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Glycosidases in Carrot Cells in Suspension Culture: Localization and Activity Change during Growth

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Localization of four glycosidases, α-galactosidase (α-Gal), β-galactosidase (β-Gal), α-glucosidase (α-Glu) and β-glucosidase (β-Glu) in suspension-cultured carrot cells was studied. Wall-bound enzymes were made soluble when the cells were converted to protoplasts by cellulase and pectinase. α-Gal was separated into two forms, designated I and II, by chromatography on a Sephadex G-200 colunm. α-Gal I was located exclusively in the cytoplasm whereas α-Gal II was found in both the cytoplasmic and cell-wall fractions. The pH optimum was in the neutral region for α-Gal I and in the acidic region for the other glycosidases, including α-Gal II. Both intact cells and protoplasts in suspension culture secreted these glycosidases, except α-Gal I, into the medium. Specific activities of the glycosidases, especially the activity of β-Gal, decreased in the early logarithmic growth phase and increased as cells went through late logarithmic and stationary phases. In protoplast culture, glycosidase activity gradually increased as cell wall regeneration proceeded.

Key words: Cell wall — *Daucus carota* — Glycosidases — Protoplast (carrot) — Suspension culture.

Cell walls of higher plants contain various hydrolytic enzymes, including glycosidases (Klis et al. 1974, Parr and Edelman 1975, Pierrot and Wielink 1977). Recently, these wall-bound glycosidases have received special attention because of the possibility that they are involved in cell wall metabolism. Murray and Bandurski (1975) found a positive correlation between the growth rate of tissue and glycosidase activity in the cell wall in pea seedlings. Similar observations also have been reported by Nevins (1970) and by Tanimoto and Pilet (1978). Keegstra and Albersheim (1970), working with suspension-cultured *Acer pseudoplatanus* cells, showed that the activity of cell-wall associated glycosidases, β-Glu and β-Gal, increased during the growth period and decreased as cell growth ceased. A possible role for some wall-degrading enzymes in the wall-loosening step of auxin-induced growth has been discussed by Masuda and his co-workers (Masuda and Shunju 1967, Tanimoto and Masuda 1968) and by Johnson et al. (1974).

In this work we studied the localization of four glycosidases, α-Gal (EC 3.2.1.22), β-Gal (EC 3.2.1.23), α-Glu (EC 3.2.1.20) and β-Glu (EC 3.2.1.21) in carrot cells in suspension culture. Activities of these enzymes were distributed both in the cytoplasmic and wall fractions and some were released into the culture medium.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; α-Gal, α-galactosidase; β-Gal, β-galactosidase; α-Glu, α-glucosidase; β-Glu, β-glucosidase.

Changes in the level of these glycosidases during the growth cycle of carrot cells also were followed.

Materials and Methods

Plant material—Carrot cells of strain GD-3, derived from a root of red carrot (*Daucus carota* L. cv. Kintoki), were used. The culture medium was prepared according to Murashige and Skoog (1962), and was supplemented with 3% sucrose and 4.5 μM 2,4-D. The cultures were maintained in 500-ml flasks containing 140 ml of the medium. The flasks were agitated on a reciprocal shaker at 155 strokes per min at 27°C. Cells were subcultured by diluting a portion of a 10-day-old culture in fresh medium.

Protoplasts were prepared by suspending the cells in Murashige and Skoog's (1962) salt solution (pH 5.5) containing 0.7 M mannitol, 2% Cellulase Onozuka R10 and 1% Macerozyme R10 (Kinki Yakult Mfg., Nishinomiya, Japan). The suspension was incubated at 30°C for 5 hr with gentle shaking. The protoplasts obtained were cultured in 500-ml Roux flasks containing 20 ml of Murashige-Skoog's liquid medium with 0.1 M sucrose, 0.5 M mannitol and 4.5 μM 2,4-D, at 27°C without shaking. Details of the procedures for the isolation and cultivation of protoplasts have been described previously (Asamizu et al. 1977, Asamizu and Nishi 1980).

