# INDUCTION OF PHENYLALANINE AMMONIA LYASE AND PISATIN IN PEA PODS BY POLY-LYSINE, SPERMIDINE OR HISTONE FRACTIONS.*

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## SUMMARY

Poly-L-lysine (1mg/ml) induces up to 10 fold increases in the activity of phenylalanine ammonia lyase (PAL) and over 100 fold increases in pisatin synthesis in excised pea pods. Poly-L-lysine in combination with the known inducers actinomycin D(AD) (10μg/ml) and bovine pancreatic ribonuclease A (RNase) (1mg/ml) complements the induction of PAL. Poly-L-arginine or spermidine when applied in combination with AD or RNase dramatically diminish induction of PAL by the latter compounds. Bovine histones of the types IIA, III and IV also antagonize this induction. These histones and spermidine, but not poly-L-arginine serve as moderately effective inducers of PAL and pisatin when applied singly. The induction of PAL activity is inhibited in the presence of cycloheximide (10μg/ml). These results suggest a direct role for certain types of histones and other proteins in the regulation of genic activity in higher plants other than as repressors.

Increased levels of PAL, EC 4.3.1.5, activity and pisatin (3-hydroxy-7-methoxy-4,5' methylenedioxy chromanocoumarane) synthesis have been observed in pea tissue when challenged by a broad range of microorganisms (1,2), and by many structurally defined microbial metabolites (3,4). The induced increases in pisatin (3) and PAL (5) (an enzyme in the pisatin pathway) can be inhibited by cycloheximide 300μg/ml or relatively high concentrations of (450μg/ml) AD. Therefore these and further characterizations (4) of this system indicate that certain genes controlling pisatin production are derepressed.

Compounds as diverse as RNase and AD (10μg/ml) have been shown to be inducers of PAL and pisatin synthesis. Potent inducers such as AD, mitomycin

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C, chromomycin A3, are believed to attach specifically to DNA and may derepress genes by changing the conformation of the DNA - thus altering the attachment of repressor components (4). However, the mode by which proteins (or polypeptides) such as RNase, phytoactin B (3) or lysozyme (Hadwiger & Schwochau, unpublished) derepress these genes remains to be elucidated.

The regulative role of certain peptides has received attention. Peptide (or amino acid) moieties have been found tightly bound to DNA isolated from E. coli (6) and animal and human tumors (7). It has been suggested that this binding may play a role in DNA structure or function or both. For instance, there are larger amounts of these moieties bound to DNA from rat liver after heptatectomy. DNA from a number of animal and human tumors also contained more of these moieties than in the respective nonmalignant tissue of reference (7). Recently, Latner and Longstaff (8) observed that crude histone extracts from calf thymus modified the activation of genes controlling synthesis of the M and H polypeptide units of lactate dehydrogenase isozymes extracted from organ cultures of mouse kidney. Furthermore, short basic clusters (5-6 amino acid residues - including lysine) from glycine and arginine rich histone stimulate the DNA-primed in vitro synthesis of RNA in the presence of RNA polymerase from Micrococcus lentus or the aggregate RNA polymerase of the Novikoff hepatoma nucleoli (9).

The present report describes the regulatory effect of polypeptides such as poly-L-lysine and poly-L-arginine as well as lysine and arginine rich histone components on the derepression of genes responsible for the synthesis of PAL and pisatin synthesis in excised pea pod tissue.

## MATERIALS AND METHODS

Materials - AD was a gift from Dr. Walter Gall, of Merck Sharp and Dohme. Poly-L-arginine • HCl (M.W.∿50,000), bovine pancreatic RNase A, poly-L-lysine hydrobromide (M.W.∿2800), poly-L-lysine hydrobromide (M.W.∿175,000) and calf thymus histones (types IIA, III and IV) were obtained from Sigma Chemical

Co. Thymus nucleohistone and spermidine·3HCL were obtained from Calbiochem.

