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SPECIFICITY BETWEEN L-LYSINE:sRNA LIGASES AND sRNA FROM WHEAT GERM AND PEA SEED

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Abstract—L-Lysine:sRNA ligase purified from either wheat germ or pea seed catalysed the incorporation of lysine into homologous sRNA at a higher rate than into heterologous sRNA. sRNA fully charged with lysine in the presence of one of the two enzymes did not accept any further lysine in the presence of the other enzyme. The patterns of adsorption and elution on calcium phosphate gel suggest that the two enzymes are two different proteins. No difference between the two enzymes was observed in the rate of heat inactivation or the rate of inhibition by *p*-hydroxymercuribenzoate.

INTRODUCTION

THE role of amino acid-RNA ligases in protein synthesis has been established.[1] These enzymes are widely distributed in plant tissues.[2–5] The degree of interaction between these enzymes and sRNA of animal tissues and micro-organisms depends upon the particular amino acid involved. For example L-valine:sRNA ligase from liver catalyses the incorporation of valine into yeast sRNA.[6] On the other hand L-leucine:sRNA ligase from *Escherichia coli* fails to catalyse the incorporation of leucine into either yeast or liver sRNA.[7] Little work has been done concerning species specificity between amino acid-RNA ligases and sRNA in plant tissues apart from preliminary observations on wheat-germ enzymes and pea-seedling sRNA.[5] The experiments described here were carried out in an attempt to compare lysine:sRNA ligases purified from pea seed and from wheat germ in relation to the species specificity between these enzymes and the sRNA from each tissue.

The crude extracts prepared from wheat germ and pea seed contained considerable amounts of valine, leucine, iso-leucine, methionine, and lysine:sRNA ligases; moderate amounts of glycine, proline, and tyrosine:sRNA ligases; and traces of alanine, phenylalanine and tryptophan:sRNA ligases. The lysine:sRNA ligase preparations used in this work (see Experimental) were tested for the above-mentioned amino acid-sRNA ligases. The enzyme prepared from wheat germ[8] contained less than 5 per cent of the valine:sRNA ligase, less than 3 per cent of the leucine:sRNA ligase and less than 6 per cent of the methionine:sRNA ligase present in the wheat-germ crude extract. The lysine:sRNA ligase prepared from pea seed contained less than 4 per cent of the valine:sRNA ligase and less than 3 per cent of the

[1] P. BERG, *Ann. Rev. Biochem.* **30**, 293 (1961).
[2] J. M. CLARK, JR., *J. Biol. Chem.* **233**, 421 (1958).
[3] G. C. WEBSTER, *Arch. Biochem. Biophys.* **82**, 125 (1959).
[4] E. MOUSTAFA and M. H. PROCTOR, *Biochim. Biophys. Acta* **63**, 93 (1962).
[5] E. MOUSTAFA and J. W. LYTTLETON, *Biochim. Biophys. Acta* **68**, 45 (1963).
[6] L. I. HECHT, M. L. STEPHENSON and P. C. ZAMECNIK, *Proc. Natl Acad. Sci.* (*U.S.*) **45**, 505 (1959).
[7] R. RENDI and S. OCHOA, *J. Biol. Chem.* **237**, 3707 (1962).
[8] E. MOUSTAFA, *Biochim. Biophys. Acta* **91**, 421 (1964).

leucine:sRNA ligase found in the crude pea-seed extract. None of the other amino acid-sRNA ligases found in the crude extracts were detectable in the enzyme preparations. Both DEAE cellulose chromatography and Sephadex G200 gel filtration were attempted in further purification of the enzymes but without success.

EXPERIMENTAL AND RESULTS

Species Specificity in the Synthesis of Lysyl-sRNA

The initial rates of lysine incorporation into sRNA of pea seed and wheat germ in the presence of the enzyme preparation from either source were measured in the presence of excess of sRNA and amino acid. The results which are shown in Table 1 indicate that the rates of reaction with both enzymes were higher in the presence of homologous than in the presence of heterologous sRNA.

