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PHYTOALEXIN PRODUCTION IN CULTURED CARROT CELLS TREATED WITH PECTINOLYTIC ENZYMES

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Key Word Index—*Daucus carota*; Umbelliferae; suspension culture; phytoalexin; 6-methoxymellein; elicitor; endo-polygalacturonase; endo-pectin lyase; pectin esterase.

Abstract—6-Methoxymellein, a phytoalexin of carrot, was produced in cultured cells upon addition of partial hydrolysates of carrot cells obtained by treatment with purified endo-polygalacturonase or endo-pectin lyase. Direct addition of these enzymes to the cell culture also stimulated the accumulation of this 6-methoxymellein. When the hydrolysates obtained by these enzymes were subsequently treated with pectin esterase, the activity for the elicitation of 6-methoxymellein production decreased appreciably. These results suggest that pectinolytic enzymes release elicitor-active cell wall fragments from carrot cells and that a certain degree of esterification of the galacturonosyl moiety in these pectic polysaccharides is required for elicitor activity.

INTRODUCTION

Phytoalexins are antimicrobial compounds produced by higher plants in response to microbial invasion. The substances which trigger phytoalexin production are termed elicitors [1].

The phytoalexin of carrot, 6-methoxymellein, is accumulated in cultured cells upon addition of partial hydrolysates of the cells obtained by treatment with a commercial pectinase or trypsin [2]. Direct addition of these enzymes to growing cell cultures also stimulates phytoalexin production since the added enzymes function to release cellular fragments which serve as endogenous elicitors. In this paper, we report that phytoalexin production in cultured carrot cells is induced by highly purified pectinolytic enzymes, endo-polygalacturonase (PG), endo-pectin lyase (PL) and pectin esterase (PE), obtained from *Aspergillus japonicus* [3–5].

RESULTS AND DISCUSSION

Partial hydrolysates of cultured carrot cells obtained by treatment with PG or PL show potent elicitor activity (Fig. 1). The activity is negligible immediately after the addition of enzymes (time 0), but increases with time suggesting that soluble fractions released from the cell homogenate by enzyme action can serve as elicitors for the production of 6-methoxymellein. Direct addition of PG or PL to carrot cell culture is also effective in phytoalexin production when applied at the correct concentration (Fig. 2). However, PE neither releases elicitor-active components from carrot cells nor shows any effect on 6-methoxymellein accumulation after addition to the cell culture (Figs 1 and 2).

These observations suggest that highly purified pectinolytic enzymes stimulate phytoalexin accumulation by releasing elicitor-active oligosaccharides from pectic polymers of carrot cell walls. The situation is analogous to that obtained by Bruce and West [6] who demonstrated that a purified galacturonase from *Rhizopus stolonifer* elicited

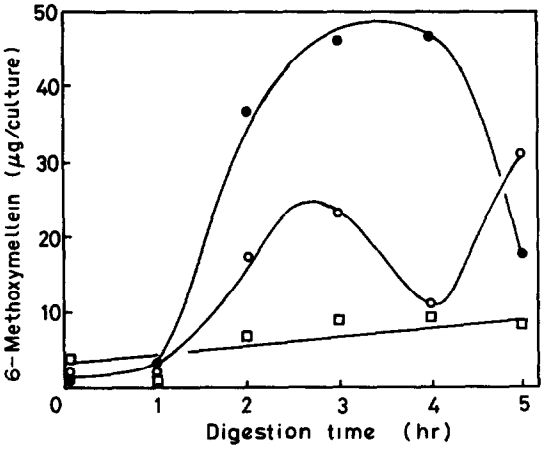

Fig. 1. Release of elicitor activity on treatment of a homogenate of cultured carrot cells with pectinolytic enzymes (12 units/ml) at 37°. The elicitor activities of digestion products were determined by measuring the production of 6-methoxymellein in carrot suspension culture after 24 hr incubation. (○) PG; (●), PL; (□), PE.

casbene synthetase in castor bean seedlings. Davis *et al.* [7] also reported that endo-polygalacturonic acid lyase from *Erwinia carotovora* induces phytoalexin production in soybean by releasing cell wall fragments.

When phytoalexin production is induced by the addition of partial hydrolysates of carrot cells, the age of the cultured cells used as elicitor source is an important factor, the hydrolysate prepared from 7-day-old cells showing the most potent elicitor activity [8]. In this growth stage, polyuronides in the cell wall of cultured carrot cells are highly esterified whereas those from the aged cultures, which are poor elicitors, consist mostly of non-esterified uronic acids [9]. These findings suggest that the elicitor activity of carrot pectic fragments is largely dependent on

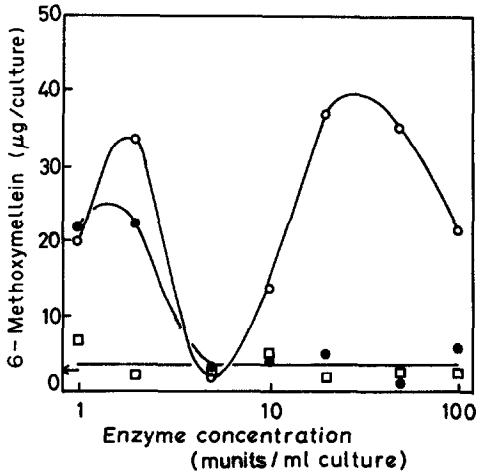

Fig. 2. Accumulation of 6-methoxymellein in cultured carrot cells on addition of pectinolytic enzymes. The arrow indicates the 6-methoxymellein content of a control culture which received only the sodium acetate buffer. (○) PG; (●), PL; (□) PE.

