.**

In several models for the regulation of protein synthesis (1, 2) a prominent role is ascribed to the multiple isoaccepting species of transfer RNA (tRNA). During the last few years, an enormous amount of data has been published concerning multiplicity of isoaccepting forms of tRNA in different cells (3–5). Also, a number of multiple aminoacyl-tRNA synthetase (synthetase) species have been found in more or less homogenous cell populations (6, 7). On the other hand, strong evidence exists in favor of a single synthetase for one amino acid (3). However, as pointed out by Peterson (4), the possibility cannot be ruled out that, in the course of extensive purification of the major enzyme species, some minor species of the enzyme could have been overlooked and "lost".

It is obvious that the synthetases may play a role in regulation of protein biosynthesis by controlling the relative amounts of free and acylated forms of their cognate tRNAs. Accordingly, an indirect involvement of tRNAs and synthetases in the regulation of developmental processes has been suggested by several authors (7, 8). The evidence for changing patterns of the isoaccepting tRNA species in growing or differentiating cells has been obtained with microorganisms (2, 9, 10), plants (11, 12), and animals (7, 13, 14). In addition, changes in chromatographic and electrophoretic patterns, or in the range of specificity of synthetases specific for one amino acid, have been shown to occur in the course of differentiation of microorganisms (10), plants (11), and animals (7, 13, 14).

In 1969, Anderson and Cherry (11) found that the leucyl-tRNA synthetase [L-leucine:tRNA ligase (AMP), EC 6.1.1.4] from soybean hypocotyls can acylate only four of the six chromatographically separable $tRNA^{Leu}$ species that are chargeable with the enzyme from soybean cotyledons. In view of the possibility that this result may reflect part of the biochemical mechanism underlying plant differentiation, we decided to investigate the differences between the enzymes from these two organs im more detail. In this report, we present results that show fractionation of the soybean cotyledon leucyl-tRNA synthetase into three fractions, which fall into two classes of exclusive, nonoverlapping ranges of specificity toward the six species of $tRNA^{Leu}$. Our results also indicate that soybean hypocotyls are deficient in one of the synthetase fractions, namely, the one responsible for specific amino acylation of peaks 5 and 6 of $tRNA^{Leu}$.

Abbreviation: HA column, a column made of a mixture of hydroxylapatite and cellulose powder at the ratio of 10:1 (w/w).

MATERIALS AND METHODS

Soybean (Glycine max L. cv. Wayne) seeds were soaked overnight in deionized water, sown in moist Vermiculite, and germinated in a dark humid chamber at 28°C. The tissue, cotyledons and hypocotyls, was harvested after four days of germination.

Transfer RNA

The phenol technique of Cherry *et al.* (15) was used for extraction and partial purification of total RNA from 4-day-old soybean cotyledons. Material precipitated with ethanol and collected by centrifugation was extracted several times with 2 M potassium acetate, pH 6.5 (1 ml/100 g tissue) by suspension with a mortar and pestle, followed by centrifugation. The extracts were chromatographed on a Sephadex G-100 column (2.5 × 90 cm) in 10 mM sodium acetate, pH 4.5, containing 10 mM $MgCl_2$ (buffer B) at room temperature. The eluate was assayed for leucine acceptance activity using partially purified, unfractionated synthetase from soybean cotyledons (see below). Fractions that exhibited acceptance activity (a mixture of tRNAs specific for leucine and, presumably, for the majority of other amino acids) were pooled, adsorbed on a 2-ml column of DEAE-cellulose in buffer B, and eluted with 1 M NaCl in the same buffer. The solution was dialyzed against a 1/10 dilution of buffer B and stored at −20°C. This tRNA was apparently free of contamination, as it gave only one (symmetrical) peak on gel electrophoresis in 2.5 and 5% acrylamide gels.

