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Gibberellic acid stimulates acid invertase secretion in pea ovary protoplasts

Juan José Estruch and José Pío Beltrán

Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Jaime Roig 11, 46010 Valencia, Spain

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Protoplasts purified from mesocarp of nonpollinated pea (*Pisum sativum* L.) ovaries released acid invertase to the incubation medium. The association of the acid invertase with microsomal fractions, and the sensitivity to energy-metabolism inhibitors and to tunicamycin, indicated the secretory nature of the release process. In the presence of GA_3 (10 μM), the protoplasts increased their invertase secretion at about 60 min, this effect being counteracted by tunicamycin but not by cycloheximide. Subcellular fractionation of GA_3-treated protoplasts showed that higher invertase secretion was the result of a promotion of invertase transfer from endoplasmic reticulum (ER) to Golgi apparatus.

Gibberellic acid; Acid invertase; Secretion; Protoplast; *Pisum sativum* L.

1. INTRODUCTION

Plant growth regulators are involved in a variety of growth and developmental processes in all plant organs, tissues, and cells [1]. But the fundamental question that remains is by what mechanism they act. Studies on the response of the cereal aleurone layer system to giberellic and abscisic acids, particularly with reference to α-amylase, have contributed to progress in the elucidation of growth regulator mode of action on protein synthesis and secretion [2].

In intact pea plants, application of GA_3 to nonpollinated ovaries promotes sucrose unloading which modifies the overall pattern of carbon allocation [3,4]. Although such effects occur concurrently with the resumption of the ovary growth, many processes have to be initiated before growth starts. Pea ovaries contain apoplastic and cell wall-bound acid invertases which are implicated in the pathway of sucrose unloading [5]. Following GA_3 application to pea ovaries and before new outgrowth is produced, the activity of such invertases is increased coinciding in time with a diminution of the activity of the intracellular acid invertase [5]. By using a secretory protoplast system and subcellular fractionation, the present work reports on the enhancement of invertase secretion underlying the GA_3 response and the involvement of the microsomal membranes in such processes.

Correspondence address: J.P. Beltrán, Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Jaime Roig 11, 46010 Valencia, Spain

Abbreviations: BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CCR, cyanide-insensitive NADH-cytochrome *c* reductase; DCCD, *N,N'*-dicyclohexyl carbodiimine; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; GA_3, gibberellic acid; GAPDh glyceraldehyde-3-phosphate dehydrogenase; IDP-ase, inosine diphosphatase; TCA, trichloroacetic acid

2. MATERIALS AND METHODS

2.1. *Plant material*

Plants of *Pisum sativum* L. cv. Alaska no. 7 were grown as described previously [4]. Flowers were emasculated two days before anthesis (day −2) and used in experiments two days after anthesis (day +2). Only first flowers were used for experimental purposes.

2.2. *Protoplast isolation from pea ovaries*

Protoplasts were obtained from nonpollinated ovaries freshly collected at different stages of development. After excising them in thin slices (about 0.1 mm), they were preincubated for 30 min in a sterilized buffer solution (BS) consisting of 0.5 M sorbitol, 0.05 mM $MgCl_2$, 0.05 mM $CaCl_2$, and 5 mM Mes-OH (pH 6.0). An enzymatic lysis of the cell walls was then performed with BS supplemented with 0.05% pectolyase and 1.5% cellulase (both previously passed through a Pharmacia Sephadex G-25 column) at 28°C for 150 min. The contents of the Petri dishes were filtered through a double layer of a 200 μm nylon net and the resulting filtrate centrifuged at 80 × *g* for 5 min. The pelleted protoplasts were washed twice with BS and finally resuspended in 1 ml of BS. The protoplasts were further purified to avoid possible contamination by the enzymes of the digestion media. One ml of protoplast suspension was layered on top of a Ficoll gradient and centrifuged (40 min, 1000 × *g*, 4°C). The gradient consisted of 2 ml of 20% (w/v) Ficoll in BS and 10% (w/v) Ficoll in BS from bottom to top. Purified protoplasts banding at the 10%/20% Ficoll interface were harvested with a Pasteur pipette. The size of these protoplasts (>36 μm), as well as as their high content in amyloplasts and chloroplasts, indicated their mesocarpic origin [6].