Extraction and purification of glycosidases—Fractionation of the cell homogenate and extraction of the glycosidases were carried out according to the procedure of Hösel et al. (1978) with some modifications. Throughout these processes, samples were kept at 0–4°C. Carrot cells from the suspension culture (ca. 70 g, fresh weight) were collected by filtration, washed with distilled water and resuspended in four volumes of 0.025 M citrate-0.05 M phosphate buffer, pH 5.0. The cells were disrupted by sonic oscillation (Tomy UR-200P, Tomy Seiko Ltd., Tokyo) for a total of 10 min with intermittent cooling. The homogenate was centrifuged at $15,000 \times g$ for 20 min. The supernatant of this centrifugation was designated Fraction I (soluble). The sediment was again disrupted by sonic oscillation three times, after which it was resuspended in distilled water. The cell-wall fragments were collected by centrifugation at $750 \times g$ for 5 min. The supernatant and sediment of this centrifugation are Fractions II (particulate) and III (cell wall). The cell-wall fraction was washed with distilled water three more times, then the cell-wall bound enzymes were extracted from the sediment by treating it with 0.1 M citrate-0.2 M phosphate buffer [McIlvaine (1921) buffer] containing 1 M NaCl, pH 5.0, for 24 hr with stirring. Each enzyme preparation was subjected to $(NH_4)_2SO_4$ precipitation and the fractions precipitated between 30 and 65% saturation were collected. The precipitate was dissolved in McIlvaine buffer containing 0.5 M NaCl and 0.02% NaN_3, pH 5.0, then chromatographed on a Sephadex G-200 column (90×4.4 cm) which had been equilibrated with the same buffer.

For the measurement of glycosidase activity in medium, the culture fluid of the cells or protoplasts was filtered through a glass-fiber filter (Whatmann GF/C), then concentrated about 50-fold by ultra-filtration using an Amicon cell (filter PM10), replacing the medium with McIlvaine buffer containing 0.5 M NaCl, pH 5.0.

Glycosidase assay—The assay for glycosidases was performed with *p*-nitrophenyl-glycosides (Nakarai Chemicals Ltd., Kyoto, Japan) as the substrate. The incuba-

tion mixture contained 0.1 ml of 25 mM *p*-nitrophenyl-glycoside solution and 0.4 ml of enzyme preparation in McIlvaine buffer, pH 5.0. The assay mixture was incubated for 60 min at 30°C, then the reaction was terminated by adding 2.5 ml of 0.2 M Na_2CO_3 at 0°C. The absorbance at 400 nm was measured against an appropriate blank. One milli-unit (mU) of enzyme activity was defined as 1 nmol of *p*-nitrophenol released per min. The amount of *p*-nitrophenol released under the assay conditions was calculated from an extinction coefficient of 18.5 mmol liter^{-1}cm^{-1}.

Protein was determined by the micro-biuret method after Itzhaki and Gill (1964) using bovine serum albumin as the standard. The dry weight of the cell wall was measured after the wall fragments in the incubation media had been collected on glassfiber filters (Whatmann GF/C), then washed with distilled water, and dried at 100°C for 60 min.

Estimation of cell volume—Cell size was measured under a microscope. Volume was calculated approximately by assuming that the cells make prolate ellipsoids of rotation.

Results

Distribution of glycosidases in carrot cell homogenate

The subcellular distributions of the glycosidases were examined after the cell homogenate had been fractionated into Fractions I (soluble), II (particulate) and

Table 1 Fractionation of the glycosidases of carrot cells in suspension culture

	Enzyme activity (mU/mg protein)			
	α-Gal	β-Gal	α-Glu	β-Glu
Fraction I				
15,000×*g* Sup.	1.14 (222)[a]	0.89 (175)	0.22 (43)	0.44 (75)
$(NH_4)_2SO_4$ Ppt. (30–65% sat.)	5.75	4.72	0.40	2.65
Fraction II				
750×*g* Sup.	3.09 (58)	6.74 (100)	0.77 (11)	4.72 (70)
$(NH_4)_2SO_4$ Ppt. (30–65% sat.)	21.86	74.28	2.53	11.52
Fraction III				
750×*g* Sed.	6.31 (44)	31.66 (216)	0.83 (6)	11.70 (82)
1st NaCl-extract	55.12 (46)	209.50 (175)	6.45 (5)	60.18 (50)
2nd NaCl-extract	3.74 (1)	32.96 (13)	2.22 (1)	12.22 (5)
Residue	0.25 (2)	0.37 (3)	0.03 (0.2)	0.21 (20)
$(NH_4)_2SO_4$ Ppt.[b] (30–65% sat.)	151.37	265.96	8.46	122.19

[a] Figures in parentheses represent the total activity (mU) per gram of fresh weight of the original whole cells.