TABLE 1. SPECIES SPECIFICITY AND REACTION RATE IN LYSYL-sRNA FORMATION

Source of enzyme	Source of sRNA	Reaction rate
Pea seed	Pea seed	5·15
Pea seed	Wheat germ	3·55
Wheat germ	Wheat germ	1·69
Wheat germ	Pea seed	1·07

Incubation was at 37° using 4 μg pea-seed enzyme or 18 μg wheat-germ enzyme per ml reaction mixture. Reaction rate was expressed in μmoles lysine incorporated in sRNA per mg enzyme per hr.

The yield of lysyl-sRNA was the same whether the homologous or the heterologous enzyme was used. This was shown by experiments in which excess enzyme was used. When the reaction was allowed to proceed to completion in the presence of sRNA and the heterologous enzyme followed by the addition of the homologous enzyme no further incorporation of lysine into sRNA was observed (Fig. 1). It was shown in separate experiments that neither the enzyme nor the sRNA preparations contained ribonucleases that could have affected the amino acid accepting ability of sRNA over the length of time used in these experiments.

In view of the considerable degree of cross-reaction between enzymes and sRNA from the two plant tissues (Fig. 1), the question of whether the two enzymes are in fact different proteins was investigated.

Dissimilarity of the Two Enzymes

During the preparation of the enzymes it was found that when the ammonium sulphate fraction prepared from pea seed was treated with half its volume of calcium phosphate gel lysine:sRNA ligase was adsorbed on the gel (see Methods section). Under these conditions the wheat-germ enzyme was not adsorbed on the phosphate gel. Wheat-germ lysine:sRNA ligase was adsorbed on the gel only when an equal volume of gel was added to the supernatant resulting from treating the ammonium sulphate fraction with half its volume of phosphate

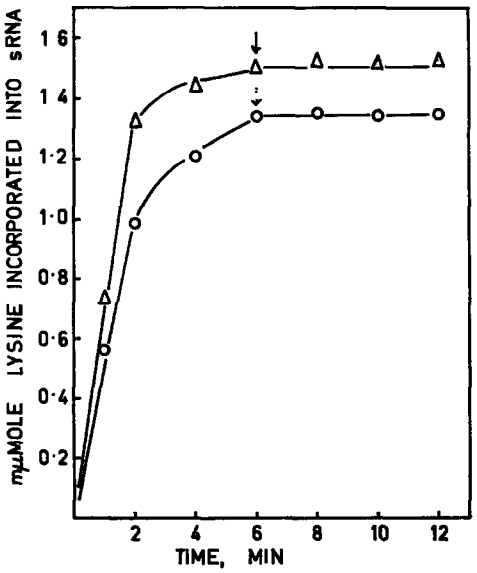

FIG. 1. SPECIES SPECIFICITY AND THE YIELD OF LYSYL-sRNA.

Incubation was at 37° using 24 μg pea-seed enzyme or 60 μg wheat-germ enzyme per ml reaction mixture.

△ Pea-seed enzyme + wheat-germ sRNA. Arrow indicates addition of 40 μg wheat-germ enzyme.

○ Wheat-germ enzyme + pea-seed sRNA. Arrow indicates addition of 16 μg pea-seed enzyme.

gel.[8] The difference in conditions under which the two enzymes are adsorbed on calcium phosphate gel does not seem to be due to the effect of impurities in either preparation. This was shown by experiments in which mixtures of the ammonium sulphate fractions prepared from wheat germ and from pea seed were treated with phosphate gel. The results of these experiments are shown in Table 2.

TABLE 2. ADSORPTION OF PEA-SEED AND WHEAT-GERM LYSINE:sRNA LIGASES ON CALCIUM PHOSPHATE GEL

Source of enzyme	^{14}C lysine incorporated into sRNA	
	First treatment with half volume of gel (counts/min)	Second treatment with equal volume of gel counts/min)
Pea seed	2212	132
Wheat germ	175	1969
Mixture of pea-seed and wheat-germ enzymes	2994	2454

Incubation was at 37° for 2 min. Results are corrected for a blank.