2.3. *Secretory pea ovary protoplast system*

Purified mesocarpic protoplasts were diluted in BS supplemented with 10 mM $MgCl_2$ and 0.1% BSA up to 10^6 protoplasts/ml. From such an incubation medium, two aliquots of 100 μl were withdrawn at appropriate times and layered on top of a gradient composed of

50 μl of 1.5 M sorbitol (bottom) and of 75 μl phenyl methyl silicone oil AP 100. The protoplasts were separated from the incubation medium by centrifugation at 12000 × g for 30 s. The upper phases were collected, dialyzed overnight against 5 mM Hepes-KOH (pH 7.0) and their acid invertase activity determined.

When used, cycloheximide (10 μM) or tunicamycin (6 μg/ml) were present in the incubation medium containing the protoplasts for 40 and 90 min, respectively. Both compounds were removed from the protoplasts by centrifugation (5 min, 80 × g) and washing with 10 vols of incubation medium. The effect of the cycloheximide treatment was tested by following the incorporation of L-[^{35}S]methionine into 10% TCA-precipitable fractions. The other compounds, FCCP (100 μM), CCCP (50 μM), NaN_3 (1 mM) and GA_3 (10 μM), were present trroughout the experiment.

2.4. *Microsomal fraction*

Purified mesocarpic protoplasts (10^6 protoplasts/ml) were allowed to lyse in extraction buffer (EB) consisting of 100 mM Tris-HCl (pH 7.8), 0.4 M sorbitol, 0.5% glycerol, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM KCl and 0.1 mM $MgCl_2$. The resulting homogenate was sonicated at 0°C with three 5 s ultrasound pulses. Then the protoplast lysate was loaded directly onto a step gradient of 27% (w/w) sucrose over 35% (w/w) sucrose in EB and centrifuged at 80000 × g for 4 h at 4°C. Fractions of 2 ml were collected using a Microcol TDC 80 fraction collector, and after dialysis overnight against 5 mM Hepes-KOH (pH 7.0), they were assayed for enzymatic activities.

2.5. *Enzyme assays*

Invertase activity was assayed as reported [5]. In microsomal fraction, CCR (ER marker) assay was performed at 25°C by measuring spectrophotometrically the reduction of cytochrome *c* at 550 nm [7]. IDP-ase (Golgi marker) was determined as described [8] and also G6PDh (cytoplasmic marker) was measured as described [9] in the same fractions.

2.6. *Protein determinations*

Protein was determined according to [10] using the stain Amidoblack 10 B and BSA as standard.

3. RESULTS AND DISCUSSION

3.1. *Establishing a secretory system with pea ovary protoplasts*

The increase of the cell wall-bound invertase activity observed after GA_3 application to nonpollinated ovaries in intact pea plants suggested that a promotion of protein secretion takes place as an early response to GA_3 [5]. A secretory protoplast system has been developed in order to characterize such a process. As can be seen in Fig. 1, protoplasts obtained from nonpollinated pea ovaries were able to release acid invertase to the medium at different levels depending on the stage of ovary development. Failure to detect G6PDh and neutral invertase activities in the medium indicated that there was very little or no breakage of protoplasts during the incubation period. The possibility of removing the activity from the incubation buffer with concanavalin A indicated the glycosylated nature of such a protein (data not shown). The treatment of the protoplasts with tunicamycin, a drug that inhibits the biosynthesis of *N*-linked glycans, slowed down their invertase export capacity (Fig. 2) suggesting that glycans may play an important role on invertase secretion (see also [11]). Phosphorylation energy has been described as a requirement for protein secretion [12,13]. When energy-metabolism inhibitors were added to the incubation medium, the outward movement of invertase drastically declined as well (Fig. 3). Subcellular localization of invertases was investigated by subjecting pea ovary protoplast homogenates to isopycnic sucrose gradients (Fig. 4). While the neutral activity remained in the non-particulate fraction (associated with a cytoplasmic marker), the distribution profile of the acid invertase coincided with the marker enzyme activities for the Golgi apparatus (IDP-ase) and ER (CCR). Indeed, one criterion to be fulfilled by secreted proteins is that they must be associated with the endomembrane system [13].

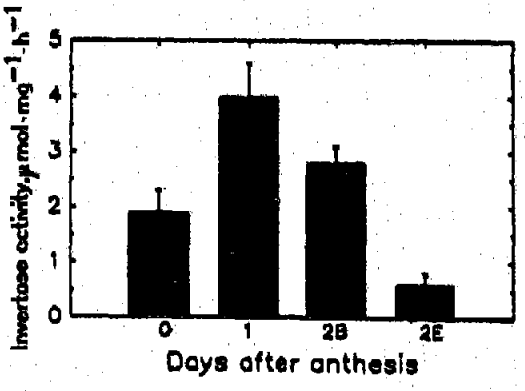

Fig. 1. Developmental stage-dependent capacity of pea ovary protoplasts to release acid invertase activity to the incubation medium. Two samples were considered for day 2 after anthesis, one taken at the beginning (2B, from 11 to 13 h) and the other at the end (2E, from 18 to 20 h). Results are the means of 5 experiments ±SE, three enzymatic assays per experiment.