Enzyme

Extraction, purification, and fractionation of the enzyme was performed at 0–4°C. Freshly harvested tissue (cotyledons or hypocotyls) was chilled on ice and ground for a few minutes with insoluble polyvinylpyrrolidone (Polyclar AT; 200 mg/g tissue) with a mortar and pestle. The grinding was continued for the next 15 min with stepwise addition of the grinding medium: 3 ml of 4/3 times concentrated, or 2 ml of 2 times concentrated, per gram of cotyledon or hypocotyls, respectively. The grinding medium (1 "time concentrated") consisted of 25 mM potassium phosphate (pH 7.8), in solution C (10 mM 2-mercaptoethanol; 10 μM phenylmethylsulfonylfluoride; 1 μM L-leucine) saturated to 30% with respect to

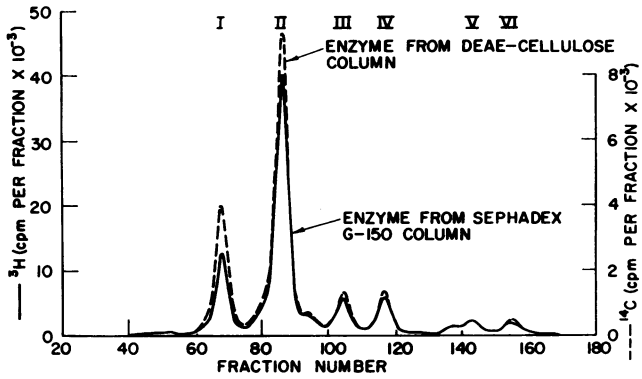

FIG. 1. Freon-column chromatography of Leu-tRNAs produced by cotyledon enzyme at two stages of purification. Cotyledon tRNA was acylated for 30 min/under the following conditions: (*a*) protein from the peak of activity from a Sephadex G-150 column (0.40 A_{280} units), 0.5 mg tRNA, and 50 μCi of [4,5-^{3}H]L-leucine per ml (*solid line*); (*b*) protein from the peak of activity from a DEAE-cellulose column (0.194 A_{280} units), 0.5 mg tRNA, and 1.63 μCi of [U-^{14}C]L-leucine per ml (*broken line*). Samples corresponding to 0.65 and 0.71 ml of reaction mixture 1 and 2, respectively, were cochromatographed.

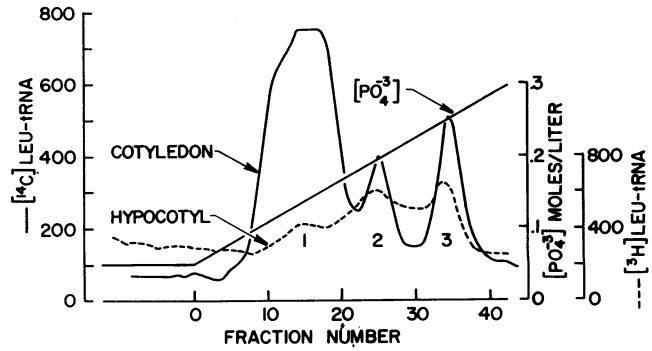

FIG. 2. Hydroxylapatite-column chromatography of leucyl-tRNA synthetases from soybean tissues. *Solid line:* data obtained with a preparation from 50 g of cotyledons assayed with [U-^{14}C] L-leucine. *Broken line:* data obtained with a preparation from 150 g of hypocotyls assayed with [4,5-^{3}H]L-leucine. Results expressed as cpm/20 min per 50 μl of enzyme.

Reaction mixtures (0.25 ml final volume) contained 0.05 ml of enzyme (fractions of the eluate). After 20 min of acylation, the entire reaction mixtures were mixed with trichloroacetic acid, filtered, and counted. The two experiments were run separately, assayed, and then the results were superimposed.

ammonium sulfate. The homogenate was strained through cheesecloth and centrifuged for 15 min at 27,000 $\times$ *g*. The supernatant was collected through Miracloth $(NH_4)_2SO_4$; was then increased to 60% of saturation (195 mg added per ml). The 30–60% fraction was collected by centrifugation and washed with 25 mM potassium phosphate (pH 7.8), in solution C saturated to 60% with $(NH_4)_2SO_4$, dissolved in 25 mM potassium phosphate (pH 7.8) in solution C, and chromatographed on a Sephadex G-150 column (2.5 $\times$ 90 cm) in the same buffer. The eluate was assayed for synthetase activity and UV absorption at 280 nm (A_{280}). About 85% of the recovered activity was adsorbed on a 20-ml column of DEAE-cellulose in 25 mM potassium phosphate, pH 7.8, in solution C. The column was washed with about 100 ml of the same buffer and the enzyme was eluted with 0.1 M phosphate, pH 7.8, in solution C. Material from the last fraction ("partially purified unfractionated synthetase") was diluted 1:1 with water and the pH was adjusted to 6.5 with 0.05 M KH_2PO_4. The solution was applied to a column (HA column) made of a mixture of 10 g of hydroxylapatite and 1 g of cellulose powder (Whatman CF 11) with small pads of cellulose at the bottom and the top of the packing. The column had previously been equilibrated with 0.05 M potassium phosphate, pH 6.5, in solution C. The column was washed with 50 ml of the above buffer, followed by a 600-ml gradient of potassium phosphate (pH 6.5), from 0.05 to 0.4 M, in solution C, as indicated in Fig. 2. Synthetase activity and A_{280} were assayed in 10-ml fractions of the eluate as above.

