Phytochemistry, Vol. 26, No. 1, pp. 81–83, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

CHANGES IN CTP:CHOLINEPHOSPHATE CYTIDYLYLTRANSFERASE PROTEIN LEVELS IN PEA STEMS TREATED WITH INDOLE-3-ACETIC ACID

MOLLY J. PRICE-JONES and JOHN L. HARWOOD

Department of Biochemistry, University College, P.O. Box 78, Cardiff, CF1 1XL, Wales, U.K.

(*Revised received* 27 *May* 1986)

Key Word Index—*Pisum sativum*; Leguminosae; indole-3-acetic acid; cytidylyltransferase.

Abstract—Indole-3-acetic (IAA) reduced the activity of CTP:cholinephosphate cytidylyltransferase in pea (*Pisum sativum*) stems by an average of 45% compared to controls 1 hr after treatment. An enzyme-linked immunosorbent assay (ELISA) was developed to measure the changes in enzyme protein levels after IAA treatment. The levels of cytidylyltransferase protein were found to be reduced significantly but only by 5%.

INTRODUCTION

Phosphatidylcholine is the major extra-chloroplastic lipid of plant tissue [1] and it is synthesized via the CDP-base pathway [2]. Studies on the effect of indole-3-acetic acid (IAA) on the incorporation of ^{14}C-labelled precursors into phospholipids of pea (*Pisum sativum* L.) stem explants showed that there was an inhibition of incorporation of [*Me*-^{14}C]choline into phosphatidylcholine within 1 hr of treatment with 10 μM IAA [3]. Measurements of *in vivo* pool sizes of phosphatidylcholine precursors and of *in vitro* activities of enzymes in the phosphatidylcholine synthetic pathway showed that the inhibition of incorporation occurred because of a decrease in the activity of the CTP:cholinephosphate cytidylyltransferase (EC 2.7.7.15) enzyme [4]. This suggested that the cytidylyltransferase was rate-limiting for phosphatidylcholine biosynthesis in this tissue. Seventy-five per cent of the pea stem cytidylyltransferase activity has been found to be present in the cytosol [5] and one possible means by which IAA reduced the enzyme's activity was by causing a decrease in the amount of soluble cytidylyltransferase enzyme protein, either by inhibiting synthesis of new enzyme protein or by increasing breakdown. We have, therefore, measured the amount of cytidylyltransferase protein to test this hypothesis.

RESULTS AND DISCUSSION

Immunological assays have been shown to give high levels of specificity and sensitivity, and systems which depend upon the capacity of antigens to bind to an inert carrier phase for subsequent attachment of the antibody, such as the ELISA, have been found to be some of the most successful [6]. An ELISA method was developed (see Experimental) for the assay of cytidylyltransferase. The results using this method to measure the pea stem cytidylyltransferase protein were found to have excellent reproducibility, and non-specific binding due to other proteins present in the cytosol did not present a problem, possibly because the total amount of soluble protein obtained from the 5% w/v homogenates used was not high (*ca* 0.5 mg/ml).

The standard curve shown in Fig. 1 indicated that over a range of 0–15 ng cytidylyltransferase protein a straight line relationship was obtained between the amount of cytidylyltransferase protein present in the antigen–antibody mixture (see Experimental) and the decrease in horseradish peroxidase activity, after correcting the values for the occurrence of non-specific binding in the assay.

This enabled us to measure the amount of soluble cytidylyltransferase protein (in the range 20–100 ng/ml) for IAA-treated and control pea stem explants and the results are shown in Table 1. Measurements of explant length showed that IAA had had a physiological effect in

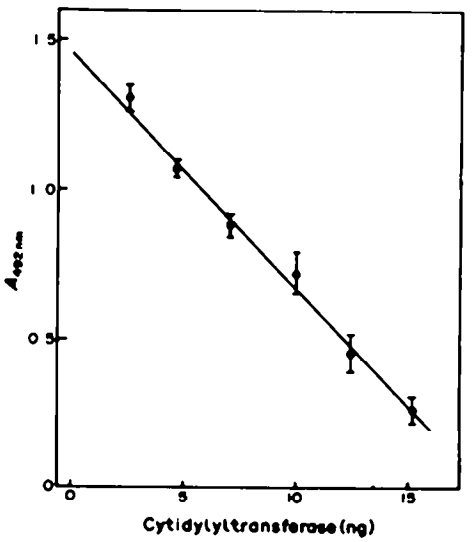

Fig. 1. Standard curve for estimation of cytidylyltransferase protein in pea stem cytosol by a competitive inhibition ELISA method. Values are means ± s.d. with duplicates for each value.