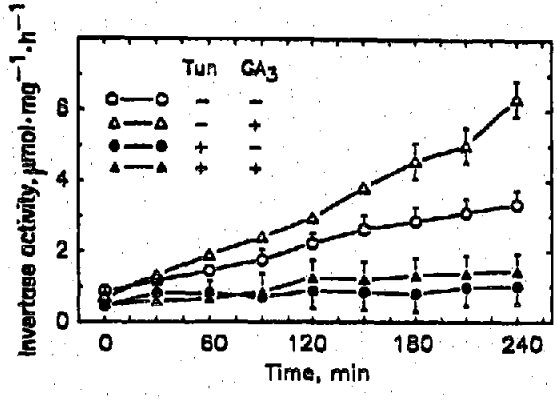

Fig. 2. Effect of GA_3 (10 μM) and/or tunicamycin on protoplasts invertase secretion to the incubation medium. Tunicamycin, when added, was present during a preincubation period of 90 min and eliminated afterwards. GA_3 was added at the time indicated as 0. Results are the means of 5 experiments ± SE, three enzymatic assays per experiment.

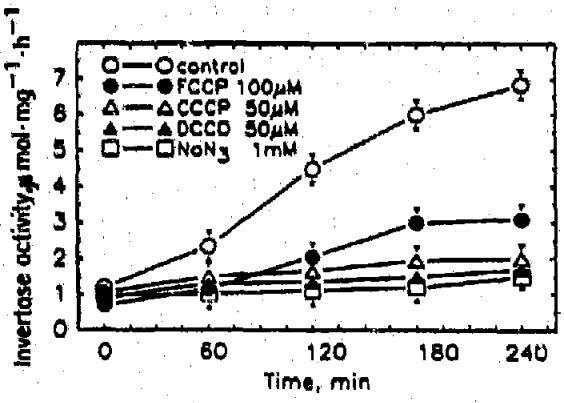

Fig. 3. Effect of energy-metabolism inhibitors on the release of acid invertase activity from mesocarpic protoplasts. The inhibitors were added to the incubation medium at the time indicated as 0. Results are the means of 5 experiments ± SE, three enzymatic assays per experiment.

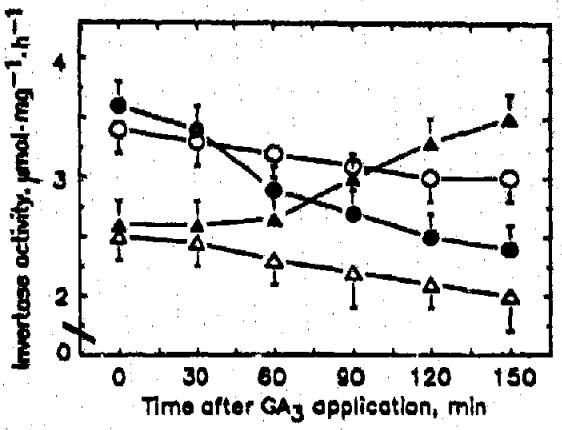

Fig. 5. Invertase specific activity associated with ER (circles) and Golgi apparatus (triangles) obtained from protoplasts treated with 10 μM GA_3 (closed symbols) or non-treated (open symbols). The microsomal fractionation was performed by discontinuous sucrose gradient centrifugation. Results are the means of 5 experiments ± SE, three enzymatic assays per experiment.

3.2. *Effect of GA_3 on invertase secretion*

Addition of GA_3 (10 μM) to purified protoplasts, obtained from the mesocarp of pea ovaries at the beginning of day +2, enhanced their invertase secretion capacity in about 60 min (Fig. 2). This enhancement could be eliminated by a pretreatment of the protoplasts with tunicamycin. Moreover, the promotion of the invertase secretion was not due to new protein synthesis because it was cycloheximide-insensitive (data not shown). Control experiments run in parallel demonstrated that preincubation periods by themselves did not affect GA_3 responses. Cereal aleurone protoplasts also have the capacity to respond to GA_3 treatment but the enhancement of protein secretion has a lag-period which varies from 4 to 20 h (see for review [2,13].

