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SHORT COMMUNICATION

MOLECULAR WEIGHT ESTIMATIONS OF SOME PYRIMIDINE-METABOLIZING ENZYMES FROM PEA COTYLEDONS BY GEL FILTRATION

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Abstract—The molecular weights of 4 pyrimidine-metabolizing enzymes from imbibed pea cotyledons were estimated by filtration on a Sephadex G-150 column to be as follows: orotidine-5′-phosphate pyrophosphorylase and orotidine-5′-phosphate decarboxylase, 62,000; uridine nucleosidase, 55,000; cytidine deaminase, 47,000.

INTRODUCTION

ALTHOUGH several enzymes involved in metabolism of pyrimidine nucleotides in plants have now been identified,[1–9] extremely little is known about their molecular weights. So far as we are aware, an estimation[9] of 50,000 for the molecular weight of thymidine kinase is the only reported estimation for such plant enzymes. We wish to report molecular weights obtained by filtration on Sephadex G-150 columns for pea cotyledon cytidine deaminase (cytidine aminohydrolase, E.C. 3.5.4.5), uridine nucleosidase (E.C. 3.2.2.3) orotidine-5′-phosphate (OMP) pyrophosphorylase (E.C. 2.4.2.10), and OMP decarboxylase (E.C. 4.1.1.23).

RESULTS AND DISCUSSION

Figure 1 shows the elution profile on Sephadex G-150 for each of the 4 enzymes. OMP pyrophosphorylase and OMP decarboxylase (upper curves) both gave peaks with a maximum V_e/V_0 (ratio of elution volume to column void volume) of 1·71. These 2 enzymes catalyze successive reactions in the conversion of orotic acid to uridine-5′-P, and the possibility cannot be eliminated from these results that the same protein contains both catalytic sites. One weak argument against this is that some OMP decarboxylase activity was eluted just before the void volume (blue dextran peak) in each of the 3 experiments in which it was studied, while OMP pyrophosphorylase did not behave this way. It is possible that the first-eluted OMP decarboxylase peak represents an artifactual aggregate of the lower molecular weight protein, with loss of its OMP pyrophosphorylase activity during aggregation. Homogenization of the seeds in the presence of 0·5 mM dithiothreitol did not change the

[1] D. WANG and E. WAYGOOD, in *Modern Methods of Plant Analysis* (edited by K. PAECH and M. TRACEY), Vol. VII, p. 421, Springer–Verlag, Berlin (1964).
[2] J. H. WOLCOTT and C. ROSS, *Plant Physiol.* **42**, 275 (1967).
[3] T. D. O'NEAL and A. NAYLOR, *Biochem. J.* **113**, 271 (1969).
[4] J. T. MAY and R. H. SYMONS, *Phytochem.* **7**, 1271 (1968).
[5] F. WANKA, *Z. Naturforschung.* **22**, 91 (1967).
[6] B. S. ACHAR, R. K. MALLER and C. S. VAIDYANATHAN, *Indian J. Biochem.* **3**, 133 (1966).
[7] D. M. FRISCH and M. A. CHARLES, *Plant Physiol.* **41**, 475 (1966).
[8] B. S. ACHAR and C. S. VAIDYANATHAN, *Arch. Biochem. Biophys.* **119**, 356 (1967).
[9] O. J. SCHWARZ and R. C. FITES, *Phytochem.* **9**, 1405 (1970).