Synthetase assay

The reaction was performed at 30°C. Unless otherwise stated, 1 ml of the reaction mixture contained: 10 μmol Tris·HCl (pH 7.8); 5 μmol $MgCl_2$; 0.5 μmol ATP; 0.2% soluble polyvinylpyrrolidone; 10 μl of radioactive leucine solution in 10 mM HCl (either [4,5-^{3}H]L-leucine, 53.9 Ci/mmol, 500 μCi/ml, or [U-^{14}C]L-leucine, 0.312 Ci/mmol, 50 μCi/ml); 0.5–1.5 A_{260} units (about 20–60 μg) of tRNA; and rate-limiting amounts of enzyme. At a given time, the tRNA-bound radioactivity was measured as follows: (*a*) 0.1 ml of the reaction mixture was pipetted onto a paper disc, dried in stream of hot air, and washed according to Mans and Novelli (16); or (*b*) the whole, or a part, of the reaction mixture was mixed with about 10 volumes of 50 mM leucine in 10% trichloroacetic acid solution, collected on a glass-fiber filter (Whatman GF/A), washed with 5% trichloroacetic acid, and dried. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer.

Reverse-phase (Freon) column chromatography

A mixture of 6 ml of methyltricaprylylammonium chloride and 125 ml of tetrachlorotetrafluoropropane (Freon-214) was washed, according to the procedure of Weiss and Kelmers (17), and dried overnight over solid NaOH. Chromosorb W was dried overnight in an oven at 105°C. It was then allowed to cool to 30°C and was coated with the dried Freon solution; was used 61 ml per 100 g of Chromosorb. The coated Chromosorb W was allowed to equilibrate while being tumbled for several days in a closed bottle. Columns (2.5 $\times$ 40 cm or 2.5 $\times$ 90 cm) were packed with a suspension of coated Chromosorb W in 0.4 M NaCl in buffer B. For Freon-column chromatography, tRNA was charged as described above but larger quantities of tRNA and radioactive leucine were used. Leucyl-tRNA was recovered as described by Anderson and Cherry (11), and applied to a Freon column. Elution was performed at room temperature with a linear gradient of NaCl in buffer B (from 0.4 to 0.8 M). Fractions of 10 ml were collected, at a flow rate of 120 ml/hr. The entire fractions were made 5% with respect to trichloroacetic acid and filtered through glass-fiber filters. Radioactivity was determined as above.

RESULTS AND DISCUSSION

Anderson and Cherry (11) showed that the total leucyl-tRNA synthetase from soybean hypocotyls failed to acylate the small quantities of $tRNA^{Leu}$ species 5 and 6 (separable on a Freon column) that can be charged with the cotyledon enzyme. To explain this difference between the enzymes, we

