Phytochemistry, 1979, Vol. 18, pp. 565–568. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0401-0565 $02.00/0

MODULATION BY GIBBERELLIC ACID AND ADENOSINE-3′,5′-CYCLIC MONOPHOSPHATE OF STARCH HYDROLYSING ACTIVITY OF COWPEA SEEDLINGS

HARISH C. KAPOOR and ROMESH C. SACHAR*
Division of Biochemistry, Indian Agricultural Research Institute, New Delhi-110012, India

(*Revised received* 6 *September* 1978)

Key Word Index—*Vigna sinesis*; Leguminosae; cowpea; starch hydrolysis; gibberellic acid; adenosine-3′,5′-cyclic monophosphate; glucose inhibition.

Abstract—In cowpea seedlings starch hydrolysing activity increases 35–50 fold on germination for 4 days. This increase in enzyme activity was inhibited by the *in vivo* addition of 1% glucose but this inhibition was completely overcome by the addition of gibberellic acid (GA_3) (10^{-5} M) and adenosine-3′,5′-cyclic monophosphate (cAMP) (10^{-5} M). At 5% glucose, GA_3 and cAMP were only partially effective. Structural analogues of cAMP failed to relieve the inhibitory effect of glucose. The inhibition by glucose is not direct but RNA and protein synthesis may be involved. Glucose appears to reduce the internal pool of cAMP which causes inhibition of RNA synthesis and decrease in starch hydrolysing activity. Exogenous application of cAMP may replenish the endogenous pool of cyclic nucleotide and thus overcome inhibition of RNA synthesis and enzyme activity.

INTRODUCTION

One event occurring shortly after imbibition of seeds is the transport of reserve food material from the storage organ of the seed to the growing embryonic axis and other active regions of the seedlings [1]. Hydrolytic enzymes play a crucial role in the mobilization of stored products. The products of hydrolysis accumulate in the endosperm and may regulate the activity of hydrolysing enzymes through feed-back mechanisms [2]. The regulation of several processes such as rooting [3–8], chlorophyll biosynthesis [9] and enzyme activity [9–13] are affected by the external addition of sugars. The significance of sugar-induced inhibition in a physiological system is, however, not clearly understood. In *Lemma gibba*, the inhibition of flowering by sucrose was overcome by the addition of adenosine-3′,5′-cyclic monophosphate (cAMP) to the nutrient medium [14]. Catabolite sugars probably repress transcription of floral DNA by lowering the levels of cAMP in the apical region [15]. According to Hillman and Posner [16], the inhibition of duckweed flowering by sugars could be due to their injurious effect on the plasma membrane. Oota and Kondo [15] surmised that any factor which affects the integrity of membrane structure will diminish flowering response of *Lemna gibba*, because the levels of cAMP in bud cells may be controlled by the membrane bound adenyl cyclase. If so, the floral inhibition brought about by sucrose and other factors should be overcome by the addition of exogenous cAMP. The present investigation deals with the regulatory role of glucose, gibberellic acid $(GA)_3$ and cAMP on starch hydrolysing activity in germinating seeds of cowpea.

RESULTS AND DISCUSSION

Glucose inhibited the development of the starch hydrolysing activity in germinating seeds of cowpea. Low concentrations of glucose (1%) inhibited the enzyme activity by 48%. This inhibitory effect of glucose was completely overcome by the addition of exogenous GA_3 (10^{-5} M). At high concentration of glucose (5%), the severe inhibition (85%) of enzyme activity was partially counteracted by GA_3 (Table 1). The observations support the view that glucose interferes with GA_3 biosynthesis.

Table 1. Glucose-induced inhibition of starch hydrolysing activity in cowpea and its counteraction by GA_3 and cAMP

Additions	Starch hydrolysing activity (enzyme units/mg protein)	% Control
Control	29	100
GA_3 10^{-5} M	96	331
cAMP 10^{-5} M	85	293
Glucose 1%	15	52
Glucose 1% + GA_3 10^{-7} M	20	69
Glucose 1% + GA_3 10^{-5} M	32	101
Glucose 1% + cAMP 10^{-5} M	28	96
Glucose 1% + cAMP 10^{-5} M	29	100
Glucose 5%	4	14
Glucose 5% + GA_3 10^{-7} M	9	31
Glucose 5% + GA_3 10^{-5} M	13	45
Glucose 5% + cAMP 10^{-5} M	12	38
Glucose 5% + cAMP 10^{-4} M	15	52
Glucose 5% + cAMP 5×10^{-4} M	21	73

The seeds were germinated with the stated treatments in the dark at 35 ± 2° for 96 hr. Enzyme activity was measured in crude extracts. The values represent an average of 3 experiments each in triplicate.