Table 1. Levels of cytidylyltransferase protein in the cytosol from IAA-treated and control pea stem explants

Expt No.	Stem length (mm)	Cytidylyltransferase activity (nmoles/min/mg protein)	Cytidylyltransferase protein (μg/mg total protein)
1. Control	7.35 ± 0.02	1.16 ± 0.03	0.284 ± 0.007
Treated	7.49 ± 0.04	0.92 ± 0.02	0.271 ± 0.005
2. Control	7.20 ± 0.03	0.65 ± 0.17	0.462 ± 0.016
Treated	7.55 ± 0.03	0.17 ± 0.06	0.442 ± 0.001
3. Control	7.24 ± 0.02	1.01 ± 0.27	0.364 ± 0.015
Treated	7.56 ± 0.04	0.48 ± 0.10	0.340 ± 0.008
Means			
Control	7.26 ± 0.04 (100%)*	0.94 ± 0.15 (100%)†	0.370 ± 0.052 (100%)†
Treated	7.53 ± 0.02 (103.7%)*	0.52 ± 0.22 (55.3%)†	0.351 ± 0.050 (94.6%)†

The cytidylyltransferase activity was assayed and the protein levels were determined as described in the Experimental. Six determinations were made for each value and the results are expressed as mean ± s.e.m.

†Significantly different at 5% level (using Student's *t*-test for paired samples).

*Significantly different at 10% level (using Student's *t*-test for paired samples).

each experiment. Mean cytidylyltransferase activity decreased in IAA-treated material by 45% and this was accompanied by a decrease in soluble cytidylyltransferase protein. However, this decrease in enzyme protein, although statistically significant, was only 5% less than that of the controls.

The results, therefore, showed that although there was a decrease in soluble cytidylyltransferase enzyme protein in IAA-treated material, it was not sufficient to account for all of the decrease in cytidylyltransferase activity. It seemed likely, therefore, that a further mechanism(s) was involved in the reduction of cytidylyltransferase activity in IAA-treated material such as allosteric regulation or changes in the subcellular distribution (cf. ref. [7]) of the enzyme.

EXPERIMENTAL

Materials. Pea (*P. sativum* cv. Feltham First) seeds were supplied by Asmer Seeds, Leicester, U.K. Phospho[*Me*-^{14}C]choline, ammonium salt (2.18 GBq/mmol) and PCS™ scintillant were obtained from Amersham International. Miracloth™ was purchased from C.P. Laboratories, Bishops Stortford, Herts, U.K.; 3 MM chromatography paper from Whatman Ltd., Springfield Mill, Maidstone, Kent, U.K.; DEAE Affi-Gel Blue from Bio-Rad Laboratories, Watford, Herts, U.K. and absolute alcohol (AR) from James Burrough, Fine Alcohols Division, London, U.K. All other chemicals were purchased from Sigma or BDH, Poole, Dorset, U.K. and were of the best available grade.

Tissue preparation. Pea seeds were grown in moist vermiculite for 10 days at 20° in an incubator with *ca* 210 μE/m^2/sec illumination. Explants (7 mm) were excised from the third internode of the pea stems and treated with IAA for 1 hr as previously described [3]. Explants were homogenized in 0.32 M sucrose, 2 mM Tris–HCl, pH 7.4, at 4° using a mortar and pestle, to give a 5% w/v homogenate, and filtered through two layers of Miracloth™, before preparation of a 105 000 *g* supernatant.

Assay for CTP: cholinephosphate cytidylyltransferase activity. Cytidylyltransferase activity was assayed and the products were separated chromatographically as previously described [8]. Assays were carried out for 1 hr at 25° and optimal conditions were found to be 2 mM $MgCl_2$, 0.25 mM ATP, 1 mM CTP, 3 mM phosphorylcholine (containing 3.7 kBq of phospho[*Me*-^{14}C]choline) and 80 mM HEPES–Tris, pH 7.5 [5].