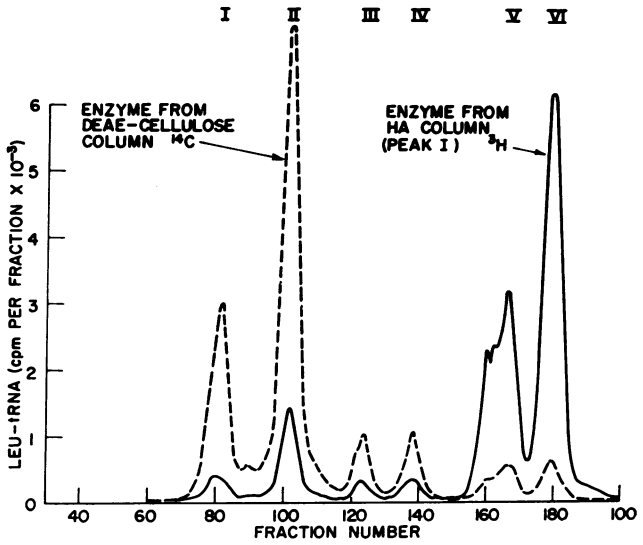

FIG. 3. Freon-column chromatography of Leu-tRNAs produced by cotyledon-enzyme 1 from the hydroxylapatite column. 0.5 mg of cotyledon tRNA was acylated with enzyme peak 1 from the HA column (0.042 A_{260} units) and 50 μCi of [4,5-^{3}H]L-leucine per ml. A sample corresponding to 0.95 ml of the above reaction mixture (*solid line*) was cochromatographed with a sample of [^{14}C]Leu-tRNA (*broken line*) produced by the unfractionated enzyme and corresponding to 0.78 ml of the reaction mixture 2 described under Fig. 1.

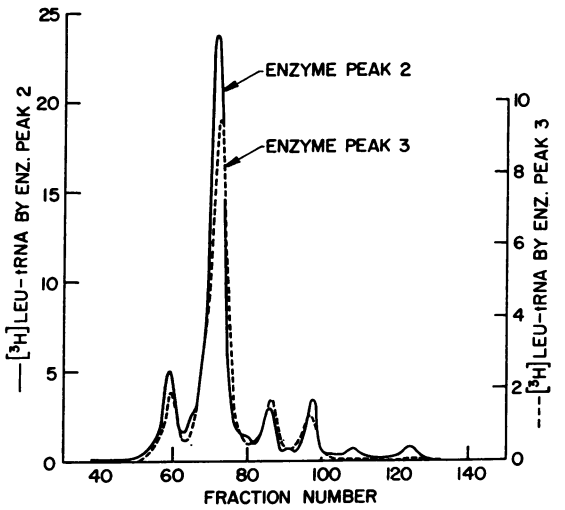

FIG. 4. Freon-column chromatography of Leu-tRNAs produced by enzymes 2 and 3 from the hydroxylapatite column. Cotyledon tRNA was acylated for 40 min with 0.3 mg of tRNA, 37.5 μCi of [4,5-^{3}H]L-leucine, and cotyledon-enzyme peak 2 about 0.01 A_{280} units) or peak 3 (about 0.005 A_{280} units). The two experiments were run separately, using samples corresponding to 0.45 ml of the above reaction mixtures. Acylation expressed as (cpm/fraction) $\times$ 10^{-3}.

adopted a hypothesis that the leucyl-tRNA synthetase activity of soybean cotyledons consists of more than one enzyme, and that one or more of these enzymes is absent from the hypocotyls. We fractionated the total, partially purified, synthetase preparation from soybean cotyledons into three fractions on a hydroxylapatite column. Under similar conditions, the hypocotyls appeared deficient in one of the three fractions.

In the development of the extraction and purification technique described above, special care was taken to avoid disproportionate loss of any of the hypothesized subspecies of the enzyme prior to the final fractionation step. The procedure is simple and relatively fast; it can be completed within 24 hr. The only time-consuming step, gel-filtration and assay of the resulting fractions, can be replaced by dialysis at the expense, however, of a substantial loss in the degree of purification. A rigorous evaluation of the quantitative aspects of the method has not been completed. Excluding the fractionation step, the apparent recovery of the activity is around 100%. This, most likely, is a result of compensation for certain losses of the enzyme by the removal of inhibitors and/or RNases in the course of its purification. An important fact is that the purification of the total enzyme does not produce any changes in its range of specificity toward $tRNA^{Leu}$ species. Fig. 1 shows the results of cochromatography, on a Freon column, of the cotyledon tRNA charged with leucine when the cotyledon enzymes purified by Sephadex and DEAE-cellulose column chromatography are used. The two enzymes display an almost identical range of specificity. Moreover, these results are similar to those obtained by Anderson and Cherry (11), using total enzyme isolated from the post-mitochondrial fraction of soybean cotyledons. This supports our conclusion that the partially purified enzyme still contains a complete set of the hypothesized leucyl-tRNA synthetase species.