Induction treatments. - Immature Alaska pea (Pisum sativum L.) pods (less than 2 cm long) were harvested while still enclosed in the blossom to minimize contamination. The pods (1 g/treatment) were immediately split and placed in covered sterile petri dishes. Treatments (1.5 ml of inducer compound) were applied to the exposed endocarp (3) and the pods were incubated in the dark and the PAL extracted after 18 hours. L-phenylalanine-U-$^{14}$C ($5.5 \times 10^5$ dpm, Sp. Act. 365 mc/mMole) was added to endocarps in experiments, since Sp. Act. of pisatin was determined to enable quantitation of the pisatin converted to anhydropisatin for detection on thin layer plates (4). When AD was utilized as an inducer, the pods were washed twice with sterile water 30 minutes after the application of AD. With the exception of the AD treatments, when more than one component was included, these were mixed prior to being applied.

Phenylalanine ammonia-lyase (PAL) extraction and assay. - One g tissue was homogenized after the appropriate incubation period in a mortar with 3 ml of 0.05M borate buffer at pH 8.8, 1 g glass beads and 0.1 g Polyclar. This and subsequent extractive operations were carried out at 2°. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 20,000 g for 10 min. The supernatant was assayed immediately according to the procedure of Koukol and Conn (10) revised as follows: The reaction mixture contained 1.5 ml enzyme homogenate, 20 µMoles of L-phenylalanine, $2.8 \times 10^5$ dpm L-phenylalanine-U-$^{14}$C and 200 µMoles of borate buffer pH 8.8 in a final volume of 2.7 ml. The mixture was incubated for 2 hr. at 37°. Enzyme activity was expressed in µMoles cinnamic acid produced per hr per g pod tissue.

Extraction and quantitation of pisatin - Pisatin was isolated as described previously (4). An ethanol extract of pod tissue was taken to dryness and further extracted with hexane or petroleum ether. The residue of the hexane extract is separated on silica gel thin layer plates. Pisatin was detected on a silica gel thin layer plate by converting it to anhydropisatin in HCl fumes. Anhydropisatin floureses under long wave UV light. Pisatin was quan

titated on the basis of its absorbance at 309 nm. Radioactivity of the purified pisatin was measured in a liquid scintillation spectrometer.

Protein synthesis. - The rate of total protein synthesis in poly-L-lysine induced pea pods was determined as described previously (4). Leucine-U-$^{14}C$ was administered to pods in 30 min pulses over a 12 hour period following application of inducer.

## RESULTS AND DISCUSSION

Histones and spermidine, compounds which have been implicated in the regulation of RNA synthesis, are effective inducers of PAL (table 1). The relative content of lysine and arginine in histones types IIA, III (lysine rich) and IV (arginine rich) may correlate with the ability of these macromolecules to induce PAL. The peptide poly-L-lysine is effective in increasing PAL activity more than 6-fold within 18 hours. It appears that the induction specificity is not due solely to the basicity of the lysine peptide since poly-L-arginine only slightly increased the activity of PAL. The free amino acids L-lysine and L-arginine (at conc of 0.01-1mg/ml) were ineffective as inducers.

Even though poly-L-lysine (M.W.$\sim$2800) was found to be the best of the polypeptide inducers, induction does not depend on consecutive lysine residues in the inducer molecule. It is now known that there are no regions homopolymeric for lysine in the amino acid sequence of calf thymus histone IV (11). However, it should be pointed out that, all peptides known to induce this system contain substantial amounts of lysine. Lysine residues in lysozyme (12), RNase (13) and calf thymus histone type IV (11) constitute 4.6, 8.1 and 10.0 percent respectively of the total amino acid residues. Also further resolution of commercially prepared bovine histones may glean very lysine rich components such as the 30% lysine fraction of lysine rich histones from rabbit thymus (14).

