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Short Communication

Responses of Enzymically Isolated Aleurone Cells of Oat to Gibberellin A_3[1]

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ABSTRACT

Oat (*Avena sativa* L.) aleurone layer cells (spheroplasts) were isolated by maceration of the aleurone layer with a mixture of commercially available cellulase and pectinase. About 20% of the cells present in intact layers were released as spheroplasts and 79 ± 9% of the spheroplast population was viable as judged by methylene blue staining. The spheroplasts became disorganized in solutions containing less than 0.4 M D-mannitol. When the spheroplasts were incubated for 48 hours, total activities of acid *p*-nitrophenyl phosphatase and acid proteinase increased and protein levels decreased. These changes were not effected by gibberellin A_3. Isolated aleurone layers incubated under the same conditions as the spheroplasts showed reduced responses to gibberellin A_3. It is concluded that the necessary presence of an osmoticum limits the value of spheroplasts as a system for studying the mechanism of action of gibberellin A_3 in the aleurone cell.

Isolated cells are useful experimental systems for studies of higher plant metabolism (6, 11, 19). It was of interest to determine if GA_3 would induce increases in enzyme activities in isolated aleurone cells (spheroplasts [18]) in the manner that it effects increased activities in the aleurone layer of several Gramineae (4, 14, 15). Should the spheroplasts respond to hormonal stimulation then they would offer a means of elucidating some aspects of the mechanism of hormone action (18). The results presented here show that while the activities of NPPase[3] and acid proteinase increase, and protein content decreases, during prolonged incubation of oat aleurone spheroplasts, these changes are not significantly effected by GA_3.

MATERIALS AND METHODS

Preparation of Aleurone Layers. *Avena sativa* L. cv. Clinton grain were soaked in water for 6 hr at 4 C and dehusked by hand. Endosperms (5) were sliced longitudinally into two endosperm pieces. One hundred endosperm pieces were incubated at 30 C and 75 rpm (Metabolyte Waterbath shaker) in 20 ml 2% w/v Meicelase P (Meiji Seika Kaisha Ltd; Tokyo) adjusted to pH 5.2 with 0.05 N HCl. After 2 hr the pieces were washed with water and transferred to 20 ml fresh enzyme solution. A further 2 hr incubation yielded aleurone layers which were washed and blotted dry. On incubation with GA_3 aleurone layers prepared in this way showed increases in NPPase and acid proteinase identical to those shown by aleurone layers prepared by hand dissection (Eastwood, unpublished).

Preparation of Aleurone Spheroplasts. Macerating medium contained 5% w/v Meicelase P, 2% w/v pectinase (Nutritional Biochem. Corp.), 1% w/v PVP 10,000 (Sigma) 0.6 M D-mannitol, 2 mM KCl, 1 mM $CaCl_2$, 20 μg/ml penicillin G and was adjusted to pH 5.6 with 0.05 N HCl. Washing medium was macerating medium minus Meicelase P and pectinase. Suspension medium contained 0.4 M D-mannitol, 10 mM $CaCl_2$, and 2 mM KCl in 5 mM acetate buffer (pH 5.6). All media were sterilized by Millipore filtration. Aleurone layers, 0.25 g fresh weight, were surface-sterilized (5) sliced under aseptic conditions into pieces approximately 1 mm^2, vacuum-infiltrated with 10 ml macerating medium, and incubated for 12 hr at 30 C and 30 rpm. After incubation the spheroplasts were separated from the tissue pieces by pouring the flask contents through nylon mesh. The spheroplast suspension was centrifuged at 60*g* for 5 min at room temperature. The pellet (spheroplasts overlain by starch grains) was dispersed in 10 ml washing medium and allowed to sediment for 10 min at room temperature. The supernatant was discarded. After five sedimentations a population of aleurone spheroplasts containing a few clusters of two to four cells was obtained and dispersed in suspension medium at 5 × 10^5 spheroplasts/ml. Spheroplast yields during maceration and purification were estimated with a hemocytometer. The viability of the spheroplast population was estimated by incubating spheroplasts in suspension medium containing 0.05% w/v methylene blue (1). Spheroplasts staining blue after 3 hr incubation were assumed to be dead.

Response of Spheroplasts and Aleurone Layers to GA_3. About 10^6 spheroplasts were incubated in the dark in 2 ml suspension medium ± 10 μM GA_3 at 25 C and 35 rpm. After incubation spheroplasts were separated from the medium and washed in three 2-ml aliquots of fresh medium by centrifugation at 60*g* for 5 min. The supernatants were combined for analysis and the washed spheroplasts were homogenized at 4 C in 2 ml 5 mM acetate buffer (pH 5.6) containing 10 mM $CaCl_2$, 2 mM KCl, and 0.08% v/v Triton X-100 using a Teflon-glass tissue disintegrator (Fisher Scientific Co.). In experiments with aleurone layers, 25 layers were incubated in the dark in either 10 ml 5 mM acetate buffer (pH 5.6) containing 10 mM $CaCl_2$ and 2 mM KCl ± 10 μM GA_3 or 10 ml suspension medium ± 10 M GA_3 at 25 C and 35 rpm. After incubation the layers were separated from the medium and washed with three 10-ml aliquots of fresh medium.