* Present address: Department of Botany, Delhi University, Delhi 110007, India.

Table 2. Effect of cAMP and its structural analogues on glucose-induced inhibition of starch hydrolysing activity in cowpea

Additions	Starch hydrolysing activity (enzyme units/mg protein)	% Control
Control	36	100
Glucose 1%	16	45
Glucose 1% + cAMP 10^{-4} M	43	121
Glucose 1% + Adenine 10^{-4} M	21	59
Glucose 1% + Adenosine 10^{-4} M	20	56
Glucose 1% + AMP 10^{-4} M	21	59
Glucose 1% + ATP 10^{-4} M	17	48
Glucose 5%	2	7
Glucose 5% + cAMP 10^{-4} M	19	52
Glucose 5% + Adenine 10^{-4} M	5	13
Glucose 5% + Adenosine 10^{-4} M	5	13
Glucose 5% + AMP 10^{-4} M	3	9
Glucose 5% + ATP 10^{-4} M	5	15

The seeds were germinated with the stated treatments in the dark at 35 ± 2° for 96 hr. Enzyme activity was assayed in crude extracts. The values represent an average of 3 experiments each in triplicate.

Lack of optimum levels of hormone could lead to repression of enzyme activity. Studies conducted by Radley [17], and Briggs and Clutterbuck [18] also revealed that accumulation of sugars may inhibit the synthesis of GA_3 which in turn prevent the synthesis of α-amylase in barley. Jones [11] gave evidence in support of the osmotic regulatory mechanism as a possible basis for glucose-induced inhibition of α-amylase activity rather than end-product inhibition, which has further been supported by Jones and Armstrong [12]. However, recent findings by Gepstain and Ilan [13] did not support the inhibition of enzyme activity through osmotic effects. Interestingly, cAMP was as effective as GA_3 in overcoming the inhibitory effect of glucose on starch hydrolysing activity in germinating seeds of cowpea (Table 1).

Substitution of cAMP by analogues such as adenine, adenosine, AMP and ATP, showed negligible counteracting effects on glucose-mediated inhibition of enzyme activity (Table 2). This indicates that the counteracting effect of cAMP on glucose-induced inhibition of starch hydrolysing activity is specific.

The effect of glucose on enzyme activity in seeds which were initially imbibed in sterile water for 6 hr was tested at intervals after germination. Transferring seeds to glucose (1%) inhibited enzyme activity by 56% in 96-hour-old seedlings. Simultaneous addition of GA_3 and cAMP with glucose (1%) completely counteracted the glucose-induced inhibition (Fig. 1). Glucose (1%) inhibition of enzyme activity was only partially overcome when cAMP (10^{-4} M) was substituted by theophylline (10^{-4} M) (Fig. 1). Theophylline was used as a phosphodiesterase inhibitor [19–23]. Similar time-course studies performed with 5% glucose in the presence and absence of GA_3, cAMP and theophylline showed partial recovery of enzyme inhibition (Fig. 2). On the basis of above results, at present it is hard to predict the precise relationships between GA_3 and cAMP although it is apparent that both are capable of reversing the inhibitory effect of glucose on enzyme activity.

Preliminary experiments suggest that protein and RNA synthesis are involved in the regulation of starch hydrolysing activity (unpublished results).

The *in vitro* addition of glucose (1%, 5%) to the crude extracts (prepared from control, GA_3 and cAMP treated seedlings) failed to inhibit starch hydrolysing activity (Table 3). This excludes the possibility of simple product inhibition of preformed enzyme molecules. It may therefore be suggested that glucose reduces the level of cAMP, which in turn affects transcription. The low transcriptional and low translational activity consequently results in the repression of starch hydrolysing

