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CHITINASE ISOENZYMES INDUCED IN CARROT CELL CULTURE BY TREATMENT WITH ETHYLENE

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Key Word Index—*Daucus carota*; Umbelliferae; ethylene; chitinase; fungal walls; isoenzyme.

Abstract—In cultured carrot cells, treatment with exogenously supplied ethylene or 1-aminocyclopropane-1-carboxylic acid induced chitinase activity. Most of the induced enzymes were secreted into the culture medium. The extracellular chitinases induced by ethylene were resolved by gel-filtration chromatography into four isoenzymes with different molecular sizes. Two of them were *exo*-hydrolases which liberated chitobiose as a sole product from insoluble chitin. Another two isomers were thought to be *endo*-chitinase because they yielded chitin oligomers of various sizes. As major molecular species of chitinase found in ethylene-treated carrot cell cultures were different from those induced by fungal walls, it was suggested that ethylene and fungal components act as independent signals in chitinase induction.

INTRODUCTION

It is well known that higher plants produce chitinase in response to infection by pathogenic microorganisms [1–3]. This chitinase has been considered to prevent the growth of microbes because its substrate chitin is an important component of mycelial walls of fungi whereas it is not found in host plant cells [4–8]. In addition, we have recently shown [9, 10] that chitinase induced in cultured carrot cells hydrolysed mycelial walls, and the resultant soluble fragments of chitin stimulated phenylpropanoid metabolism in the host cells which caused the enhancement of lignification of these cells. Chitinase activity can also be induced in a number of plants by exogenously supplied ethylene or 1-aminocyclopropane-1-carboxylic acid (ACC), a direct precursor of ethylene [3, 11, 12]. It was also demonstrated that appreciable amounts of ethylene are generated from plant cells during the infection process [13]. It has been hypothesized, therefore, that infection-induced ethylene plays a key role in chitinase induction in diseased plant cells.

We have previously reported [10] that chitinase induced in cultured carrot cells after treatment with mycelial walls of *Chaetomium globosum* is composed of four isoenzymes of *endo*-hydrolases. The occurrence of several isoforms of chitinase was also reported in melon [14] and potato [15] plants. In the present experiment, we characterized chitinase isoenzymes induced in carrot cells by ethylene, and compared their properties with those induced by fungal cell walls to understand the possible role of ethylene as an inducer during infection.

RESULTS

Chitinase induction by ethylene

Cultured carrot cells produced appreciable amount of chitinase when they were exposed to exogenously supplied ethylene at concentrations more than 1 nl/ml air of the culture (Fig. 1). Most of the induced activity appeared to be secreted from the cells and was found only in the medium. Extracellular chitinase activity increased with increasing concentrations of ethylene. Addition of ACC to carrot cell cultures also induced chitinase activity in a dose-dependent manner. The amount of chitinase seemed to be dependent on the concentration of ethylene generated from ACC. Almost all the enzyme activity induced by ACC was also found in the extracellular fluid as in ethylene-treated carrot culture. Viability of carrot cells after treatment of the cells with ethylene or ACC was determined after 24 hr incubation. No appreciable difference in viable cell number was observed between the treated and non-treated control culture (*ca* 90%, data not shown), suggesting that possible occurrence of extracellular enzymes caused by the destruction of cellular structures can be neglected.

Chitinase induction by fungal components

Intra- and extracellular chitinase activities induced by the addition of mycelial walls of *C. globosum* are shown in Fig. 2. The enzyme induction occurred even with very small amounts of mycelial walls (0.1 mg/culture). When the amount of mycelial walls was relatively small (less than 3 mg/culture), most of the induced chitinase was secreted into the medium, while the activity of intracellular enzyme increased by the addition of larger amounts of the walls. Carrot cells treated with mycelial walls generated small amount of ethylene. From the results shown in Fig. 1, however, the concentration of ethylene after treatment with mycelial walls seemed to be insufficient to induce chitinase in carrot cells. We have done a similar experiment in the presence of 0.5 mM aminoethoxyvinylglycine (AVG), a well known inhibitor of ethylene-biosynthesis, by which ehylene evolution was depressed to less than 10% of the control. However, chitinase induced in the presence of AVG was essentially similar to those without the inhibitor (data not shown). We, therefore, considered that the contribution of ethylene in the induction of chitinase shown in Fig. 2 was, if any, very small.