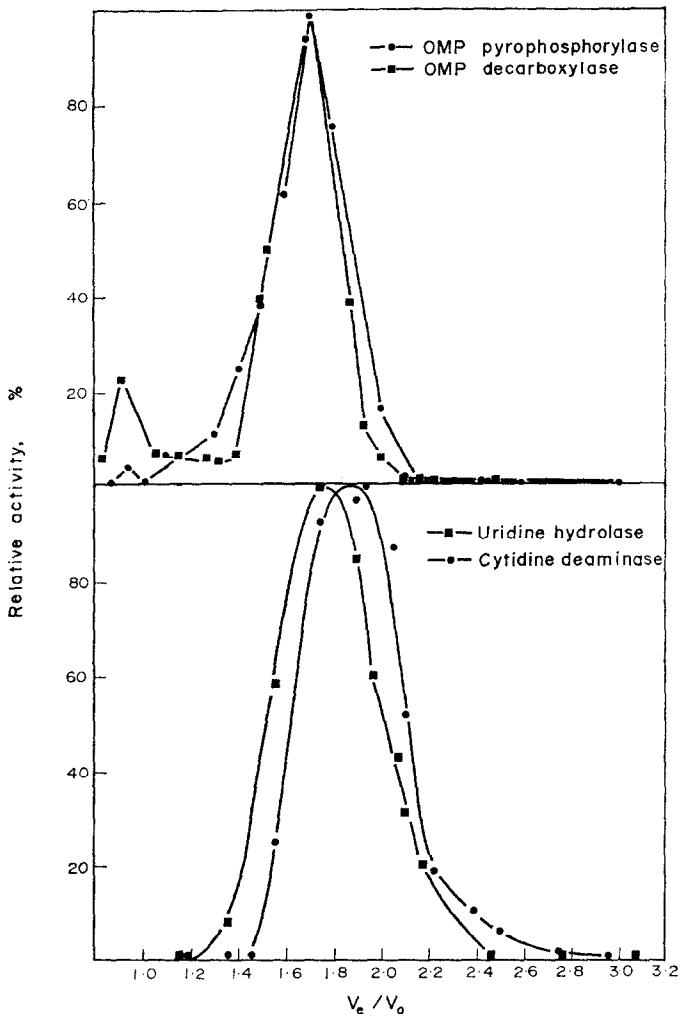

FIG. 1. UPPER CURVES SHOW ELUTION PROFILES FOR OMP PYROPHOSPHORYLASE AND OMP DECARBOXYLASE. COLUMN VOID VOLUME (V_0) WAS 49 ml. VALUES ARE MEANS FROM 2 EXPERIMENTS FOR OMP PYROPHOSPHORYLASE AND 3 EXPERIMENTS FOR OMP DECARBOXYLASE. PEAK VALUES CORRESPONDED TO RELEASE OF 0·066 mμmoles CO_2 FOR OMP DECARBOXYLASE. LOWER CURVES SHOW ELUTION PROFILES FOR CYTIDINE DEAMINASE AND URIDINE HYDROLASE, EACH CURVE A MEAN OF 2 EXPERIMENTS. MAXIMUM VALUES CORRESPONDED TO FORMATION OF 6·8 mμmoles OF URIDINE BY CYTIDINE DEAMINASE AND 2 mμmoles OF URACIL BY URIDINE HYDROLASE.

elution profile for OMP pyrophosphorylase. That the 2 enzymes from calf thymus indeed represent different proteins was indicated by Kasbekar *et al.*,[10] who found that although the 2 activities could not be separated by Sephadex columns, starch gel electrophoresis did resolve them. Nevertheless, even if the 2 enzymes are different proteins they are likely closely situated within the cell, since OMP is not normally found in plant tissues but is decarboxylated rapidly upon formation.[11] The elution profiles for cytidine deaminase and uridine

[10] D. K. KASBEKAR, A. NAGABHUSHANAM and D. M. GREENBERG, *J. Biol. Chem.* **239**, 4245 (1964).
[11] C. ROSS, *Biochim. Biophys. Acta* **87**, 564 (1964).

nucleosidase are shown in the lower curves of Fig. 1. The peak V_e/V_0 for the former occurred at about 1·88, and that for uridine nucleosidase at about 1·78. That the uracil-forming activity assigned to uridine nucleosidase is probably not due to a uridine phosphorylase was shown by measuring the conversion of uridine to uracil in the presence and absence of 0·03 M potassium phosphate. Uracil formation due to activity in tubes representing V_e/V_0 values from 1·2 to 3·1 (eluted with Tris-HCl) was the same with or without phosphate. Uridine phosphorylase thus does not appear to be present in pea cotyledons, as is also true for mung bean seedlings.[8] To our knowledge this enzyme has not been reported in higher plants, although it occurs in many other non-photosynthetic organisms[12] and results from tracer experiments suggested its presence in pinto bean leaves.[13]

The molecular weight of each enzyme was estimated from the straight line graph relating V_e/V_0 to log molecular weight of 5 proteins for which molecular weights are known. The values for OMP pyrophosphorylase and OMP decarboxylase were thus calculated to be about 62,000, uridine nucleosidase, 55,000, and cytidine deaminase, 47,000.

The use of this method has the primary uncertainty that the enzyme being studied has the same shape to molecular weight relation as the proteins used for calibration, and some elongated enzymes depart from this relation.[14] Our results should thus be considered as somewhat approximate. The estimated molecular weight of cytidine deaminase lies between the values obtained by gel filtration for the sheep liver enzyme[15] (37,000) and that from baker's yeast[16] (57,000). We are not aware of any molecular weight estimations for the other enzymes with which our values could be compared.