[b] Bound enzymes were put through $(NH_4)_2SO_4$-fraction after being made soluble by treatment with NaCl (1.0 M).

III (cell wall); results are listed in Table 1. The activities of the glycosidases tested were detected in all the fractions, but the distribution patterns differed. The activities of α-Gal and α-Glu were, for the most part found in the soluble fraction whereas those of β-Gal and β-Glu were distributed equally between the soluble and cell wall fractions. When the activities were expressed on a protein basis, however, the crude cell wall had the highest specific activity for the hydrolysis of *p*-nitrophenyl glycosides. Treatment of the wall preparation for 24 hr with 1 M NaCl made most of the glycosidase soluble.

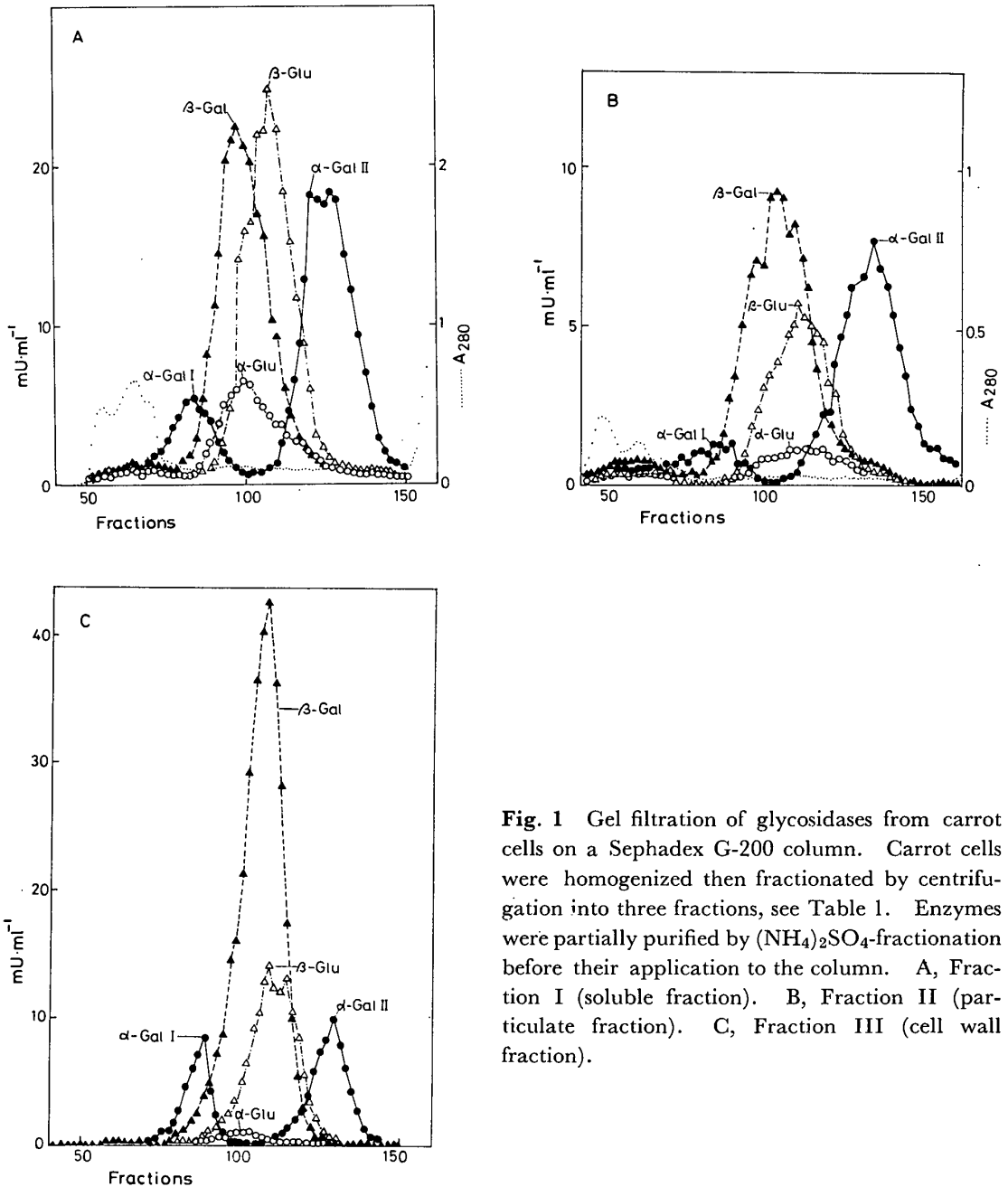

Fig. 1 Gel filtration of glycosidases from carrot cells on a Sephadex G-200 column. Carrot cells were homogenized then fractionated by centrifugation into three fractions, see Table 1. Enzymes were partially purified by $(NH_4)_2SO_4$-fractionation before their application to the column. A, Fraction I (soluble fraction). B, Fraction II (particulate fraction). C, Fraction III (cell wall fraction).

Chromatography of glycosidase from the soluble and wall fractions

Sephadex G-200 chromatography of the soluble fraction after $(NH_4)_2SO_4$-fractionation revealed the presence of at least two forms of α-Gal, designated here as α-Gal I (high molecular weight) and II (low molecular weight), (Fig. 1A). A similar chromatographic pattern also was observed for the α-Gal from the particulate and wall fractions (Fig. 1B and C). The other three glycosidases, however, showed single peaks in the gel filtration profile (Fig. 1).

Carrot cells in suspension culture were treated with cell-wall lytic enzymes so that their wall-bound enzymes would be released into the medium (Ueda et al. 1974). The cells were converted completely to protoplasts after a 5-hr incubation with 2% Cellulase Onozuka and 1% Macerozyme (Asamizu et al. 1977). These protoplasts were separated from the incubation medium by low speed centrifugation ($50 \times g$, for 2 min). The glycosidases remaining in the protoplasts and those released into the medium were chromatographed as described above. Fig. 2 gives the elution patterns of the α-Gal from the protoplasts and the medium after cell-wall digestion. The cell-wall lytic enzymes used contained a high level of β-Glu activity, but α-Gal was present at a much lower level as compared to the enzyme activity derived from carrot cells, and it showed a different elution pattern (Fig. 2). The results shown

