MECHANISM OF OSMOTIC REGULATION OF HYDROLASE SYNTHESIS IN ALEURONE CELLS OF BARLEY: INHIBITION OF PROTEIN SYNTHESIS

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Summary

The osmotic regulation of gibberellic acid-enhanced hydrolase synthesis in aleurone cells of barley is mediated via a general inhibition of protein synthesis. This inhibition of protein synthesis occurs both in the absence and in the presence of gibberellic acid. Osmotica do not specifically inhibit gibberellic acid elicited responses in aleurone cells as was thought in the past.

Introduction

When aleurone cells of barley and other cereal grains are challenged with the hormone gibberellic acid (GA_3) they synthesize and secrete several hydrolytic enzymes: α-amylase (5), protease (7) ribonuclease and β-glucanase (2). This system has been used to study the mechanism by which GA_3 enhances the synthesis of specific enzymes and facilitates their secretion into the endosperm cells. The response of the tissue to GA_3 is inhibited when the cells are placed in an osmoticum such as polyethylene glycol or mannitol (8). It has been suggested (8) that the osmotica do not affect the general metabolism of the cells (such as respiration or protein synthesis) but specifically inhibit GA_3-elicited responses. Jones and Armstrong(9) have presented evidence that such an osmotic regulation may normally occur in germinating barley seeds. The starch-containing tissue (starchy endosperm) which lies adjacent to the aleurone cells completely desintegrates during germination as a result of the hydrolytic activity of these secreted enzymes. Large amounts of glucose and maltose are produced when the starch is hydrolyzed and the resulting osmotic stress, equivalent to 500 milliosmoles (9), on the aleurone cells may inhibit the further production of these enzymes. It has recently been shown by Hsiao (6) that water stress causes a disaggreg-

ation of polysomes in corn leaves. This led us to examine the possibility that the osmotic regulation of enzyme synthesis in aleurone tissue is mediated via a general inhibition of protein synthesis.

Materials and Methods.

Preparation of aleurone layers. Aleurone layers from seeds of *Hordeum vulgare* L. cv. Himalaya, were prepared and incubated as described by Chrispeels and Varner (4).

Enzyme Activities. At the end of the 24 hr. incubation, 3.5 ml of 10mM acetate buffer pH 4.8 containing 20 mM $CaCl_2$ was added to the incubation medium and this was decanted; this solution contained the secreted enzymes. The aleurone layers were rinsed once and then homogenized in 5.0 ml of the same buffer. The homogenate was centrifuged at 3000 x g for 5 min. and this extract contained the intracellular enzymes. The activity of α-amylase was measured by the disappearance of the ability of a soluble starch solution to form a blue complex with iodine. This method has been described in detail in an earlier paper (4). One unit of activity represents a change in absorbance at 620 mM of 1.0 O.D unit per min. The activity of β-glucanase was measured by incubating (30 min at 37°) the crude enzyme with a 0.5% solution of laminarin in 10 mM acetate pH 4.8 containing 20 mM calcium chloride. The formation of reducing ends was measured with the Nelson-Somogyi copper reagent as described by Ashwell (1). One unit of activity represents an increase in absorbance at 540 nm of 1.0 OD unit over the 30 min. incubation period.

Incorporation studies. Five aleurone layers per treatment were incubated with 2 μc of ^{14}C-mixed amino acids (New England Nuclear Corp. NEC 445) for 1.5 or 2 hr. The aleurone layers were rinsed 3 times with a cold, dilute (1%) solution of casein hydrolysate and homogenized in 5 ml of acetate buffer (0.01 M pH 4.8 containing 20 mM $CaCl_2$). The homogenate was centrifuged at 2000 x g for 5 min to remove cell walls and cellular debris. Uptake of labeled amino acids into the tissue was determined by mixing 0.2 ml of the cleared homogenate with 10 ml of Aquasol (New England Nuclear Corp.). Incorporation into proteins was determined by mixing a 0.5 ml aliquot of the cleared homogenate with 0.5 ml of a dilute casein hydrolysate solution and 1.0 ml of 15% TCA. The precipitated proteins were collected on a bacterial membrane filter (Type B6 from Schleicher and Schuell) which was washed with 5% TCA and dried. Radioactivity was determined with a Beckman Liquid Scintillation Spectrometer. Ethylene glycol and polyethylene glycol 400 were obtained from J. T. Baker Chemical Co. and laminarin from Pierce Chemical Co.

Results

The effect of different concentrations of polyethylene glycol and ethylene glycol on the GA_3-enhanced synthesis of α-amylase and β-glucanase is shown in Table I. Increasing the concentration of osmoticum results in an increasing inhibition of enzyme synthesis. The inhibition of α-amylase synthesis is more pronounced than the inhibition of β-glucanase synthesis. The effect of the same two osmotica on the uptake and the incorporation of

Table I. Effect of ethylene glycol and polyethylene glycol on the synthesis of α-amylase and β-glucanase by aleurone layers.

Five aleurone layers were used per treatment. The numbers represent the total enzyme activity (intracellular and secreted) in enzyme units as defined in "Materials and Methods".

