Phytochemistry, 1974, Vol. 13, pp. 383 to 387. Pergamon Press. Printed in England.

INVERTASE IN CULTURED *DAUCUS CAROTA* CELLS

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(*Received* 10 *June* 1973. *Accepted* 11 *September* 1973)

Key Word Index—*Daucus carota*; Umbelliferae; carrot; suspension culture; invertase; cell wall; enzyme solubilization; protoplast.

Abstract—Invertase activity of cultured carrot cells was distributed between cell wall and supernatant fractions of the cell homogenate. The enzyme associated with the cell wall fraction was solubilized by alkaline NaCl solution and the proportions found in the cell wall and soluble fractions depended on the concentration of NaCl. Formation of protoplasts by the action of cellulase and pectinase was accompanied by release of 50–60% of the invertase activity from the cells.

INTRODUCTION

THE INVERTASES of higher plants have been shown to exist in at least two separable forms in plant cells. Ricardo and ap Rees[1] have reported that two invertases, acid and neutral enzymes, can be extracted from carrot root and that the acid invertase was distributed between the cell wall and supernatant fractions of root homogenate whereas the neutral enzyme was located predominantly in the cytoplasm. Copping and Street[2] have also reported the presence of neutral and acid enzymes in cultured sycamore cells. The activities of both enzymes were higher in the cell walls than in the soluble fraction. However, these authors have pointed out that the invertase content of the cell wall fraction was strongly influenced by the conditions of extraction. Therefore, it is still uncertain whether the invertase is localized in the cell wall *in vivo* or if the apparent binding is an artifact. In the present work, we have studied the localization of invertases in cultured carrot cells and attempted to distinguish cell wall invertase from intracellular enzyme by fractionation of cell homogenate and by a technique in which cells were converted to protoplasts by cell wall lytic enzymes.

RESULTS

Changes in sugar content in culture medium

Carrot cells in a suspension culture can use either sucrose or glucose as carbon source. When the culture was initiated from early stationary phase cells, active cell growth was observed after a lag of about 2 days. Sucrose in the medium was rapidly hydrolyzed after inoculation of the cells (Fig. 1). Little or no sucrose was detectable after 12 hr of incubation by which time about 8% of the sugar had been utilized. Since no invertase activity was found in the culture filtrate, this fact suggests the localization of invertase on the cell surface.

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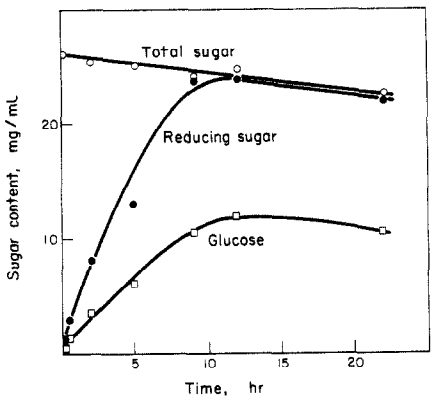

FIG. 1. UTILIZATION AND HYDROLYSIS OF SUCROSE IN CULTURE MEDIUM BY CARROT CELLS.

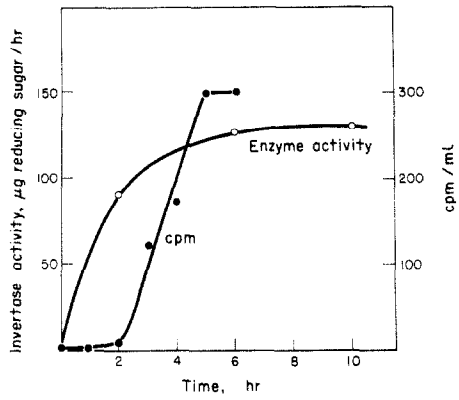

FIG. 2. LIBERATION OF INVERTASE AND OF LABELED PROTEIN FROM PROTOPLASTS OF CARROT CELLS.

(For Fig. 2. Protein released from the protoplasts was precipitated by the addition of TCA (10%) to the culture filtrate. The radioactivity was determined by a liquid scintillation counter. Invertase activity present in the lytic enzymes as a contaminant was subtracted from the total activity.)