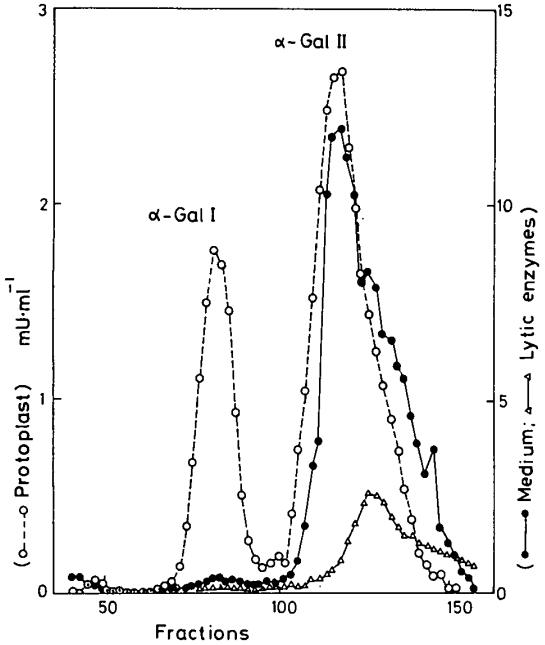

Fig. 2

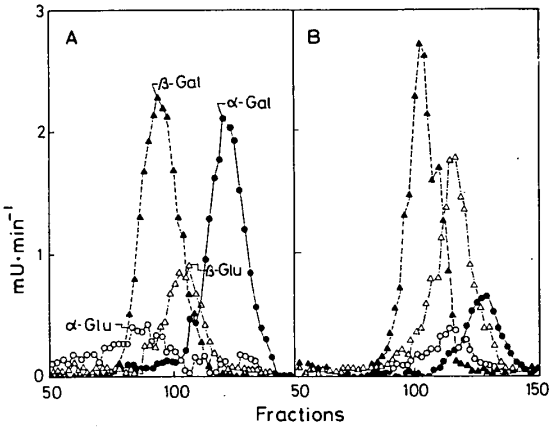

Fig. 3

Fig. 2 Gel filtration on a Sephadex G-200 column of the α-Gal in protoplasts and that released into the medium after cell-wall digestion. Enzymes were partially pruified by $(NH_4)_2SO_4$-fractionation before column chromatography. The elution pattern for α-Gal in a combined mixture of Cellulase Onozuka R10 (2%) and Macerozyme R10 (1%) was obtained with a separate column under the same conditions. -○-, Protoplast. -●-, Medium. -△-, Lytic enzymes alone.

Fig. 3 Gel filtration on a Sephadex G-200 column of the extracellular glycosidases excreted from cells (A) and protoplasts (B). Enzymes from culture media of carrot cells (10-day-old) or protoplasts (24-hr-old) were concentrated by ultra-filtration then applied to the column without further purification.

in Fig. 2 are evidence that α-Gal I and II are located inside the protoplast, and that only α-Gal II is liberated into the medium upon wall digestion. Other glycosidases in the medium and in the protoplast, except β-Glu which was not determined, showed chromatographic patterns similar to those in Fig. 1.

Enzymes excreted by cells and protoplasts

Glycosidase activity was found in the medium in which cells or protoplasts had been grown. These extracellular enzymes were concentrated using Amicon PM10, then fractionated by Sephadex G-200 chromatography (Fig. 3). The elution profile shows that α-Gal in the culture filtrates of cells and of protoplasts consisted mainly of α-Gal II. β-Gal was predominant among the extracellular glycosidases.

Effect of pH on glycosidase activity

Fig. 4A and B show the pH dependence of the glycosidases prepared from the

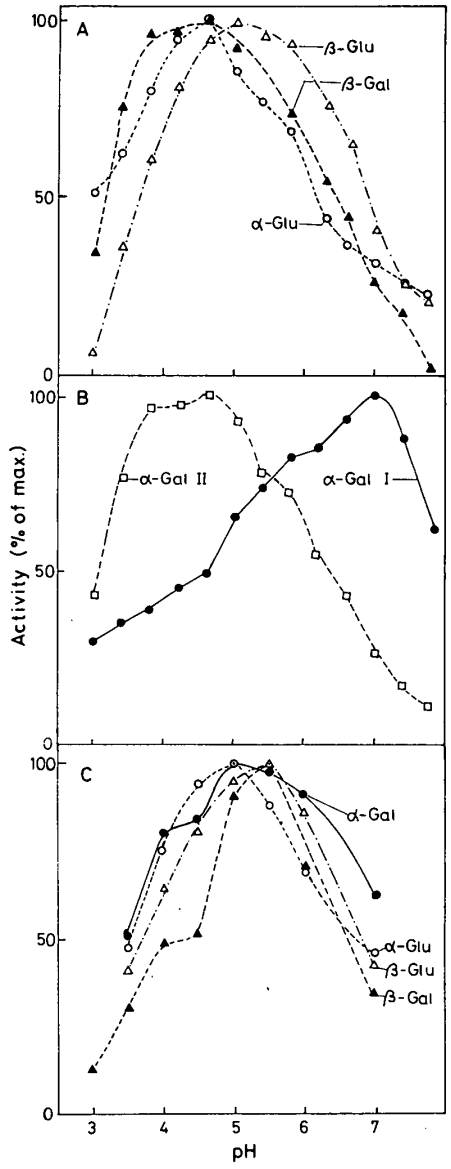

Fig. 4 Effect of pH on the activity of glycosidases from carrot cells. A and B, Activity in the cytoplasmic fraction. C, Activity in the cell wall fraction. McIlvaine buffer was used for the entire pH range. Actual pH values were measured after the reaction.