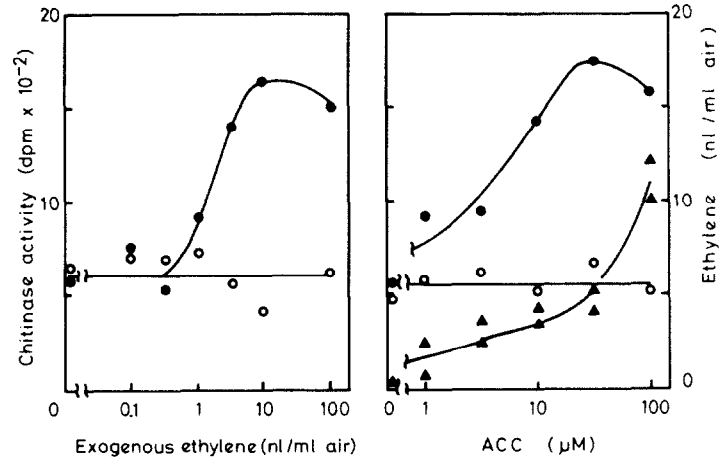

Fig. 1. Induction of chitinase in carrot cell culture by exogenously supplied ethylene or ACC. Ethylene or ACC was added to 10-day-old carrot cell culture (5 ml) in a screw-capped test tubes at various concentrations. After 24 hr incubation, cells and medium were separated by filtration, and intra- (○) and extracellular (●) chitinase activities were determined radiochemically. Ethylene generated from ACC was determined by GC 24 hr after the addition (▲). Three replicates were prepared for each treatment.

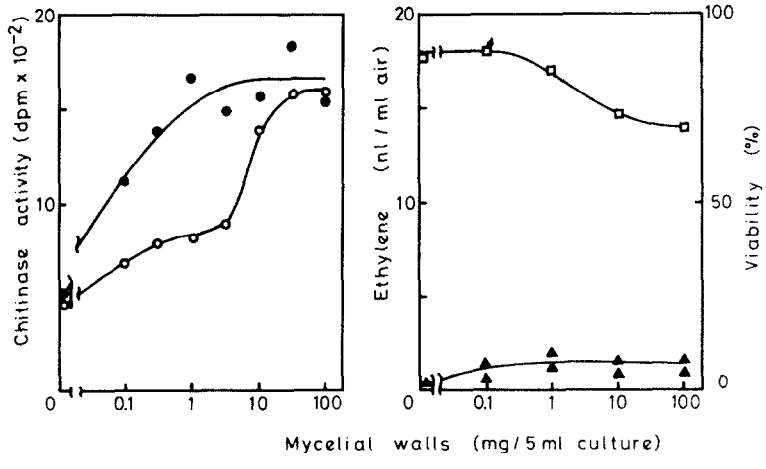

Fig. 2. Induction of chitinase in carrot cell culture by mycelial walls of *C. globosum*. Varied amounts of the mycelial walls were added to 5 ml of carrot cell culture in a closed tube. After 24 hr incubation, intra- (○)and extracellular (●) chitinase activities were determined. Viability of the carrot cells (□) and generated ethylene (▲) was also determined by the dye exclusion test and by GC, respectively.

Characterization of chitinase isoenzymes

Because the majority of induced chitinase activity was secreted to the external medium, our attention was focused on the extracellular enzymes. The extracellular chitinases induced by ethylene were partially purified with ion exchange resins and fractionated on a Sephadex G-75 column (Fig. 3). Extracellular chitinases induced by ethylene were composed of four major isoenzymes of which the M_r were estimated to be *ca* 28 700, 23 000, 17 500 and 15 500 (Fig. 3, I–IV).

The products of these isoenzymes were compared by analysing the radiolabelled GlcNAc oligomers liberated from insoluble [^{3}H]-chitin, and typical results are shown in Fig. 4. In the reaction mixtures of the isoenzymes of II and III in Fig. 3 (23 000 and 17 500), GlcNAc oligomers of various molecular size were observed after 2 hr hydrolysis (Fig. 4a), and amounts of each oligomer increased at about the same rate during further incubation (Fig. 4b). By contrast, in the hydrolysis products of chitinase I and IV (M_r 28 700 and 15 500), only the dimer of GlcNAc was observed (Fig. 4c, d). These results indicate that the former two isoenzymes are *endo*-chitinases, and the latter two are *exo*-chitinases liberating chitobiose as the sole product. In neither case would significant amounts of radioactivity be observed at the postion corresponding to GlcNAc monomer or lower M_r fractions, indicating that chitin deacetylase and *N*-acetylglucosaminidase were absent from these enzyme preparations.

