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THE NATURE OF THE INCREASE IN RIBONUCLEASE ACTIVITY IN GERMINATING SEEDS OF COWPEA TREATED WITH GIBBERELLIC ACID OR CYCLIC-AMP

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Key Word Index—*Vigna sinensis*; Leguminosae; cowpea; RNAase; GA_3; cAMP; protein synthesis.

Abstract—There was a two- to three-fold stimulation of RNAase activity by the application of GA_3 and cAMP to cowpea seedlings. The increase in activity was inhibited by the administration of actinomycin D, cycloheximide, cordycepin, 5-fluorouracil and amino acid analogues. Purification of labelled RNAase revealed that GA_3 and cAMP enhanced the RNAase activity predominantly by its fresh synthesis.

INTRODUCTION

Ribonuclease is widely distributed in plants, micro-organisms and animal cells. GA_3 has been shown to stimulate the RNAase activity in barley aleurone layer [1, 2], barley seedlings [3] and cowpea seedlings [4]. *De novo* synthesis of RNAase has been demonstrated in GA_3-treated barley aleurone cells by means of density labelling techniques [2, 5]. Since very little is known about the hormonal regulation of RNAase in higher plants, the present study has been undertaken to study the modulation of RNAase in cowpea seedlings. In this plant both cAMP and GA_3 have been reported to stimulate the incorporation of labelled precursors into RNA and protein fractions. The enhanced enzyme activity was possibly linked with qualitative and quantitative changes in RNAase isoenzymes [4].

In the present study, the possible mode of stimulation of RNAase by GA_3 and cAMP has been investigated (a) through the use of amino acid analogues and (b) by following the uptake of ^{3}H-labelled leucine into RNAase.

RESULTS AND DISCUSSION

Both GA_3 and cAMP caused a 2- to 3-fold stimulation of RNAase activity in 96-hr-old seedlings (Table 2). This stimulation of enzyme activity could be due either to activation of pre-existing enzyme molecules or to *de novo* synthesis of the enzyme. Crude extracts prepared from the control, GA_3- and cAMP-treated seedlings gave identical thermal inactivation curves of RNAase activity at 64°. This indicated that stimulation of RNAase activity by hormone or cyclic nucleotide is not brought about by a conformational change in the already existing enzyme molecules. To elucidate the mode of action of GA_3 and cAMP, the effect of inhibitors of RNA and protein synthesis on RNAase activity was studied. The stimulation of RNAase elicited by GA_3 and cAMP was extremely sensitive to the action of actinomycin D (Table 1). Strong inhibition of RNAase synthesis was also observed in seedlings treated with cordycepin (Table 2). These results suggested that fresh transcription is necessary for the stimulation of RNAase activity, both in the control as well as in the hormone or cyclic nucleotide-treated seedlings. 5-Fluorouracil proved to be an effective inhibitor of RNAase synthesis. Addition of 5-fluorouracil to GA_3- and cAMP-treated seedlings showed an identical pattern (Fig. 1). The inhibition of RNAase by cycloheximide can be taken as an indication for the requirement of protein synthesis as a pre-requisite for enzyme stimulation (Table 1). These observations cannot,

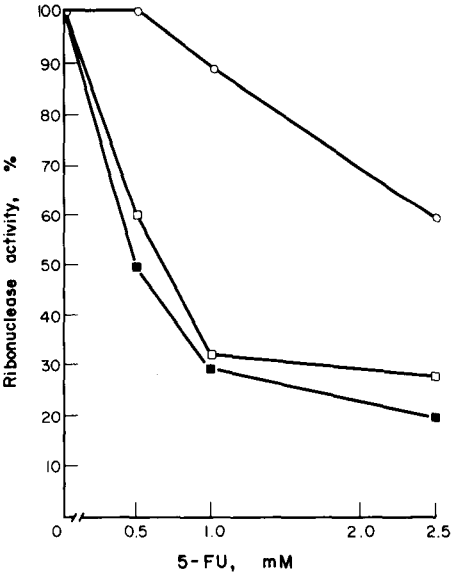

Fig. 1. Effect of 5-fluorouracil (5-FU) on the level of RNAase activity in dark-grown seedlings of cowpea. RNAase activity measured in crude extracts. Values are averages of the values obtained in two experiments each of which was performed in duplicate. ○—○, H_2O control; □—□, cAMP-treated; ■—■, GA_3-treated.

Table 1. Inhibitory effects of actinomycin D (Act D) and cycloheximide (CHI) on GA_3- and cAMP-stimulated production of RNAase activity in dark-grown seedlings of cowpea

Additions	RNAase activity* (unit/mg protein)†	(% control)
None (control)	1.00	100
GA_3 (10^{-5} M)	2.88	288
cAMP (10^{-5} M)	3.05	305
Act D (25 μg/ml)	0.60	60
Act D (50 μg/ml)	0.48	48
GA_3 (10^{-5} M) + Act D (25 μg/ml)	0.66	66
GA_3 (10^{-5} M) + Act D (50 μg/ml)	0.44	44
cAMP (10^{-5} M) + Act D (25 μg/ml)	0.55	55
cAMP (10^{-5} M) + Act D (50 μg/ml)	0.43	43
CHI (5 μg/ml)	0.51	51
GA_3 (10^{-5} M) + CHI (5 μg/ml)	0.41	41
cAMP (10^{-5} M) + CHI (5 μg/ml)	0.58	58

* Measured on crude extract.

† Average of three separate experiments each of which was performed in duplicate.