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[3] Abbreviations: NPPase: *p*-nitrophenyl phosphatase; NaNPP: *p*-nitrophenyl phosphate di-sodium salt; NP: *p*-nitrophenol; DOPA: dihydroxyphenylalanine.

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The washings and incubated medium were combined for analysis and the tissue was homogenized at 4 C in 10 ml spheroplast homogenizing medium using a Polytron homogenizer.

Assay Procedures. NPPase was measured as the F^--sensitive (8) release of NP from NaNPP at 30 C in 50 mM acetate buffer (pH 5). Acid proteinase was assayed by the release of Congo red from Congo-coll (Calbiochem.) (13) at 30 C in 50 mM acetate buffer (pH 5). Peroxidase activities of the macerating enzymes were measured with DOPA (16) at 30 C and pH 5.6 in washing medium. Protein was measured with the Folin phenol reagent (12) using BSA (Sigma, fraction V) as a standard. DNA was extracted and measured by the method of Holdgate and Goodwin (7) using calf thymus DNA (Sigma, fraction V) as a standard.

RESULTS AND DISCUSSION

Maceration of Aleurone Layers. When aleurone layers were incubated in macerating medium, spheroplasts, similar in appearance to those of barley (18) were released from the tissue. After a 5-hr lag period release was linear with time for at least 7 hr. The mean yield of spheroplasts was $4.6 \pm 0.5 \times 10^5$/ 0.25 g fresh weight tissue (about 100 layers). DNA distribution between layers and spheroplasts indicated that 20 ± 2% of the layer cells were released and that each spheroplast contained, on average, 41 ± 13 pg DNA. Roughly 40% of the spheroplast population was lost during purification and methylene blue staining showed that 79 ± 9% of the freshly prepared, purified spheroplasts were viable.

Responses of Spheroplasts to GA_3. When spheroplasts were incubated in suspension medium ± 10 μM GA_3 for 48 hr there were significant increases in the total activities of NPPase and acid proteinase and a significant decrease in protein level in comparison with freshly isolated spheroplasts (Table I). There were no significant differences between spheroplasts incubated plus GA_3 and spheroplasts incubated minus GA_3. Over the incubation period dead spheroplasts increased to 48 ± 7% of the population with no significant differences between survival in medium minus GA_3 and medium plus GA_3. These results agree with those obtained for the production of α-amylase by barley aleurone spheroplasts (10) in which it was noted that the external osmoticum necessary for spheroplast survival was probably inhibiting GA_3-induced protein synthesis. In the case of oat spheroplasts it was similarly impossible to study responses to GA_3 in the absence of the osmoticum as in media containing less than 0.4 M D-mannitol the cell matrices became disorganized and cell death resulted. Cells isolated by enzymic maceration may be damaged by peroxidases contaminating the macerating enzymes (17). In the present case such damage seemed unlikely in view of the low peroxidase activities of Meicelase P and pectinase (0.02 and 0.04 units/g, respectively, at pH 5.6 and 30 C) and the high population viability of freshly prepared spheroplasts. This view was confirmed by the use of peroxidase-free enzymes (17) to isolate spheroplasts which were also found not to respond to GA_3.

Table I. Responses of Spheroplasts to GA_3.

Spheroplasts were isolated as in Materials and Methods and incubated for 48 hr in suspension medium ± GA_3. Data are means of three experiments each with five replications.

	NPP ase[1] units/10^6 Spheroplasts		
	Spheroplasts	Medium	Total
Prior to Incubation :	2.53	...	2.53
Incubation for 48 hr; no GA_3 :	2.21	1.12	3.33
Incubation for 48 hr; 10 um GA_3:	2.29	1.24	3.53
	Acid proteinase[2] units/10^6 spheroplasts		
	Spheroplasts	Medium	Total
Prior to Incubation :	36.7	...	36.7
Incubation for 48 hr; no GA_3 :	67.3	8.7	76.0
Incubation for 48 hr; 10 um GA_3:	71.0	14.0	85.0
	Protein mg/10^6 spheroplasts		
	Spheroplasts	Medium	Total
Prior to Incubation :	1.61	...	1.61
Incubation for 48 hr; no GA_3 :	0.96	0.37	1.33
Incubation for 48 hr; 10 um GA_3:	0.84	0.30	1.14

[1] One unit is the release of 1 umol NP/hr at 30 C and pH 5.

[2] One unit acid proteinase is the release of 1 ug Congo red/hr at 30 C and pH 5.

Table II. Effect of Mannitol on Responses of Oat Aleurone Layers to GA_3.

Aleurone layers prepared as under Materials and Methods were incubated in either 5mm sodium acetate ± GA_3 or in spheroplast suspension medium ± 10 um GA_3.

Units of enzyme activity as per Table I. Data are means of three experiments each with three replications.