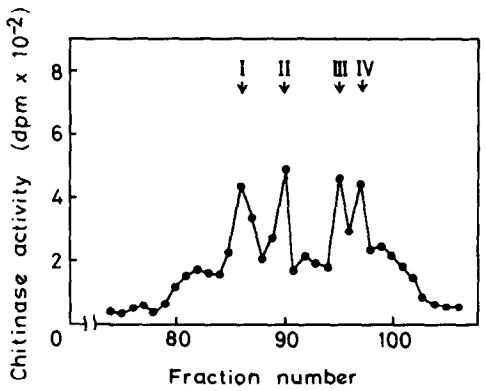

Fig. 3. Fractionation of chitinase isoenzymes on a Sephadex G-75 column. Extracellular chitinases induced by incubating carrot culture for 24 hr with 100 nl/ml air of ethylene were partially purified by batch treatment with ion exchange resins, and applied to a Sephadex G-75 column. Chitinase activities of the collected fractions were determined radiochemically. The column was calibrated before each run, and M_r of the peaks of enzyme activity indicated by arrows were estimated.

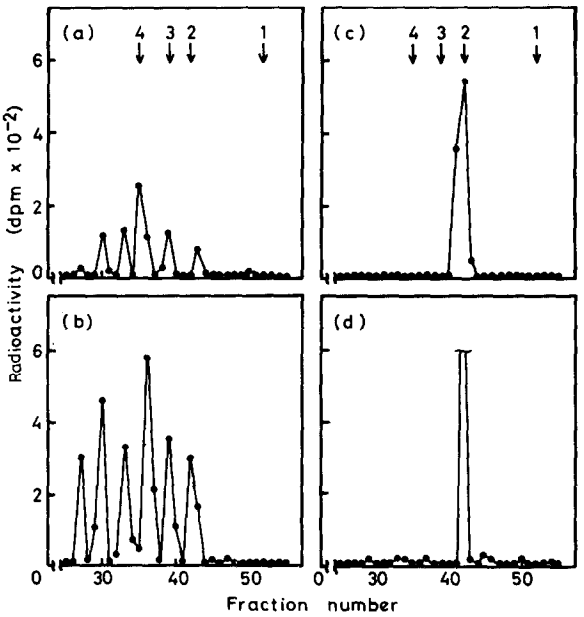

Fig. 4. Analysis of oligosaccharides liberated from insoluble chitin by chitinase isoenzymes. Chitinase isoenzymes were incubated with [^{3}H] chitin at 37°, and the solubilized oligomers were fractionated on a Bio Gel P-2 column. Results obtained with chitinase II in Fig. 3 are shown in (a) and (b), and those with chitinase I in (c) and (d). Reaction products were analyzed after 2 hr (a, c) and 6 hr (b, d) of incubation. Arrows indicate fractions in which authentic GlcNAc oligomers (mono-, di-, tri- and tetramers as indicated by numbers) were eluted from the column.

DISCUSSION

We previously reported that secretion of chitinase by carrot cells treated with fungal walls was strongly inhibited by monensin and colchicine [16] which are known to inhibit the transport process through the Golgi body. We also reported [16] that most of the intracellular chitinase once accumulated in the carrot cells were allowed to secrete into the medium even in the presence of cycloheximide. The present experiments clearly show that carrot cells treated with mycelial walls of less than 0.1 mg/ml secreted appreciable amounts of chitinase activity to the external medium. The viability of these cells incubated with this amount of the walls was comparable to the control culture (Fig. 2) indicating that the extracellular chitinases did not result from the destruction of cellular structures by hypersensitive cell death. Cultured carrot cells treated with ethylene or ACC also secreted most of the induced chitinase into the medium (Fig. 1). These results suggested that the chitinase induced by fungal components and ethylene are primarily secretory enzymes and intracellular occurrence of chitinase may be observed when the enzyme production is very rapid. Broglie *et al.* [17] reported that nucleotide sequence of cDNA clones of chitinase from bean encodes a 27-residue amino-terminal signal peptide which is characteristic of secretory proteins.

We have previously shown [10] that the carrot cells incubated with mycelial walls produce four different chitinases whose M_r were estimated to be *ca* 29 000, 20 500, 17 500 and 12 500. These enzymes were all *endo*-hydrolases and liberated GlcNAc oligomers of various sizes. In ethylene-induced chitinases, however, we found two isoenzymes which exhibit exo-hydrolase activity (Fig. 4). The enzymes release GlcNAc dimer (chitobiose) as sole product from chitin. *exo*-Chitinases are usually found in microorganisms [18] and have been scarcely reported in higher plants. However, any microbial contamination was not observed in our carrot cell culture when inspected under a microscope. Roby and Esquerré-Tugayé reported [14] that melon plant infected by *Colletotrichum lagenarium* produced both *endo*- and *exo*-chitinases, but they did not examine the effect of ethylene on the production of these enzymes. Our results indicate that molecular species of chitinase predominantly produced in ethylene-treated carrot cells were different from those induced by fungal components, and that ethylene and fungal components act as independent signals in chitinase induction during infection.