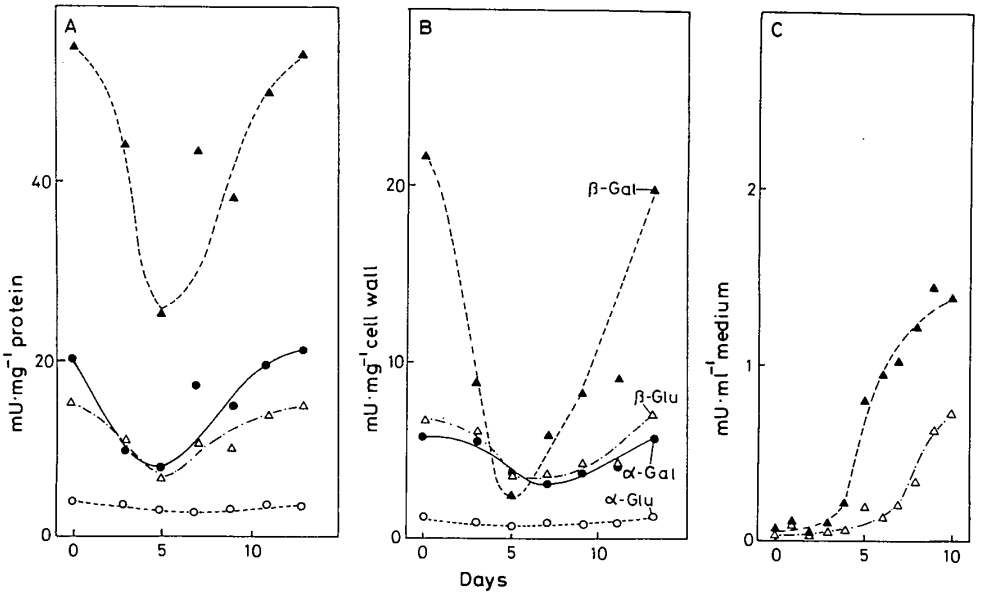

Fig. 5 Changes in the activity of glycosidases during culture growth. A, Cytoplasmic enzymes. Enzyme preparations were partially purified by $(NH_4)_2SO_4$-fractionation before the assay. B, Enzymes from the isolated cell wall. C, Extracellular enzymes.

soluble fraction. All had maximum activity in the acidic range (pH 4–5), except α-Gal I which had an optimum at pH 7. Glycosidases from the isolated wall had pH optima in the acidic range (Fig. 4C). The pH optima for the extracellular enzymes of normal cells and of protoplasts were almost the same; 5.0 for α-Gal, 4.5 for β-Gal and 5.5 for β-Glu. The activity of α-Glu in the medium was very low, therefore, the pH optimum could not be determined.

Change in glycosidase activity during the culture of carrot cells and protoplasts

Carrot cells in suspension culture multiplied rapidly during the first 5–6 days with a doubling time of about 2 days (Okamura et al. 1975). Cell size increased after the rate of cell division fell suggesting that cell wall synthesis is greater in aged cells. The average cell volume on day 5 was approximately 2,000 μm^3 whereas that on day 10 was 27,000 μm^3.

Glycosidase activities in both the cytoplasmic (Fig. 5A) and cell wall fractions (Fig. 5B) change significantly during the growth cycle. The specific activities of these enzymes decreased sharply in the early log phase, reached minimum values at about day 5, then increased again. The change was most notable for the β-Gal activity in the cell wall fraction (Fig. 5B). The extracellular activities of β-Gal and β-Glu were very low in the early log phase, but increased abruptly after 4–5 days of cultivation (Fig. 5C).

Cell numbers in the protoplast cultures began to increase about 4 days after inoculation (Asamizu and Nishi 1980). Cell wall synthesis started without a noticeable lag although the rate of wall deposition was very low during the initial stage (Asamizu et al. 1977, Asamizu and Nishi 1980). The specific activity of glycosidases increased gradually during the culture of protoplasts (Fig. 6A). Excretion of enzymes into the culture fluid by protoplasts began shortly after the start of incuba-