The concentration of ammonium sulphate necessary to elute lysine:sRNA ligase prepared from pea seed from phosphate gel is lower than that necessary to elute the wheat-germ enzyme.

This was shown by the following experiment. Each of the ammonium sulphate fractions prepared from the two plant tissues was adsorbed on phosphate gel and the gels combined and washed with phosphate buffer containing mercaptoethanol. The gel was then eluted with sucrose–phosphate buffer containing mercaptoethanol and increasing concentrations of ammonium sulphate. This was repeated with the pea-seed enzyme alone and the wheat-germ enzyme alone (ammonium sulphate fractions) and the results are shown in Fig. 2. It will be seen that the pea-seed enzyme is eluted at an ammonium sulphate concentration lower than that necessary to elute the wheat-germ enzyme.

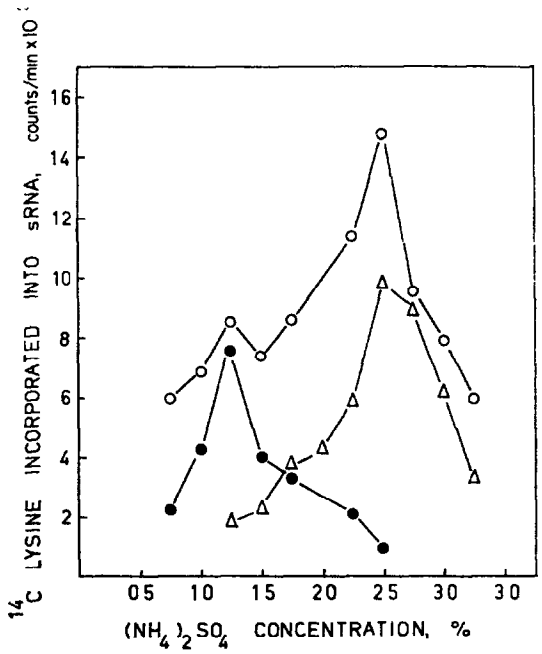

FIG. 2. ELUTION OF WHEAT-GERM AND PEA-SEED LYSINE:sRNA LIGASES FROM CALCIUM PHOSPHATE GEL.

Details of the experiment are described in the text. Incubation was at 37 for 2 min using 0·05 ml of each eluate.

– Wheat-germ enzyme.
● Pea-seed enzyme.
Mixture of wheat-germ and pea-seed enzymes.

The difference in patterns of adsorption and elution on phosphate gel suggested that the lysine:sRNA ligases from wheat germ and pea seed are different proteins.

Stability of the Enzymes and Effect of p-*Hydroxymercuribenzoate*

The high degree of cross reaction between the enzymes and sRNA from the two tissues plus the fact that the rate of reaction was higher with the homologous than with the heterologous sRNA made further experiments necessary to establish whether or not the active centres of the two enzymes are identical. It has been shown by various investigators that several amino acid:sRNA ligases are labile enzymes containing sulphydryl groups in the

active centres.[8–10] Experiments were, therefore, carried out to ascertain whether or not there is any difference in the stability or the rate of inhibition by *p*-hydroxymercuribenzoate in the case of the two enzymes used in this work.

Both enzymes lose activity to the same extent when incubated alone at 37°. In both cases about 75 per cent of the activity was lost after 5 min incubation at 37° (Table 3) and after 10 min all the enzyme activity was lost.