Distribution of invertase activity in cell

When the distribution of invertase activity was examined in homogenates of carrot cells, about 55–60% of the total activity was found in the cell wall fraction even after repeated extractions by acetate and borate buffer (Table 1). Intracellular invertase activity was found to be distributed in a sedimentable and a soluble fractions. The former fraction consisted mainly of cytoplasmic particles. The enzyme activity in the cell wall fraction was higher than that of the cytoplasmic fraction, regardless of the culture age. We tried to solubilize the cell wall bound enzyme by treating cells with cellulase and pectinase. Most of the cells were converted to protoplasts after about 2 hr treatment. Liberation of invertase started shortly after the addition of the lytic enzymes and continued for about 5–6 hr (Fig. 2). When ^{14}C-leucine was added to the medium, it was readily incorporated into the cells and labeled protein was released after about 2 hr. It was therefore assumed that the enzyme activity released during the initial 2 hr of the treatment represents invertase originally localized in the cell wall. Activity released afterwards may involve enzymes which were newly synthesized and secreted from the protoplasts. Since the cell number was unchanged during the initial 2 hr, the enzyme activity recovered in the medium did not originate from the broken protoplasts. On the basis of the experiments, surface localized invertase was estimated to be at least 50–60% of the total enzyme.

TABLE 1. DISTRIBUTION OF INVERTASE ACTIVITY BETWEEN VARIOUS FRACTIONS OF CARROT CELL HOMOGENATE

Growth phase	Enzyme activity (μmol sucrose hydrolyzed/hr) Whole homogenate	Soluble fraction	Particulate fraction	Cell wall fraction
Log	15	2·5	0·5	8·4
Stationary	15	0·3	3·6	9·1

TABLE 2. SUCCESSIVE EXTRACTIONS OF INVERTASE FROM CELL WALL PREPARATION WITH BORATE BUFFER CONTAINING VARIOUS CONCENTRATIONS OF NaCl

Expt	NaCl conc. (M)	Invertase activity (%) Solubilized Extractions 1st	2nd	3rd	4th	Cumulative value	Bound
	0	0	—	—	—	0	100
	0·1	16·0	8·0	2·5	0	26·5	39·0
A	0·25	12·0	2·0	0	—	40·5	2·8
	0·5	0·8	0	—	—	41·3	2·4
B	1·0	47·0	0	—	—	47·0	7·0

Dissociation of invertase from cell wall fraction

Enzyme activity in the cell wall fraction was removed by treatment with NaCl solution at pH 8·5. Table 2 shows the results of successive extractions of the enzyme with NaCl solutions of various concentrations. Since the enzyme was rather labile in the alkaline NaCl solution, samples were kept in ice cold water and in each extraction the cell wall suspension was vigorously shaken for 30 sec at room temp. When the cell wall pellet extracted with 0·1 M NaCl in borate buffer was treated again with the same buffer, activity was again recovered in the soluble fraction. No further enzyme was extracted with 0·1 M NaCl solution after the 3rd extraction, but extra enzyme was liberated when the sample was treated with 0·25 and 0·5 M NaCl. Although much of the original activity was lost during the treatments, the results suggest the presence of an enzyme fraction easily extractable with NaCl at low concentration and other fractions bound more firmly. When the cell wall preparation was extracted with 1 M NaCl, nearly 50% of the enzyme initially present in the pellet was recovered in the soluble fraction by a single treatment. Borate buffer alone was ineffective in releasing invertase from the cell wall fraction. Cytoplasmic insoluble enzyme was similarly extracted by borate buffer containing 1 M NaCl. It has been known that the experimental results on the distribution of plant invertase were influenced by pH of the buffer used for the extraction[1,2]. In the present case, no enzyme was released with NaCl solution in acetate buffer at pH 4·7.

TABLE 3. REVERSIBLE BINDING OF SOLUBILIZED INVERTASE TO CELL WALL FRAGMENTS BY REDUCTION OF NaCl CONCENTRATION*

Addition of cell wall fragments	Invertase activity (μmol sucrose hydrolyzed/hr) Supernatant fraction	Pellet fraction
−	3·43	0·18
+	0·37	4·23

* Cell wall preparation was treated with alkaline NaCl solution (1 M) as in Table 2. The enzyme solution was then dialysed against borate buffer (pH 8·5, 50 mM) in the presence or absence of cell wall fragments.