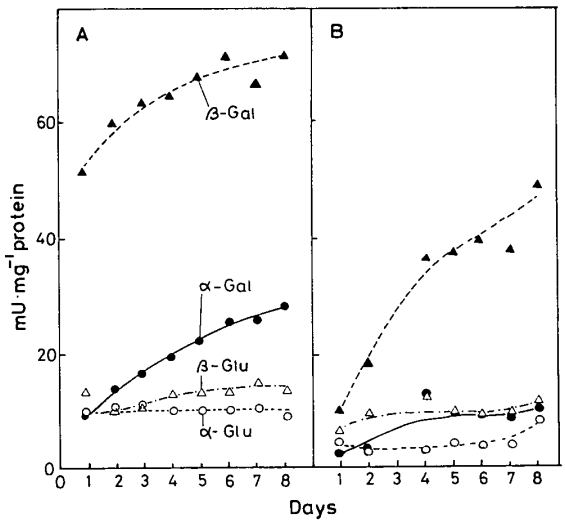

Fig. 6 Changes in the activity of glycosidases during the culture of regenerating protoplasts. A, Enzymes in protoplasts. Enzyme preparations were partially purified by $(NH_4)_2SO_4$-fractionation before the assay. B, Extracellular enzyme excreted from the protoplasts.

tion (Fig. 6B). The increase in the extracellular activity of β-Gal especially was marked.

Discussion

Enzymes in plant cell walls usually have been studied by fractionation of the cell homogenate by differential centrifugation. Enzyme activity associated with insoluble residues of the cell homogenate, however, does not necessarily represent the in vivo wall-bound enzyme. The amount of enzyme precipitated with the cell wall in the crude homogenate may vary considerably depending on the pH and the ionic strength of the extraction medium (Jaynes et al. 1972, Parr and Edelman 1975, Copping and Street 1972). Ueda et al. (1974) found that formation of protoplasts by cell-wall lytic enzymes was accompanied by the release of 50–60% of the invertase activity from carrot cells. They showed that the technique is useful for isolating surface-localized enzymes. In this study we examined the localization of glycosidases in carrot cells by fractionation of the cell homogenate and by removing the cell wall enzymatically. The gel-filtration profile showed that only one of the two forms of α-Gal was released by cell-wall digestion. The result indicates that the enzyme activity in the incubation medium is not due to leakage of cytoplasmic protein and that a part of the α-Gal II is located outside the plasmalemma in vivo. The activity of α-Gal I observed in the cell-wall fraction of the cell homogenate (Table 1) might be due to cytoplasmic contamination.

The liberation of wall polymers into the medium from carrot cells and protoplasts in suspension culture has been reported (Asamizu and Nishi 1980). The results of experiments shown in Fig. 3 and 5 showed that glycosidases also are excreted from the cells. Little α-Gal I activity was found in the medium which suggests that the extracellular enzymes were liberated from the cell wall. The release of enzymes into the medium was paralleled by increases in the specific activities of intracellular and wall glycosidases (Fig. 5). The extracellular enzymes may represent part of the wall enzymes which have been transported across the plasmalemma together with the other cell wall polymers, but which were not deposited on the cell surface.

Glycosidase activity in both the soluble and wall fractions changed considerably during the growth cycle of carrot cells. It is unlikely that the increase in enzyme activity in the late growth phase is caused by a release from the glucose repression because in the 5-day-old culture, the medium still contained about a half of the sugar originally supplied (Okamura et al. 1975). It is more likely that this increase is related to the rapid expansion of the surface area of the cells during the late log and stationary phases. In a previous paper (Asamizu et al. 1977), we showed that the cellulose content per cell initially decreased when carrot cells were transferred to fresh medium. A minimum was reached on day 6, after which the cellulose content increased 4- to 5-fold during the next 10 days. That the cell wall expanded after the period of rapid cell division also was indicated from the change in cell size. Fairly similar growth patterns have been found in various plant cell cultures (Henshaw et al. 1966, Fletcher and Beevers 1970).

Several lines of evidence suggest that the wall-bound glycosidases are involved in the growth of cell wall (Keegstra and Albersheim 1970, Johnson et al. 1974, Wallner and Walker 1975, Tanimoto and Igari 1976). Enzymatic cleavage of certain linkages in the wall polymers may alter the rigidity of the wall and permit its extension. The increases in the specific activities of glycosidases, especially the activity of β-Gal, that take place during cell expansion suggest that these enzymes play a role in the growth of the cell walls of carrot cells.

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