Preparation of antibody. The cytidylyltransferase enzyme was purified from pea stems as previously described [5]. A male New Zealand White rabbit was immunized using an initial injection of 150 μg enzyme protein, followed by a second injection of 100 μg protein. IgG was separated from the rabbit serum using a DEAE Affi-Gel Blue column, eluted with 28 mM NaCl, 20 mM Tris–HCl, pH 8.0. Fractions containing IgG were dialysed to remove NaCl.

Enzyme-linked immunosorbent assay. Cytidylyltransferase enzyme protein levels were measured in the 105 000 *g* supernatant from pea stems, using a competitive inhibition enzyme-linked immunosorbent assay (ELISA) method [9]. The wells of the assay plate were coated overnight at 4° with 200 μl purified cytidylyltransferase at a concn of 5 mg/ml in 50 mM Na_2CO_3 buffer, pH 9.6. After coating, the wells were washed for 3 × 3 min with 0.5% Tween 20 in 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 0.14 M NaCl, pH 7.4 (phosphate buffered saline; PBS), to remove unadsorbed enzyme. Non-specific binding sites on the wells were blocked by incubation with 200 μl 1% bovine serum albumin in PBS for 1 hr at room temp., and wells were washed as before. A 10^6 dilution of IgG containing the anti-cytidylyltransferase antibody was mixed with different dilutions of the 105 000 *g* supernatant from pea stems or known amounts of purified cytidylyltransferase (in order to obtain a standard curve), and 200 μl of the mixture was incubated in the wells for 2 hr at room temp. (The anti-cytidylyltransferase antibody bound to the cytidylyltransferase coating the wells, but the presence of unbound cytidylyltransferase in the incubation with the antibody reduced binding to the coating antigen, because binding will also occur to the unbound antigen in the antigen–antibody mix. The amount of binding of antibody to the coating antigen was, therefore, inversely proportional to the amount of free antigen in the 105 000 *g* supernatant.) Wells were washed as before and 200 μl goat anti-rabbit IgG-horseradish peroxidase conjugate diluted 1/500 in 0.05% Tween 20 in PBS was incubated in the wells for 2 hr at room temp., before washing again. Finally, 100 μl 0.5 M Pi–citrate buffer containing 3.5 mM H_2O_2 and 2.2 mM *O*-phenylenediamine was added to each well and incubated at room temp. for 20 min. The reaction was stopped by the addition of 100 μl 2.5 M H_2SO_4 and the absorbance was read at 492 nm in a Titertek™ Multiskan plate

reader (Flow Laboratories Ltd., Irvine, Ayrshire). Controls were run omitting either coating antigen, antibody or enzyme conjugate, and also using non-immune rabbit serum.

Protein determinations. Protein was estimated by the method of Bradford [10], using bovine serum albumin as standard.

Acknowledgement - The financial support of the A.F.R.C. is gratefully acknowledged.

REFERENCES

1. Harwood, J. L. (1980) in *Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds) Vol. 4, pp. 1–55. Academic Press, New York.
2. Mudd, J. B. (1980) in *Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds) Vol. 4, pp. 250–282. Academic Press, New York.
3. Moore, T. S., Price-Jones, M. J. and Harwood, J. L. (1983) *Phytochemistry* **22**, 2421.
4. Price-Jones, M. J. and Harwood, J. L. (1983) *Biochem. J.* **216**, 627.
5. Price-Jones, M. J. and Harwood, J. L. (1985) *Phytochemistry* **24**, 2523.
6. McLaren, M. L., Lillywhite, J. E. and Au, A. C. S. (1981) *Med. Lab. Sci.* **38**, 245.
7. Vance, D. E. and Pelech, S. L. (1984) *Trends Biochem. Sci.* **9**, 17.
8. Infante, J. P. and Kinsella, J. E. (1978) *Biochim. Biophys. Acta* **526**, 440.
9. Voller, A., Bidwell, D. A. and Bartlett, A. (1976) *Bull. W. H. O.* **53**, 55.
10. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.