Whilst the capacity of invertase secretion by protoplasts prepared from nonpollinated pea ovaries declined throughout day +2 (see Fig. 1), this tendency could be reversed by incubating the protoplasts with GA_3. Microsomal membranes obtained from pea ovary protoplasts with discountinuous sucrose gradients enabled us to study the re-establishement of the GA_3-associated invertase release at a subcellular level (Fig. 5). Treatment with GA_3 provoked an increment of invertase activity in the region corresponding to the position of the Golgi apparatus and a decrease of the same activity where the ER-marker enzyme banded as well (Fig. 5). These changes were independent of the presence of tunicamycin suggesting that they do not reflect a different degree on invertase glycosylation induced by GA_3

At the same time, higher invertase activity was detected in the protoplast incubation medium (Fig. 2). The Golgi apparatus and ER-marker enzyme levels remained unchanged during the period of activation of invertase secretion, in agreement with electron microscopic evidence [6] which indicates that the ER of pea ovaries did not undergo important changes at least up to 6 h after GA_3 treatment. Hence, we conclude that GA_3 stimulates the invertase traffic from the ER to the Golgi apparatus on its way to the cell exterior.

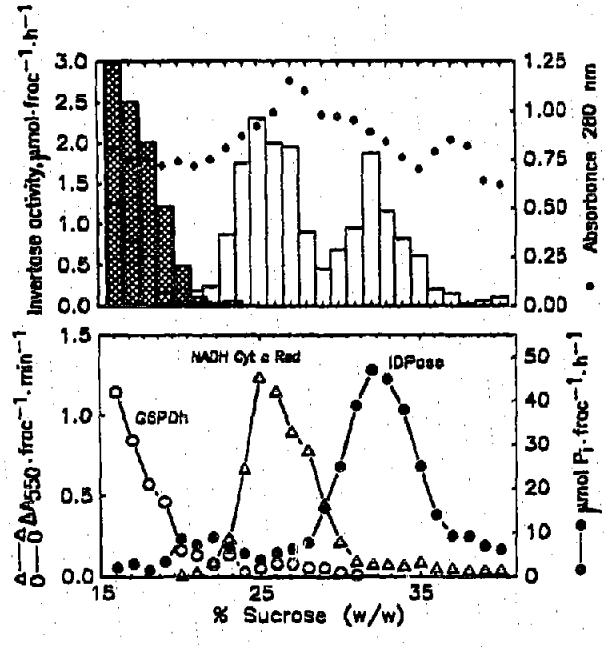

Fig. 4. Localization of ER, Golgi apparatus and cytoplasmic marker enzymes, and neutral (▩) as well as acid invertase (□) activity in subcellular fractions from pea mesocarpic protoplasts separated by isopycnic density gradient centrifugation.

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REFERENCES

[1] Davies, P.J. (1988) in: Plant Hormones and their Role in Plant Growth and Development (Davies, P.J. ed.) pp. 1-11, Kluwer Academic Publishers, Dordrecht.
[2] Fincher, G.B. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 305-346.
[3] Pereté, J.G. and Beltrán, J.P. (1987) Physiol. Plant 69, 355-360.
[4] Jahnke, S., Bier, D., Estruch, J.J. and Beltrán, J.P. (1989) Planta 180, 53-60.
[5] Estruch, J.J. and Beltrán, J.P. (1991) Physiol. Plant. (in press).
[6] Vercher, Y., Molowny, A., Lopez, C., García-Martínez, J.L. and Carbonell, J. (1984) Plant Sci. Lett. 36, 87-91.
[7] Rasi-Caldogno, F., De Michaelis, M.I. and Pugliarello, M.C. (1982) Biochem. Biophys. Acta 693, 287-295.
[8] Ray, P.M., Shininger, T.L. and Ray, M.M. (1969) Proc. Natl. Acad. Sci. USA 64, 605-612.
[9] Lehr, G.W. and Waller, H.D. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H.V. ed.) vol. 2, pp. 636-643, Verlag Chemie International, FL.
[10] Neuhoff, V., Philipp, K., Zimmer, H.-G. and Mesecke, S. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1657-1670.
[11] Olden, K., Parent, J.B. and White, S.L. (1982) Biochem. Biophys. Acta 650, 209-232.
[12] Baleh, W.E. (1989) J. Biol. Chem. 264, 16965-16968.
[13] Jones, R.L. and Robinson, D.G. (1989) New Phytol. 111, 567-597.