Table 2. Effect of cordycepin on GA_3- and cAMP-stimulated production of RNAase activity in dark-grown seedlings of cowpea

Additions	RNAase activity* (unit/mg protein)†	(% control)
None (control)	1.00	100
Cordycepin (5×10^{-4} M)	0.54	54
GA_3 (10^{-5} M)	2.15	215
GA_3 (10^{-5} M) + cordycepin (5×10^{-4} M)	0.50	50
cAMP (10^{-5} M)	2.10	210
cAMP (10^{-5} M) + cordycepin (5×10^{-4} M)	0.61	61

* Measured on crude extract.

† Average of three separate experiments each of which was performed in duplicate.

however, be regarded as a conclusive evidence for the *de novo* synthesis of RNAase.

Administration of six amino acid analogues to the seedlings showed a progressive inhibition (30–55%) of RNAase activity with increasing concentration of analogues (0.5–1 mM). However, the GA_3- and cAMP-stimulated enzyme activity was completely inhibited by this treatment (Table 3). The inhibitory effect of these amino acids analogues was completely reversed by the addition of corresponding amino acids (2 mM) (Table 3). This clearly indicated that the stimulation of RNAase by GA_3 and cAMP in germinating cowpea seedlings required fresh protein synthesis.

Purification of labelled RNAase revealed a preferential stimulation of the incorporation of [^{3}H]leucine into RNAase in GA_3- and cAMP-treated seedlings (Table 4). To ascertain whether the [^{3}H]leucine was incorporated directly, the protein hydrolysate was subjected to paper chromatography and the leucine spot was analysed. The results indicated that quite a substantial amount (*ca* 70%) of the incorporation was direct. This further supported the findings that stimulation of RNAase activity by GA_3 and cAMP is attributable to its fresh synthesis rather than its activation. One may conclude that both GA_3 and cAMP stimulate RNAase synthesis by almost a similar mechanism.

EXPERIMENTAL

Seed germination. The seeds were surface-sterilized with 0.1% $HgCl_2$ for 5 min and rinsed thoroughly with sterile H_2O. The seeds were pre-soaked in H_2O for 4 hr and germinated in the dark at 35° ± 2° in Petri dishes containing white sand for 96 hr. Chloramphenicol (20 μg/ml) was added to prevent bacterial contamination. Any compound or mixture of compounds under investigation was present throughout the germination period. According to the literature each of the amino acids tested can be incorporated into proteins [6].

Preparation of enzyme extract. All extraction procedures were carried out at 4°. Seedlings (10 g) were homogenized in 20 ml 0.05 M Pi buffer (pH 6.5) and the homogenate centrifuged at 10000 g for 20 min.

Table 3. Inhibitory effect of amino acid analogues on the GA_3- and cAMP-stimulated production of RNAase activity in dark-grown seedlings of cowpea and its reversal by the corresponding amino acids

Additions	RNAase activity*† (% control)
None (control)	100
Aa‡ analogues (0.5 mM each)	75
Aa analogues (1.0 mM each)	55
Aa (2.0 mM each)	130
Aa analogues (1.0 mM each) + aa (2.0 mM each)	105
GA_3 (10^{-5} M)	270
GA_3 (10^{-5} M) + aa analogues (0.5 mM each)	110
GA_3 (10^{-5} M) + aa analogues (1.0 mM each)	70
GA_3 (10^{-5} M) + aa analogues (1.0 mM each) + aa (2.0 mM each)	295
cAMP (10^{-5} M)	240
cAMP (10^{-5} M) + aa analogues (0.5 mM each)	115
cAMP (10^{-5} M) + aa analogues (1.0 mM each)	65
cAMP (10^{-5} M) + aa analogues (1.00 mM each) + aa (2.0 mM each)	260

* Measured on crude extract.

† Average of three separate experiments each of which was performed in duplicate.

‡ aa, amino acid. The amino acids and analogues used were: tryptophan and 7-azatryptophan, proline and thioproline, phenylalanine and *o*-fluorophenylalanine, tyrosine and 3,5-dinitrotyrosine, methionine and ethionine, arginine and canvanine.

Enzyme assay. The 10000 *g* supernatant (crude extract) (containing 2–4 mg protein/ml) was assayed for RNAase activity by the procedure given in ref. [7]. One enzyme unit is equivalent to $\Delta E_{260nm} \times 30$, where ΔE_{260nm} is the change in E_{260nm} after an interval of 4 min. Protein was estimated by the procedure of ref. [8].

Incorporation of [^{3}H]*leucine.* The effect of GA_3 and cAMP was tested on [^{3}H]leucine incorporation into RNAase. The seedlings were grown in the dark in the presence of GA_3 and cAMP and [^{3}H]leucine (2 μCi/ml, 7600 mCi/mmol) was added after 48 hr germination. The seedlings were harvested after 52 hr. RNAase was extracted and purified as described below.

Table 4. Effect of GA_3 and cAMP on the incorporation of [^{3}H]leucine into RNAase in dark-grown seedlings of cowpea

Additions	Activity* (unit/mg protein)	L-[^{3}H]Leucine incorporation (cpm/mg protein)†
None (control)	140	480
GA_3 (10^{-5} M)	420	1360
cAMP (10^{-5} M)	390	940

* Measured on purified preparation.

† Activity of ^{3}H-labelled leucine from hydrolysed protein.

Purification of RNAase. RNAase was isolated and partially purified by following the procedure of ref. [9]. This partially purified preparation of RNAase was further purified by adsorbing it on alumina C_γ and eluting with 0.01 M β-glycerophosphate. This process was repeated 5× [10]. The purified RNAase was hydrolysed in 6 M HCl in sealed tubes under vacuum. [^{3}H]leucine was recovered from the hydrolysate by 2D-PC (BuOH–HOAc–H_2O, 4:1:5 and PhOH–H_2O, 4:1) and assayed for radioactivity in a liquid scintillation spectrometer.

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