	NPP ase units/25 aleurone layers					
	5mm Acetate			0.4m D-mannitol		
	Tissue	Medium	Total	Tissue	Medium	Total
Prior to Incubation :	4.4	...	4.4	4.4	...	4.4
Incubation for 48 hr; no GA_3 :	4.0	1.6	5.6	4.2	0.2	4.4
Incubation for 48 hr; 10 um GA_3:	8.4	3.6	12.0	6.7	1.0	7.7
	Acid proteinase units/25 aleurone layers					
	5mm Acetate			0.4m D-mannitol		
	Tissue	Medium	Total	Tissue	Medium	Total
Prior to Incubation :	164	...	164	164	...	164
Incubation for 48 hr; no GA_3 :	140	100	240	120	60	180
Incubation for 48 hr; 10 um GA_3:	110	350	460	150	140	290

Response of Enzymically Isolated Aleurone Layers to GA_3. Oat aleurone layers incubated for 48 hr in 5 mM acetate buffer minus GA_3 showed some increased NPPase and acid proteinase activities (Table II). In the presence of 10 μM GA_3 the increases were much greater, with much of the GA_3-induced activities appearing in the incubation medium, a situation resembling phosphatase activity in barley aleurone layers (2). When the layers were incubated in spheroplast suspension medium the GA_3-induced increases in enzyme activities were much reduced in comparison with those obtained in layers incubated in acetate buffer. The GA_3-induced increases in enzyme activity in oat aleurone layers display an osmotic sensitivity similar to that noted for barley aleurone layers (3, 9). As isolated cells are intrinsically more susceptible to osmotic effects than the same cells in tissue (19) it is probable that osmotic inhibition accounts, in part at least, for the spheroplasts' lack of response to GA_3. In view of this technical limitation it must be concluded that, as with barley, aleurone spheroplasts do not offer a promising system for investigations of the mechanism of action of GA_3 in the aleurone layer.

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LITERATURE CITED

1. ALPER RE, JL DAINKO, F SCHENK 1967 Properties of yeast cell ghosts obtained by ribonuclease action. J Bacteriol 93: 759-763
2. ASHFORD AE, JV JACOBSEN 1974 Cytochemical localization of phosphatase in barley aleurone cells: the pathway of gibberellic-acid-induced enzyme release. Planta 120: 81-105
3. CHRISPEELS MJ, AJ TENNER, KD JOHNSON 1973 Synthesis and release of sucrose by the aleurone layer of barley: regulation by gibberellic acid. Planta 113: 35-46
4. CHRISPEELS, MJ, JE VARNER 1967 Gibberellic acid-enhanced synthesis and release of α-

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amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol 42: 398–406
5. Doig RL, AJ Colborne, G Morris, DL Laidman 1975 The induction of glyoxysomal enzyme activities in the aleurone cells of germinating wheat. J Exp Bot 26: 387–398
6. Edwards GE, SS Lee, TM Chen, CC Black 1970 Carboxylation reactions and photosynthesis of carbon compounds in isolated mesophyll and bundle sheath cells of *Digitaria sanguinalis* (L.) Scop. Biochem Biophys Res Commun 39: 389–395
7. Holdgate DP, TW Goodwin 1976 Quantitative extraction and estimation of plant nucleic acids. Phytochemistry 4: 831–844
8. Hubscher G, GR West 1965 Specific assays of some phosphatases in sub-cellular fractions of small intestinal mucosa. Nature 205: 799–800
9. Jones RL 1969 Inhibition of gibberellic acid-induced α-amylase formation by polyethylene glycol and mannitol. Plant Physiol 44: 101–104
10. Jones RL JE Armstrong, L Taiz 1973 Barley aleurone protoplasts; some observations on their structure and physiology. *In* I Tempe, ed, Protoplastes et Fusion de Cellules Somatiques Vegetales. Inst Nat Rech Agron, Paris pp 109–117
11. Kannan S 1969 Factors related to iron absorption by enzymically isolated leaf cells. Plant Physiol 44: 1457–1460
12. Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
13. Mount MS, DF Bateman, HG Basham 1970 Induction of electrolyte loss, tissue maceration and cellular death of potato tissue by an endopolygalacturonate *trans*-eliminase. Phytopathology 60: 924–931
14. Naylor JM 1966 Dormancy studies in seed of *Avena fatua*. 5. On the response of aleurone cells to gibberellic acid. Can J Bot 44: 19–32
15. Phillips ML, LG Paleg 1972 The isolated aleurone layer. *In* D Carr, ed, Proceedings of the Seventh International Conference on Plant Growth Substances. Canberra Australia pp 396–406
16. Polis BG, HW Shmukler 1953 Crystalline lactoperoxidase. I. Isolation by displacement chromatography. II. Physicochemical and enzymic properties. J Biol Chem 201: 475–500
17. Schenk RV, AC Hildebrandt 1969 Production of protoplasts from plant cells in liquid culture using purified commercial celluloses. Crop Sci 9: 629–631
18. Taiz L, RL Jones 1971 The isolation of barley-aleurone protoplasts. Planta 101: 95–100
19. Takebe I, Y Otsuki, S Aoki 1968 Isolation of tobacco mesophyll cells in intact and active state. Plant Cell Physiol 9: 115–124

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