Molarity of osmoticum	Ethylene Glycol		Polyethylene Glycol	
	α-amylase	β-glucanase	α-amylase	β-glucanase
0.0	23.0	40.0	36.0	69.0
0.2	18.0	38.6	16.0	48.6
0.4	9.5	43.5	4.0	38.5
0.6	4.0	29.6	1.5	12.0
0.8	1.6	16.8	0.5	4.0

labeled amino acids into proteins is shown in Table II. Both osmotica inhibit incorporation much more strongly than uptake, suggesting that the inhibition of incorporation cannot simply be accounted for by an inhibition of uptake. In the experiment with polyethylene glycol we measured the capacity for protein synthesis in the presence of GA_3 by labeling from 3 1/2 to 5 hr after the start of the incubation. This is the time period just prior to the start of α-amylase synthesis. In the experiment with ethylene glycol we measured the capacity for protein synthesis in the absence of GA from 15 1/2 to 17 hr after the start of incubation, the period during which α-amylase synthesis normally occurs most rapidly if GA_3 is present. This experiment was done in the absence of GA_3 because the hormone causes protein hydrolysis with the resulting release of free amino acids starting about 8 hr after its addition. This release of amino acids makes the interpretation of labeling data difficult. The differences in the degree of inhibition obtained with EG and PEG may be due to differences in the osmotica or to the length of the incubation prior to labeling. Subsequent experiments showed that there were differences between osmotica, especially

Table II. Effect of ethylene glycol and polyethylene glycol on the incorporation of ^{14}C-labeled amino acids into cellular proteins.

Five aleurone layers were used per treatment, the tissue was incubated with 2 μc of radioactive amino acids (mixed) for 90 min. Osmotica were added at the start of the incubation. Label was added 3 1/2 hr later (polyethylene glycol) or 15 1/2 hr later (ethylene glycol). Incubation in polyethylene glycol was done in the presence of 1mM GA_3. Numbers represent counts per minute taken up into the tissue or incorporated into protein.

Polyethylene glycol			Ethylene glycol		
Molarity	Uptake	Incorporation	Molarity	Uptake	Incorporation
0.0	202,000	28,000	0.0	690,000	72,500
0.2	203,500	26,200	0.25	407,000	25,000
0.4	165,000	19,500	0.50	340,000	14,250
0.6	115,000	2,500	0.75	262,000	5,750
0.8	117,000	250	1.00	237,000	3,250

Table III. Effect of ethylene glycol on the synthesis of β-glucanase in the absence and presence of GA_3.

Five aleurone layers per treatment were incubated in the absence or presence of 1 μM GA_3. Enzyme activity (secreted and intracellular) was measured after 24 hr. Numbers represent the total amount of enzyme synthesized during the incubation period.

	Enzyme activity	
	$-GA_3$	$+GA_3$
control	38.2	73.5
0.8M ethylene glycol	13.1	19.6

between those which permeate the cells quite readily (such as ethylene glycol and poly ethylene glycol 400) and those which do not (such as sorbitol and mannitol). At the same time we observed a marked progression of inhibition with time of incubation in osmoticum. For example, when the

cells were labeled from 3 1/2 to 5 hr after the start of incubation with 0.5 M ethylene glycol we observed a 24% inhibition of protein synthesis, but when the cells were labeled from 15 1/2 to 17 hr after the start of incubation, inhibition was 60%. After 24 hr in ethylene glycol protein synthesis was inhibited by 76%. This progression could account for the differential sensitivity of α-amylase and β-glucanase synthesis to the osmotica. Indeed, β-glucanase is synthesized continuously throughout the incubation period, but little α-amylase is synthesized during the first 8 hr.

The next question which we sought to answer was whether the effects of these osmotica are specific for GA3-elicited responses. The effect of ethylene glycol on the synthesis of β-glucanase both in the absence and in the presence of GA_3 is shown in Table III. Treatment with osmoticum inhibits the basal level of enzyme synthesis as well as the GA_3-enhanced synthesis. Similar results were obtained with 2 other osmotica, mannitol and sorbitol. They also inhibited the synthesis of these 2 enzymes and the incorporation of amino acids into proteins. The drastic inhibition of amino acid uptake in the presence of these osmotica made it difficult to measure incorporation into protein.

Discussion

Our data on the effects of osmotica on GA_3-enhanced enzyme synthesis are in general agreement with those obtained by Jones (8). In addition we have shown that the osmotica also inhibit GA_3-independent enzyme synthesis. The data furthermore show that the effect of the osmotica is mediated via a general inhibition of protein synthesis. Treatment with osmotica results in water stress and water stress has been shown to decrease the protein synthetic capacity of tobacco leaves (3). More recently Hsiao (6) has shown that water stress causes a disaggregation of polysomes in corn leaves. Treatment with osmotica may well have a similar effect on aleurone cells thereby impairing their capacity for protein synthesis.

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References.

1. Ashwell, G. In: Methods in Enzymology. p. 73-105. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York (1957).
2. Bennett, P. A. and M. J. Chrispeels. Plant Physiol. 49: 445 (1972).
3. Ben-Zioni, A., C. Itai, and Y. Vaadia. Plant Physiol. 42: 361 (1967).
4. Chrispeels, M. J. and J. E. Varner. Plant Physiol. 42: 398-406 (1967).
5. Filner, P. and J. E. Varner. Proc. Natl. Acad. Sci. U. S. A. 58: 1520 (1967).
6. Hsiao, T. C. Plant Physiol. 46: 281 (1970).
7. Jacobsen, J. V. and J. E. Varner. Plant Physiol. 42: 1596 (1967).
8. Jones, R. L. Plant Physiol. 44: 101 (1969).
9. Jones, R. L. and J. E. Armstrong. Plant Physiol. 48: 137 (1971).