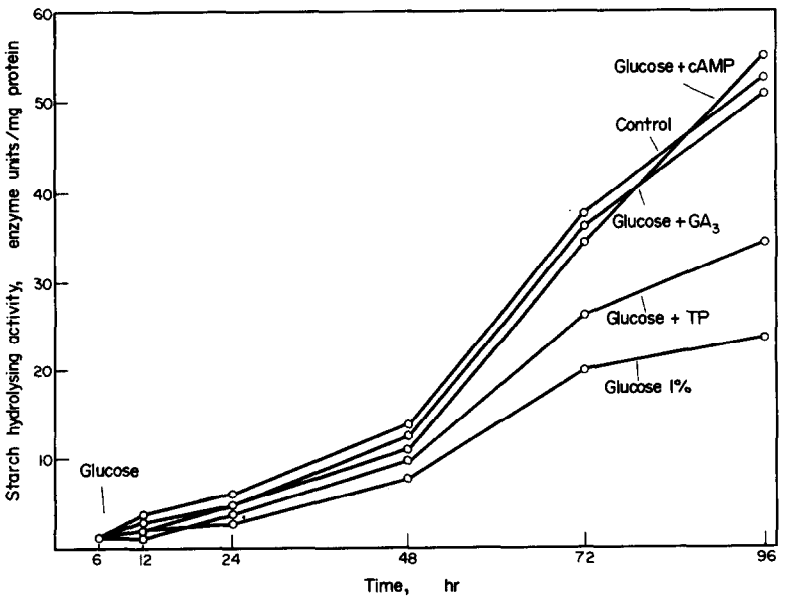

Fig. 1. Time-course studies showing the effect of glucose (1%) in the presence and absence of GA_3 10^{-5} M, cAMP 10^{-5} M or theophylline (TP) 10^{-5} M on starch hydrolysing activity in cowpea.

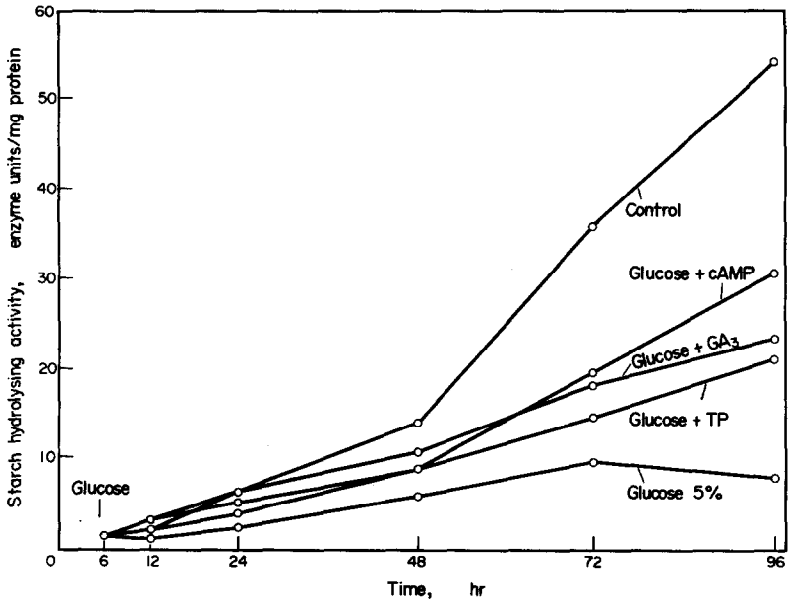

Fig. 2. Time-course studies showing the effect of glucose (5%) in the presence and absence of GA_3 10^{-5} M, cAMP 10^{-5} M or theophylline (TP) 10^{-5} M on starch hydrolysing activity in cowpea.

activity. Exogenous addition of cAMP to glucose-treated seedlings could compensate for the low internal pool of cyclic nucleotide. This may lead to increased transcription which is reflected in the stimulation of enzyme activity. The above suggestion draws much of its support from the bacterial, yeast and fungi systems where glucose and other carbon sources repress several degrading enzymes [24–28]. There the phenomenon has been described as catabolite repression and cAMP has been reported to regulate the enzyme activity by affecting transcription or translation. Although cAMP has not yet been reported to occur in cowpea, its presence has been reported in a number of other plants [29–31].

Table 3. Effect of *in vitro* addition of glucose to crude extracts prepared from GA_3 and cAMP-treated cowpea seedlings

Additions	Starch hydrolysing activity (enzyme units/mg protein)	% Control
Crude extract (H_2O)	21	100
Crude extract (H_2O) + Glucose 1%	21	100
Crude extract (H_2O) + Glucose 5%	22	103
Crude extract (GA_3 10^{-5} M)	56	270
Crude extract (GA_3 10^{-5} M) + Glucose 1%	58	280
Crude extract (cAMP 10^{-5} M)	40	190
Crude extract (cAMP 10^{-5} M) + Glucose 1%	42	200
Crude extract (GA_3 10^{-5} M) + Glucose 5%	58	280
Crude extract (cAMP 10^{-5} M) + Glucose 5%	40	190

The seeds were germinated in dark at 35 ± 2° for a period of 96 hr. GA_3 and cAMP were present throughout the period of germination. Glucose was added to crude extracts and pre-incubated in cold for 48 hr before assaying starch hydrolysing activity.

EXPERIMENTAL

Seed germination. Cowpea seeds (*Vigna sinesis*) were surface-sterilized with a 0.1% soln of $HgCl_2$ for 5 min and rinsed thoroughly with sterile H_2O. The seeds were placed for germination in the dark at 35 ± 2° in Petri dishes containing white sand. Chloramphenicol (20 μg/ml) was added to prevent bacterial contamination.