EXPERIMENTAL

Enzyme source. Alaska pea seeds (*Pisum sativum* L.) were soaked 3 min in 1% NaOCl, rinsed with water, and germinated under aseptic conditions on moistened filter paper in Petri dishes at about 26° for 12–36 hr. Twenty excised cotyledons were homogenized at about 4° in 10 ml of 0·05 M potassium phosphate buffer, pH 7·4. Homogenates were squeezed through 4 layers of cheesecloth and centrifuged at 27,000 *g* for 15 min at 6°. The supernatant was used in gel filtration studies.

Sephadex gel filtration. A 2·5 × 32 cm column of Sephadex G-150 (superfine) obtained from Pharmacia was equilibrated in 0·05 M potassium phosphate (pH 7·4) at 2°. Usually 1·0 ml of supernatant protein solution containing 0·1 g of sucrose was layered over the column and eluted at a flow rate of about 1·5 ml/hr with the same phosphate buffer at 2°. Approximately 40 fractions (4 ml) were collected.

Proteins of known molecular weights used to construct the elution volume (V_e) versus log molecular weight curve were cytochrome c, myoglobin, ovalbumin, bovine serum albumin and γ-globulins. Eluted proteins were detected by the method of Lowry *et al.*[17] and by measuring the absorbance of eluates at 412 nm. The column void volume (V_0) was determined with blue dextran (Pharmacia).

Enzyme assays. OMP decarboxylase activity was determined by measuring the release of $^{14}CO_2$ from OMP-7-^{14}C (New England Nuclear Corp.), essentially as described before.[2] Reaction media contained 1·0 ml of eluted enzyme solution, 0·05 μc of OMP-7-^{14}C, unlabeled OMP to make a final concentration of 9 μM, KF (final concentration, 46 mM), and potassium phosphate buffer (6 mM), pH 6·4, in a total volume of 1·63 ml. Reactions occurred at 29° for 15 min before terminating with 0·5 ml HOAc.

OMP pyrophosphorylase activity was analyzed by measuring $^{14}CO_2$ release from orotate-7-^{14}C in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) and sufficient OMP decarboxylase to remove $^{14}CO_2$ from OMP being formed.[2] OMP decarboxylase was supplied by adding 0·2 ml of eluted enzyme from 4 or 5 tubes previously determined to contain most of this enzyme. An additional 1·0 ml of eluted enzyme collected in various tubes provided OMP pyrophosphorylase for analysis. Final concentrations of other components in a total reaction volume of 2·6 ml were orotate-7-^{14}C (0·1 μc), 1·0 μM; KF, 40 mM; $MgCl_2$, 3·8 mM. Reactions occurred at 24° for 15 min before terminating with HOAc.

[12] E. W. YAMADA, *J. Biol. Chem.* **243,** 1649 (1968).
[13] C. ROSS, *Plant Physiol.* **40,** 65 (1965).
[14] P. ANDREWS, *Biochem. J.* **96,** 595 (1965).
[15] N. V. MCFERRAN, M. SMYTH and B. A. ORSI, *Biochem. J.* **114,** 8P (1969).
[16] P. L. IPATA, G. CERCIGNANI and E. BALESTRERI, *Biochemistry* **9,** 3390 (1970).
[17] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193,** 265 (1951).

Uridine nucleosidase was detected by measuring the amount of labeled uracil produced from uridine-2-^{14}C (International Chemical and Nuclear Corp.). Reaction tubes contained 0·5 ml of eluted enzyn e solution and 0·1 ml (2 μc) of labeled uridine, 40 mc/mM. After incubating 1 hr at 30° reactions were stopped by adding 0·2 ml HOAc and boiling for 1 min. The coagulated protein was centrifuged off and aliquots of the supernatant solutions were chromatographed with carrier uridine and uracil on paper strips in *n*-BuOH-HCO_2H-H_2O (77:10:13, v/v). The UV-absorbing marker spots were cut out and ^{14}C present in uridine (R_f 0·18) and uracil (R_f 0·37) was determined by liquid scintillation counting. Corrections were made for the small amount of uracil present in uridine blanks (reactions stopped at time zero).

Cytidine deaminase activity was also determined chromatographically. To tubes containing 0·1 ml (1·2 μc) of cytidine-2-^{14}C, 24·5 mc/mM, 0·5 ml of eluted enzyme solution was added. After 1 hr of incubation at 30°, ^{14}C present in marker spots of chromatographically separated cytidine (R_f 0·08), uridine, and uracil was analyzed as for uridine hydrolase. Corrections were made for small amounts of labeled uridine and uracal present in cytidine blank tubes (reactions stopped at time zero).

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