The induction of PAL (table 1) by poly-L-lysine complements inducers such as AD and RNase while poly-L-arginine and spermidine in combination with these

Table 1. In vivo effect of poly-L-lysine, poly-L-arginine, spermidine and various histone preparations on the level of phenylalanine ammonia lyase in pea pod tissue.

| Treatment[a] | Cinnamic acid μ Moles[b] | % of Control |
|---|---|---|
| Control | .015 | 100 |
| Poly-L-lysine | .101 | 655 |
| Poly-L-arginine | .020 | 127 |
| Histone Type III (lys rich) | .061 | 395 |
| Histone Type IV (arg rich) | .037 | 235 |
| Histone Type IIA | .075 | 479 |
| Spermidine | .075 | 481 |
| AD | .216 | 1391 |
| AD + poly-L-lysine | .257 | 1635 |
| AD + poly-L-arginine | .155 | 998 |
| AD + histone type III (lys rich) | .170 | 1095 |
| AD + histone type IV (arg rich) | .132 | 850 |
| AD + histone type IIA | .152 | 974 |
| AD + spermidine | .077 | 496 |
| RNase | .093 | 600 |
| RNase + poly-L-lysine | .224 | 1442 |
| RNase + poly-L-arginine | .076 | 490 |
| RNase + histone type III (lys rich) | .095 | 612 |
| RNase + histone type IV (arg rich) | .084 | 540 |
| RNase + histone type IIA | .084 | 540 |
| RNase + spermidine | .064 | 413 |

[a]The final concentration of each component in the treatments was 1 mg/ml solution, except for AD. When AD was administered it was applied at -30 minutes and was washed from the pods immediately before the standard 1.5 ml treatment was applied as described in methods.

[b]PAL activity is expressed in μMoles cinnamic acid produced/hr/g fresh pod tissue.

inducers is antagonistic. The relative abilities of RNase, AD, poly-L-lysine, poly-L-arginine, spermidine and histones to penetrate the cell membranes of

pea pod endocarp tissue is not known. The high molecular weight poly-L-lysine ((M.W.∿150,000) is less effective as an inducer of PAL (table 2) than is the 2800 M.W. poly-L-lysine. This may reflect the relative abilities of the two compounds, to penetrate the cell membrane. The possibility that the action of these externally applied components is restricted to the cell membrane (15) cannot presently be eliminated.

The induction of PAL has been shown to require newly synthesized RNA and protein (4,5). Cycloheximide (10μg/ml) inhibits the poly-L-lysine induced increase of PAL activity (table 2) (cycloheximide also inhibits the induction of PAL by spermidine and the various histone fractions). Poly-L-lysine also

Table 2. The effect of cycloheximide and molecular weight on poly-L-lysine induced synthesis of PAL in pea pod tissue.

| Treatment[a] | Cinnamic acid μMoles[b] |
|---|---|
| Control | n.d.[c] |
| Poly-L-lysine-M.W.∿2800 | .114 |
| Poly-L-lysine-M.W.∿150,000 | .044 |
| Poly-L-lysine-M.W.∿2800 + cycloheximide | .003 |
| Histone Type III (lysine rich) | .019 |
| Pancreatic ribonuclease A | .064 |

[a]Treatments were mixed in 1.5 ml water and applied to pods. The final concentration of each component was 1 mg/ml with the exception of cycloheximide. Cycloheximide was added 10 minutes subsequent to poly-L-lysine in a concentration equaling 10 μg/ml of the total inducer solution.

[b]Enzyme was extracted and assayed as described in methods after 17 hours incubation in the dark at 22° C. Enzyme activity is expressed in μMoles cinnamic acid/hr/g fresh pod tissue.

[c]n.d. = non-detectable above the value obtained in the reaction vessel with TCA precipitated enzyme.

stimulates the net rate of protein synthesis in pea pods 9 hours after application.