TABLE 3. EFFECT OF INCUBATION FOR 5 MIN AT 37° ON LYSINE:sRNA LIGASES FROM PEA SEED AND WHEAT GERM

Treatment	Rate of reaction	
	Pea-seed enzyme	Wheat-germ enzyme
Incubation alone	1·25	0·30
Incubation with $MgCl_2$	1·49	0·29
Incubation with lysine + $MgCl_2$	1·65	0·31
Incubation with ATP + $MgCl_2$	1·99	0·31
Incubation with lysine + ATP + $MgCl_2$	5·05	1·10
Control	4·95	1·01

Incubation was at 37°. Reaction rate was expressed in μmoles lysine incorporated into sRNA per mg enzyme per hr.

The instability at 37° of the two enzymes was reduced in each case when both ATP and lysine were added to the enzyme (Table 3). It will be seen that neither ATP nor the amino acid alone could reduce the loss of enzyme activity during preincubation at 37°.

The rate of inhibition of the two enzymes by *p*-hydroxymercuribenzoate is almost the same (Fig. 3).

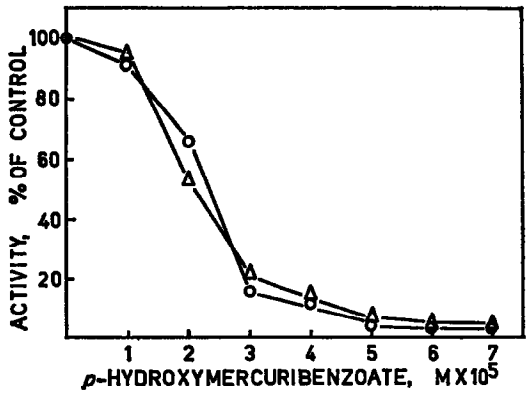

FIG. 3. INHIBITION OF WHEAT-GERM AND PEA-SEED LYSINE:sRNA LIGASES BY *p*-HYDROXYMERCURIBENZOATE.

Incubation was at 37° using 4 μg pea-seed enzyme or 18 μg wheat-germ enzyme per ml reaction mixture.

△ Wheat-germ enzyme.
○ Pea-seed enzyme.

[9] E. H. ALLEN, F. GLASSMAN and R. S. SCHWEET, *J. Biol. Chem.* **235**, 1061 (1960).
[10] K. OGATA, H. NOHARA, K. ISHIKAWA, T. MORITA and H. ASOAKA, In *Protein Biosynthesis* (Edited by R. J. C. HARRIS), p. 163. Academic Press, New York (1961).

DISCUSSION

Cross reactions between lysine:sRNA ligases and sRNA in the two tissues used in this work have been demonstrated. The yield of lysyl-sRNA is the same whether the homologous or the heterologous enzyme is used. The rate of reaction, however, is higher when the homologous sRNA is used than when the heterologous sRNA is used. This may reflect a difference in the part of the sRNA that combines with the enzyme rather than a difference in the structure of the active centres of the two enzymes.

The pattern of adsorption and elution on calcium phosphate gel shows that the lysine: sRNA ligases described in this paper are different proteins. The two enzymes, however, do not differ in the rate of inhibition by *p*-hydroxymercuribenzoate which suggests that the role of the sulphydryl groups in the active centre of the two enzymes is similar.

Both enzymes are unstable to the same extent when preincubated at 37°. They become more stable when ATP and lysine are added. This suggests that the amino acid adenylate-enzyme complex known to be formed as an intermediate in the reaction[11, 12] is more stable than the free enzyme.

MATERIALS AND METHODS

Plant Materials

Freshly milled wheat germ (variety "Gabo", New Zealand grown) was obtained from the Manawatu Milling Company, Palmerston North. This material can be stored at −20° for a year without any significant loss of enzyme activity.

Pea seed (variety "Greenfeast") was washed with running water, soaked in distilled water for 8 hr and spread on damp filter paper for 24 hr at 30°. No root or shoot had appeared at this stage of germination.