Fractionation of the partially purified enzymes from the two tissues on HA columns is shown in Fig. 2. In the case of the cotyledon enzyme (*solid line*), the profile is typical in that three peaks of activity are always present. The relative magnitude of the peaks depends to a certain degree on the time elapsed between extraction and fractionation of the enzyme and on the presence of phenylmethylsulfonylfluoride*. In contrast, the hypocotyl preparation (*broken line*) gives only two major peaks of activity, which correspond to peaks 2 and 3 of the cotyledon synthetase. The peak eluting at a concentration of 0.14 M phosphate is practically absent here. In our hands, the hypocotyl preparations have shown poor stability when applied to the HA column. For this reason, this result cannot be considered as a decisive proof of the absence of this enzyme from the hypocotyl tissue. However, under no conditions have we observed this peak in the hypocotyl preparation, even when certain purification steps were omitted to make the procedure more rapid. Therefore, considering that also a crude hypocotyl enzyme displayed only traces of the activity characteristic of this peak (11), we believe that the profiles shown in Fig. 2 reflect an approximate composition of the synthetase activity in these two tissues.

The range of specificity of the individual enzyme peaks was examined using the technique of Freon-column chromatography. Fig. 3 shows the result of cochromatography of the leucyl-tRNAs obtained with total enzyme from the DEAE-cellulose (*broken line*) and the first cotyledon peak from the HA column (*solid line*). The enzyme eluting first from the HA column acylates tRNAs 5 and 6 exclusively (the presence of small amounts of peaks 1 through 4 can be ascribed to con-

* The extraction, purification, and fractionation of the cotyledon enzyme in the absence of leucine and phenylmethylsulfonylfluoride gave similar results; however, the omission of the latter resulted in lower recovery of peak 2.

tamination of enzyme 1 with the neighboring enzyme fraction). In similar experiments, the cotyledon synthetase fractions 2 and 3 and the two hypocotyl enzymes all revealed specificity for the remaining four $tRNA^{Leu}$ species producing leucyl-$tRNA^{Leu}$ peaks 1 through 4 (Fig. 4).

The same pattern of specificity was found (data not shown) when uncharged tRNA was chromatographed on a Freon column and the eluate was assayed for leucine acceptor activity with isolated fractions of the cotyledon synthetase: enzyme fraction 1 charged only two tRNA peaks, located in the area of leucyl-tRNA peaks 5 and 6, while fraction 3 preferentially acylated tRNA peaks corresponding to leucyl-tRNA peaks 1 through 4. Fraction 2 was not tested.

The Michaelis constant for the total cotyledon tRNA in the aminoacylation reaction with the enzyme fraction 1 (K_m = 2.5 A_{260} units/ml) was about twenty times higher than that with the other two enzymes. However, in terms of relative molar concentrations, the affinity of the three enzymes for their cognate tRNAs appear to be of the same order of magnitude if we consider that, in the unfractionated tRNA, species 5 and 6 account for only 8–11% of the leucine acceptor activity, while species 1–4 together make up the remaining 90% (calculated from the data in Fig. 1). This would argue against the possibility that one of the enzymes erroneously attaches leucine to tRNAs specific for an amino acid other than leucine†. This possibility, although unlikely, cannot be excluded until the tRNAs involved are available in sufficient purity to directly test their specificity.

The data are insufficient to decide whether the two synthetase fractions with apparently identical specificities (Fig. 4) represent two unrelated proteins with distinct amino acid sequences or two forms (native or artifactual) of a common polypeptide structure. So far, the results shown above (identical K_m values for the total tRNA, and identical chromatographic properties and relative proportions of the leucyl-tRNAs produced by the two enzymes) are in favor of the idea that the two enzymes differ only in some structural features not related to their catalytic function, but resulting in a different elution pattern from the HA column. However, more profound differences, including the possibility of the existence of separate sets of substrates ($tRNA^{Leu}$ 1–4) for the two enzymes, are also possible (19).

To our knowledge, the above results represent the first instance of isolation from a higher plant of an organ-specific aminoacyl-tRNA synthetase (peak 1, *solid line* in Fig. 2) with a distinct and strictly defined specificity (Fig. 3). It is interesting that a similar enzyme can hardly be detected in the hypocotyls, a tissue that has been shown (12) to contain small amounts of both of its cognate tRNAs. Further studies on the properties of this cotyledon-specific amino acid activating system: its intracellular localization, its response to hormone treatments, codon recognition by the individual $tRNA^{Leu}$ species, and their possible involvement in the biosynthesis of cotyledon-specific proteins, promise a new insight into the mechanism of plant differentiation.

† An elevated K_m would be expected for the tRNA mischarged in such an erroneous reaction (18).

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