the degree of esterification. To confirm this, partial hydrolysates prepared by PG or PL were hydrolysed by PE and tested for elicitor activity. The results (Table 1) indicate that the elicitor activity of the hydrolysates is reduced markedly by treatment with PE and suggest that a certain degree of esterification of the galacturonosyl moiety in the elicitor molecule is required for the induction of 6-methoxymellein production in cultured carrot cells. Contrary to our observation, however, Nothnagel *et al.* [10] isolated non-esterified dodeca-α-1,4-D-galacturonide from the cell wall of soybean as the active elicitor, and Jin and West [11] reported that trideca-α-1,4-D-galacturonide is the most active elicitor of casbene synthesis in castor bean seedlings. Jin and West also reported that elicitor activity is decreased by methyl esterification of carboxylate groups. The effect of chemical modification of polyuronide fragments may vary according to the plant species. Characterization of the elicitor for 6-methoxymellein production in cultured carrot cells is now in progress.

Table 1. Effect of PE treatment of elicitor on phytoalexin production in cultured carrot cells

	6-Methoxymellein (μg/culture)	
Treatment	PG hydrolysate	PL hydrolysate
−PE	22.1	42 1
+PE	3.8	12.4

Crude elicitor fractions obtained by partial hydrolysis of carrot cell homogenate with PG or PL (12 units/ml) for 3 hr were further incubated with 10 units/ml PE for 1 hr. Control experiments (−PE) were carried out with heat denatured PE.

EXPERIMENTAL

Enzymes. Purified PG, PL and PE were generous gifts from Dr. S. Ishii, Central Research Laboratories, Kikkoman Corporation, Noda, Chiba, Japan.

Preparation of elicitor-active fraction. Carrot cells were cultured in Murashige and Skoog's synthetic medium [12] with agitation on a reciprocal shaker at 27° [13]. Partial hydrolysis of the cells by PG or PL was carried out using the method of ref. [2] with some modifications. Cultured carrot cells (7-day-old, 4 g fr. wt) were harvested by filtration and suspended in 10 ml NaOAc buffer (0.1 M, pH 5.2). The cells were killed by autoclaving and homogenized by a sonicator. The homogenate was incubated at 37° with 120 units of the enzymes and 1.5 ml aliquots were removed at various intervals to test the elicitor activity. The reaction was terminated by boiling for 20 min. The hydrolysates were then centrifuged at 10 000 *g* for 20 min and the supernatants sterilized by autoclaving before addition to the cell culture.

When phytoalexin production was triggered by the direct addition of these enzymes to the culture, enzyme solns at various concns were sterilized by filtering through a Millipore filter (0.22 μm).

Production and determination of 6-methoxymellein. The hydrolysate or enzyme soln (1 ml each) was added to a 10-day-old carrot culture and the 6-methoxymellein content determined after 24 hr incubation. The procedures used for the extraction and purification of 6-methoxymellein from cultured carrot cells were as described in ref. [14]. Partially purified materials were subjected to TLC on silica gel developed with C_6H_6–MeOH (50:1). The amount of 6-methoxymellein was determined by a scanning method (λ_S 265 nm and λ_R 400 nm).

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REFERENCES

1. Grisebach, H. and Ebel, J. (1978) *Angew. Chem. Int. Ed.* **17**, 635.
2. Kurosaki, F. and Nishi, A. (1984) *Physiol. Plant Pathol.* **24**, 169.
3. Ishii, S. and Yokotsuka, T. (1972) *Agric. Biol. Chem.* **36**, 1885
4. Ishii, S. and Yokotsuka, T. (1975) *Agric. Biol. Chem.* **39**, 313
5 Ishii, S., Kino, K., Sugiyama, S. and Sugimoto, H. (1979) *J Food Sci.* **44**, 611.
6. Bruce, J. R. and West, C. A. (1984) *Plant Physiol.* **74**, 989.
7. Davis, K. R., Lyon, G. D., Darvill, A. G. and Albersheim, P. (1984) *Plant Physiol.* **74**, 52
8. Kurosaki, F., Futamura, K. and Nishi, A. (1985) *Plant Cell Physiol.* **26** (in press).
9. Asamizu, T., Nakayama, N. and Nishi, A. (1984) *Planta* **160** 469
10. Nothnagel, E. A., McNeil, M., Albersheim, P and Dell, A (1983) *Plant Physiol.* **71**, 916.
11. Jin, D. F. and West, C. A. (1984) *Plant Physiol.* **74**, 989.
12. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.
13. Okamura, S., Sueki, K. and Nishi, A. (1975) *Physiol. Plant.* **33**, 251.
14. Kurosaki, F. and Nishi, A. (1983) *Phytochemistry* **22**, 669.