Preparation of sRNA. Wheat-germ sRNA was prepared as described previously.[5] Pea-seed sRNA was prepared by grinding 400 g pea seed with 500 ml 0·025 M tris buffer (pH 7·1) and 700 ml water-saturated phenol in a commercial Waring blendor, model CB-4, at full speed for 3 min at room temperature. The mixture was transferred to an end runner mill and ground for 30 min at 2–4°. The mixture was then subjected to the same procedure used to prepare sRNA from wheat germ.

sRNA from both plant tissues was purified by using the method of Kirby[13] as modified by Ralph and Bellamy.[14]

Purification of lysine:sRNA ligases. The wheat-germ enzyme was prepared as described previously[8] except that the solution used to elute the enzyme from the phosphate gel contained 3·25% ammonium sulphate instead of 7%.

Pea-seed enzyme was prepared by grinding 75 g of soaked seed in a chilled mortar with 40 ml sucrose buffer (0·01 M phosphate buffer, pH 7·1; 0·4 M sucrose) at 2–4°. The homogenate was left at 2–4° for 30 min with occasional stirring and then centrifuged at 7000 *g* for 30 min. The supernatant was centrifuged at 30,000 *g* for 30 min and resultant supernatant was dialysed against 800 ml sucrose buffer for 18 hr at 2–4°. This extract (96 ml), designated "crude dialysed extract", can be stored at −20° for several weeks without significant loss of enzymic activity.

[11] A. T. NORRIS and P. BERG, *Proc. Natl Acad. Sci. (U.S.)* **52**, 330 (1964).
[12] U. LAGERKVIST and J. WALDENSTRÖM, *J. Biol. Chem.* **240**, PC 2264 (1965).
[13] K. S. KIRBY, *Biochem. J.* **64**, 405 (1956).
[14] R. K. RALPH and A. R. BELLAMY, *Biochim. Biophys. Acta* **87**, 9 (1964).

The crude dialysed extract was made 0·05 M with mercaptoethanol and then fractionated by the addition of solid ammonium sulphate at pH 7·1. The fraction obtained between 40 and 55 per cent saturation was dissolved in 48 ml sucrose–phosphate buffer. This enzyme preparation (ammonium sulphate fraction) can be stored for 2 days at 2–4°.

The ammonium sulphate fraction was made 0·05 M with mercaptoethanol and 24 ml of phosphate gel were added drop by drop with stirring and the mixture left at 2–4° for 10 min. The mixture was centrifuged and the precipitate washed with 48 ml of 0·1 M phosphate buffer (pH 7·1) containing 0·005 M mercaptoethanol. The gel was then eluted with 48 ml of a solution containing 0·01 M phosphate buffer (pH 7·1), 0·4 M sucrose, 2% ammonium sulphate and 0·005 M mercaptoethanol. The eluate was made 0·05 M with mercaptoethanol and to each 1 ml 0·35 g ammonium sulphate was added (pH 7·1). The solution was stirred for 15 min at 2–4° and then centrifuged at 20,000 *g* for 15 min. The precipitate was dissolved in 24 ml of sucrose–phosphate buffer. The enzyme was about 100 fold purified by this method and contained 0·075 mg protein per ml.

Determination of enzyme activity. This was as described previously.[8] The reaction mixture was made up in tubes which were chilled in ice until incubation was started. The reaction mixture contained, per ml, 20 μmoles tris (pH 7·1, 37°), 10 μmoles $MgCl_2$, 0·036 μmoles L-[U-^{14}C]lysine (about 170,000 counts/min), 4 μmoles ATP, 4 mg sRNA and the required amount of enzyme.

When the effect of incubation at 37° was studied 15 μg pea-seed enzyme or 40 μg wheat-germ enzyme were preincubated for the appropriate time. The tube was then chilled in ice and the rest of the reaction mixture was added before incubation.

Preparation of phosphate gel. The method described by Kunitz[15] was used.

Determination of protein. The method described by Lowry *et al.*[16] was used.

Radioactive material. L-[U-^{14}C]Lysine was obtained from the Radiochemical Centre, Amersham, Great Britain.

[15] M. KUNITZ, *J. Gen. Physiol.* **35**, 323 (1952).
[16] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).