In vitro, poly-L-lysine is known to react with DNA stoichiometrically (one lysine residue per nucleotide and is selective under certain conditions for the A-T rich fractions) possibly causing a perturbation of the DNA secondary structure (16). The basic groups of the poly-L-lysine side chains are thought to neutralize the phosphate groups on the DNA (17). If this in vitro complex between polylysine and DNA exists in intact cells the action of polylysine in activating the PAL gene(s) may be consistent with the hypothesized induction via changes in DNA conformation (4). Conformation changes of DNA hypothetically alter the repressor complexing properties of certain segments of the DNA (4).

The effects of polyamines on RNA synthesis in vitro are well known. Studies with spermidine suggest that it increases RNA synthesis by RNA polymerase (18,19) and also increases amino acid incorporation into protein in in vitro systems (20,21).

Histones have been shown to inhibit DNA-primed RNA synthesis in vitro (22,23). These studies have been interpreted as showing that histones may be involved in the regulation of gene expression. However, it has recently been reported that inhibition of RNA polymerase systems in vitro by glycine-arginine rich histones may involve, not simply the neutralization of negative charges of the DNA by the positive charges of the histone, but the nonspecific precipitation of DNA-histone complexes from solution (9).

PAL catalyzes the conversion of phenylalanine to cinnamic acid a reaction 5 or more metabolic steps prior to the biosynthesis of pisatin (24). It was therefore of interest to determine the effect of these histones and homopolymeric peptides on the net synthesis of pisatin. The data in table 3 indicate that poly-L-lysine is also superior to poly-L-arginine in stimulating the production of pisatin. However, the accumulative effects of these two peptides in combination with other inducers are not consistent with those observed for the induction of PAL activity. Poly-L-arginine, which suppresses the induction of PAL, may

Table 3. In vivo effect of poly-L-lysine, poly-L-arginine spermidine and various histone preparations on the production of pisatin in excised pea pods.

| Treatment[a] | µg Pisatin per g pod |
|---|---|
| Control | n.d.[b] |
| Poly-L-lysine | 130 |
| Poly-L-arginine | 35 |
| Histone type III (lysine rich) | 24 |
| Histone type IV (arginine rich) | 27 |
| Histone type IIA | 52 |
| Nucleohistone | n.d.[b] |
| Spermidine | 40 |
| AD[c] | 166 |
| RNase | 221 |
| AD + poly-L-lysine | 209 |
| AD + poly-L-arginine | 275 |
| AD + histone type III (lysine rich) | 164 |
| AD + histone type IV (arginine rich) | 270 |
| AD + histone type IIA | 257 |
| AD + spermidine | 82 |
| AD + RNase | 193 |
| RNase + poly-L-lysine | 73 |
| RNase + poly-L-arginine | 150 |
| RNase + histone type III (lysine rich) | 148 |
| RNase + histone type IV (arginine rich) | 166 |
| RNase + histone type IIA | 163 |
| RNase + nucleo histone | 139 |
| RNase + spermidine | 46 |

[a]The final concentration of each component in the treatment was 1 mg/ml solution, (except for AD at 0.01mg/ml). When AD was administered it was applied -30 minutes and was washed from the pods before the standard 1.5ml treatment was applied as described in methods. Pisatin production was measured 40 hours after induction.

[b]n.d. = non detectable under U.V. light after conversion to anhydropisatin--see methods.

[c]AD = actinomycin D.

favorably affect other steps in the pisatin pathway. Levels of PAL activity are low, but detectable in "non induced" pea pod tissue, and appear to be sufficient to provide cinnamic acid at levels adequate for pisatin production when the rest of the system is activated.

In pea pod tissue, the in vivo action of histone fractions results in gene activation, analogous to the histone induced synthesis of the lactate dehydrogenase isozymes M or H in mouse tissues (8). No valid explanation of this action is possible at present. Recent studies (25,26,27,28) on the interaction of lysine-rich histones and DNA point out the specificity and importance of these complexes. We are reporting a biological assay which may be useful in selecting histone fractions or other polypeptide fragments which function in gene activation in pea tissue.

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