EXPERIMENTAL

Carrot cell culture. Carrot cells (*Daucus carota* L. cv Kintoki, Y-1 strain) were grown on a reciprocal shaker (120 strokes /min) in the synthetic medium of ref. [19] according to the method of ref. [9].

Induction of chitinase by ethylene. Induction of chitinase by ethylene was carried out in a closed atmosphere, and three replicates were always prepared for each treatment. 5 ml of cultured carrot cells (10-day-old) were transferred into screwcapped test tubes (30 ml). C_2H_4 was directly introduced into the closed test tubes by a gas-tight syringe. ACC was dissolved in 50 μl of Na-citrate buffer (0.1 M, pH 5.0) at various concns, and filter-sterilized through a Millipore filter (0.22 μm) before the addition to the tubes. They were incubated at 26° for 24 hr, and a set of replicates (3 tubes) were combined. Cells and medium were separated by filtration, and the cells were homogenized in the citrate buffer by sonication. The homogenates were then centrifuged at 10 000 *g* for 20 min, and the vol. of the supernatants was adjusted to 15 ml to serve as the preparation of intracellular

chitinase. Medium was dialysed against the citrate buffer overnight, and the vol. was adjusted to 15 ml before the measurement of extracellular chitinase activity.

Induction of chitinase by fungal components. Mycelial walls of *C. globosum* were prepared from 3-day-old shake culture of the fungus grown in an yeast-malt medium according to the method of ref. [20] with a slight modification as described previously [9]. Before the addition to carrot suspension culture, the wall preparations were suspended in 400 μl of Na-citrate buffer (0.1 M, pH 5.0) and sterilized by autoclaving. Cells were cultured at 26° for 24 hr, and intra- and extracellular chitinases were determined as described above.

Chitinase assay. Chitinase activity was measured radiochemically according to ref. [21] as described previously in detail [7]. [^{3}H] Labelled chitin was prepared from chitosan by acetylation with [^{3}H] acetic anhydride, and the sp. act. of synthesized chitin was 57 600 dpm/mg. Radioactivity of the solubilized [^{3}H] GlcNAc oligomers was measured after 2 hr incubation with the enzyme preparations.

Viability of carrot cells. Viability of cultured carrot cells was determined microscopically by the dye exclusion method as described previously using Rhodamine 6G (50 μg/ml) [16]. The results are presented as means from 5 replicate measurements, and 100 cells were assessed in each determination.

Determination of C_2H_4. Gas sample (1 ml) was taken from the incubation tubes, and C_2H_4 was determined by GC (equipped with FID) on a 2 m glass column packed with Al_2O_3 at 60°.

Chromatographic separation of chitinase isoenzymes. Extracellular chitinases were analysed on a Sephadex G-75 column after being partially purified with ion exchange resins. Culture filtrates of carrot cells were transferred to a cellophane tube and dialysed against Na-citrate buffer (0.1 M, pH 5.0). The dialysed soln was mixed with CM-Sephadex (5 ml packed vol.) and stirred for 30 min. Non-adsorbed materials were recovered by filtration and dialysed against Tris–HCl buffer (0.1 M, pH 7.6). After dialysis, the soln was mixed with 5 ml of DEAE-Sephacel, and the non-adsorbed fraction was again recovered by filtration. This fraction was further dialysed against H_2O and lyophilized, redissolved in a small amount of the citrate buffer. It was then applied to a column of Sephadex G-75 (84 × 1.8 cm) which was previously calibrated with M_r markers (blue dextran, 2 000 000; bovine serum albumin, 66 000; carbonic anhydrase, 29 000; cytochrome *c*, 12 400; aprotinin, 6 500; vitamin B_{12}, 1 355). The column was eluted with the citrate buffer (1.3 ml fractions, flow rate 17 ml/hr), and the enzyme activity in these fractions was determined radiochemically.

Analysis of hydrolysed products of chitin. To determine the mode of hydrolysis of chitinase isoenzymes, GlcNAc oligomers liberated from [^{3}H] chitin by enzymes were analysed by gel-filtration chromatography on a Bio-Gel P-2 column (120 × 1 cm). Chitinase isoenzymes (enzyme activity: *ca* 3 000 dpm equivalent under the standard assay condition) purified as above were incubated with 100 μl of [^{3}H] chitin suspension for 2 or 6 hr at 37°. The reaction was terminated by boiling for 20 min, and insoluble matter was removed by centrifugation (700 *g*, 5 min). The solubilized products were applied to the column and eluted with water (1.5 ml fraction, flow rate 4.3 ml/hr). Aliquots (1 ml) were taken from each fraction to determine radioactivity. Authentic GlcNAc oligomers (Seikagaku Kogyo) were chromatographed in the same way for assessment of the peaks of radioactive oligomers.

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