The effect of the salt concentration on the distribution of invertase acitivity was reversible. In the experiments shown in Table 3, cell wall preparation was treated with alkaline NaCl solution as above. The activity was found predominantly in the supernatant fraction.

The solubilized enzyme thus obtained was dialyzed against buffer solution in the presence and absence of cell wall fragments. As shown in the table, the enzyme activity was again found in a sedimentable fraction when the cell wall fragments were present.

Gel-filtration

Soluble enzyme was eluted from a Sephadex G 100 column as a single peak (fractions 13 and 14) and the elution pattern was very similar to those of enzymes solubilized from the cell wall and particulate fractions with alkaline NaCl solution. These partially purified enzymes, as well as crude preparations, showed optimal pH around 4·7 and no neutral enzyme[1-4] was found.

DISCUSSION

It has been suggested that much of the invertase in several plant tissues is localized in the cell wall. These results were obtained by studying the distribution of invertase activity in cell homogenates. Ricardo and ap Rees[1] have studied invertases in disks of carrot root and reported that the pH and the chemical composition of the fractionation medium strongly affect the activities of invertase in cell wall and soluble fractions. Similar results have been reported for other storage organs[3, 5] and for cultured sycamore cells.[2] The results presented here show that in cultured carrot cells part of the invertase is localized outside the plasma membrane and probably in the cell wall. Intracellular invertase occurs in soluble and insoluble forms. The latter may be associated with cytoplasmic particles or membrane. It could be concerned with the sucrose metabolism at the tonoplast or other cellular organelles. However, the possibility is not excluded that the insoluble enzyme is an artifact of extraction. Our results indicate that the properties of the cell wall bound enzyme are similar to those of cytoplasmic enzymes. Failure to detect the neutral enzyme in our carrot cells may be due to the fact that we worked with cultured cells which grow rapidly and have a high demand for sucrose hydrolysis. It has been postulated[1, 3] that the neutral invertase is responsible for intracellular sucrose metabolism in cells which have differentiated and have less sugar demand.

EXPERIMENTAL

Material. Cultured carrot cells (Strain GD-3) were grown in a suspension synthetic medium prepared according to Murashige and Skoog.[6] The general techniques for the maintenance of the culture were as previously described.[7]

Fractionation of the cells. Harvested cells were washed and disrupted with ultra sonics in acetate buffer (pH 4·7, 50 mM). Cell wall pellet obtained by centrifuging at 1000 *g* for 10 min still contained cytoplasmic jelly and many particles. When the pellet was resuspended in borate buffer (pH 8·5, 40 mM) and centrifuged at 1000 *g*, these cytoplasmic materials were separated from the cell wall fragments. The supernatant obtained by the 1st and 2nd centrifugations were combined and further centrifuged at 10000 *g* for 30 min. Invertase activity was found both in the pellet spun down by the high speed centrifugation (particulate fraction) and in the supernatant fraction.

Preparation of protoplast. Protoplasts of the cultured carrot cells were obtained by treating the cells with pectinase and cellulase as described by Takebe *et al.*[8] Commercial enzyme preparations, Macerozyme and Cellulase Onozuka, were purchased from Kinki Yakult Manuf. Co., Japan. Enzyme solution was sterilized by filtration through a Millipore filter. Carrot cells were suspended in the growth medium containing 1% pectinase, 2% cellulase and 0·4 M mannitol and were incubated at 28° with gentle shaking. After incubation for 2–3 hr, cells were

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converted to spherical protoplasts and the cell wall became invisible under a phase-contrast microscope. Lysis upon dilution was complete.

Enzyme assay and determination of sugars. The assay mixture contained 0·1 ml of enzyme preparation and 0·9 ml acetate buffer (pH 4·7, 50 mM) with 250 μmol sucrose. Reaction was carried out at 28°. Reducing sugar was determined by the method of Nelson[9] and Somogyi.[10] Glucose was measured using commercial Glucostat.

Gel-filtration. A column of Sephadex G100 (1·25 × 30 cm) was equilibrated with 40 mM borate buffer (pH 8·5) containing 0·1 M NaCl. Enzyme preparation (1 ml) was eluted with the same buffer at a flow rate of 9·6 ml/hr at 4° and collected in 1·5 ml fractions.

Acknowledgement—The authors wish to thank Prof. N. Sugano for his helpful suggestions and criticism of the manuscript.

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