Preparation of enzyme extract. All extraction procedures were carried out at 4°. Seedlings were normally harvested after a period of 96 hr, unless stated otherwise, and washed with sterile H_2O. A sample (10 g) of seedlings was homogenized in a 15 ml of 50 mM Pi buffer, pH 6.5. The homogenate was centrifuged at 10000 *g* for 20 min. The supernatant, crude extract was collected and used for measuring the starch hydrolysing activity.

Assay of starch hydrolysing activity. The method of ref. [31] was adapted for measuring the activity. The incubation mixture comprised 1 ml of 0.15% hydrolysed starch soln. The reaction was carried out at 25° for 5 min and terminated by adding 1 ml of 50 mM HCl. The final vol. of the incubation mixture was made to 7 ml by adding 4 ml of H_2O. The reaction mixture was centrifuged in the cold at 3000 *g* for 5 min in order to remove acid precipitable material which may interfere with assay system. The blue colour was developed by mixing 1 ml of I_2 reagent. HCl was omitted from the I_2 reagent. The reaction mixture processed as above at zero time served as blank. The change in *A* of 0.01 at 610 nm/mg portein/min represents one enzyme unit.

Protein was estimated by the procedure of ref. [32].

REFERENCES

1. Nakano, M. and Asahi, T. (1972) *Plant Cell Physiol.* **13**, 101.
2. Anderson, J. W. and Fowden, L. (1969) *Plant Physiol.* **44**, 60.
3. Moore, K. G., Cobb, A. and Lovell, P. H. (1972) *J. Exp. Botany* **23**, 65.
4. Lovell, P. H., Illsey, A. and Moore, K. G. (1974) *Physiol. Plant.* **31**, 231.
5. Stenlid, G. (1957) *Physiol. Plant.* **60**, 807.

6. Ferguson, J. D., Street, H. E., and David, S. B. (1958a) *Ann. Botany* **22**, 513.
7. Ferguson, J. D., Street, H. E. and David, S. B. (1958b) *J. Exp. Botany* **22**, 524.
8. Nanda, K. K., Jain, M. K. and Malhotra, S. (1971) *Physiol. Plant.* **24**, 387.
9. Pamplin, E. J. and Chapman, J. M. (1975) *J. Exp. Botany* **26**, 212.
10. Li, S. L. and Redei, G. P. (1969) *Plant Physiol.* **44**, 225.
11. Jones, R. L. (1969) *Plant Physiol.* **44**, 101.
12. Jones, R. L. and Armstrong, J. E. (1971) *Plant Physiol.* **48**, 137.
13. Gepstain, S. and Ilan, I. (1974) *Plant Cell Physiol.* **15**, 399.
14. Oota, Y. (1972) *Plant Cell Physiol.* **13**, 195.
15. Oata, Y. and Kondo, T. (1974) *Plant Cell Physiol.* **15**, 403.
16. Hillman, W. S. and Pasner, H. B. (1971) *Plant Physiol.* **47**, 586.
17. Radley, M. (1967) *Planta* **75**, 164.
18. Briggs, D. E. and Clutterbuck, V. J. (1973) *Phytochemistry* **12**, 1047.
19. Rast, D., Skrivanova, R. and Bachofen, R. (1973) *Phytochemistry* **12**, 2669.
20. Hall, K. A. and Galsky, A. G. (1973) *Plant Cell Physiol.* **14**, 565.
21. Basile, D. V., Wood, H. N. and Braun, A. C. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3055.
22. Gilbert, M. L. and Galsky, A. G. (1972) *Plant Cell Physiol. (Tokyo)* **13**, 867.
23. Galsky, A. G., Gilbert, M. L., Miller, J. and Potempa, L. A. (1972) *Plant Physiol.* **49** (Suppl.), 31.
24. Pastan, I. and Perlman, R. L. (1971) *Nature (London) New Biol.* **229**, 5.
25. Schlandeer, G. and Dellwag, H. (1974) *Eur. J. Biochem.* **49**, 305.
26. Wiseman, A. and Lim, T. A. (1974) *Biochem. Soc. Trans.* **2**, 932.
27. Kalyanaraman, V. S. (1971) *Biochem. Rev. (India)* **42**, 48.
28. Brown, E. G. and Newton, R. P. (1973) *Phytochemistry* **12**, 2683.
29. Raymond, P., Narayanan, A. and Pradet, A. (1973) *Biochem. Biophys. Res. Commun.* **53**, 1115.
30. Kessler, B. and Levinstein, R. (1974) *Biochim. Biophys. Acta* **343**, 156.
31. Filner, P. and Varner, J. E. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1520.
32. Lowry, C. H., Rosebrough, N. J., Färr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.