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Invertase Activity and the Kinetin-Stimulated Enlargement of Detached Radish Cotyledons[1]

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HAROLD F. HOWARD[2] AND FRANCIS H. WITHAM
Department of Biology (H. F. H.) and Horticulture (F. H. W.), The Pennsylvania State University, University Park, Pennsyvlania 16802

ABSTRACT

Cytokinin treatment is known to promote expansion of light-grown excised radish (*Raphanus sativus* L. cv Crimson Giant) cotyledons. This expansion, at least in part, seems to be related to an increased accumulation of osmotically active reducing sugars. Kinetin treatment did not cause increased levels of isocitrate lyase activity over the controls, but stimulated increased levels of two invertase forms, designated types I and II. Type I was soluble and type II was insoluble after homogenization in 10 millimolar tris(hydroxymethyl)aminomethane-HCl (pH 7.0). Both types were soluble after homogenization in 300 millimolar NaCl. At low salt concentration, type II was retained on a diethylamioethyl-cellulose column and type I was not. Type II was then eluted from the column at high salt concentration. Types I and II exhibited pH optima of 5.3 and 4.3, Michaelis constants of 4.96 and 1.23 millimolar sucrose, and molecular weights of 65,000 and 57,000 daltons, respectively. The kinetin promotion of reducing sugar accumulation may be related to increased levels of the two invertase forms, but is probably not a result of direct cytokinin-stimulated glyoxysomal activity.

Cytokinins are known to promote the expansion of excised cotyledons (2, 7).

Excised radish cotyledons incubated in darkness were shown to accumulate K and this accumulation was greatly enhanced by cytokinin treatment (8). However, when the cotyledons were incubated in the light, K accumulation was not observed. Rather, light was shown to promote the accumulation of reducing sugars in excised radish cotyledons and this reducing sugar accumulation was further promoted by cytokinin treatment (6). A high correlation between reducing sugar accumulation and cotyledon expansion indicates that, in light, reducing sugars were the major osmotically active component.

Huff and Ross (6) suggested that the increased reducing sugar levels may result from the conversion of lipids to sugars. Thus, cytokinin treatment could influence the levels of glyoxysomal enzymes. In support of this hypothesis, Penner and Ashton (9), working with peanut and squash cotyledons, observed that a hormonal stimulus from the embryonic axis is required for optimum isocitrate lyase activity. Exogenously supplied cytokinin seemed to replace that stimulus.

Rijven and Parkash (11) reported that sucrose increased expansion of kinetin-treated fenugreek cotyledons when incubated in darkness, but only after the first 24 h of incubation. This and other experiments indicate that in light-grown cotyledons, sucrose may be the source of reducing sugar. Hence, reducing sugar accumulation may be dependent on invertase activity. The experiments reported here deal with the influence of cytokinin treatment on isocitrate lyase and invertase activities in radish cotyledons incubated in light.

MATERIALS AND METHODS

Cotyledon Preparation and Incubation. Radish seed (*Raphanus sativus* L. cv 'Crimson Giant') was obtained from the W. Atlee Burpee Company. Seed of approximately the same size (those retained on a 2-mm wire mesh sieve) were sown on distilled H_2O-moistened Whatman No. 1 filter papers contained in plastic trays (10 × 20 cm). These trays were then enclosed in polyethylene bags and the seed germinated in darkness at 25°C for 38 h. For the experiments, the inner cotyledon from each seedling was excised and placed adaxial surface down on Whatman No. 1 filter paper in a 9-cm Petri dish. Ten cotyledons of uniform size were placed in each dish which contained 5 ml of 2.0 mM K-phosphate (pH 6.0) with or without 10 mg/l kinetin (Sigma Chemical Co.). The Petri dishes were then incubated for various times at 25°C in constant white fluorescent light (450 lux). Under these conditions, kinetin increased cotyledon fresh weight by 21, 63, and 81% over the controls at 24, 48, and 72 h, respectively (3).

Isocitrate Lyase Extraction and Assay. The following procedure is a modification of that used by Hock and Beevers (4). Twenty detached cotyledons were incubated for appropriate time periods and homogenized in a Teflon pestle tissue homogenizer (Kontes) containing 10 ml of isolation buffer (500 mM mannitol, 10 mM K-phosphate [pH 7.5], 10 mM EDTA, and 0.08% BSA). The homogenate was centrifuged at 500*g* for 10 min and the pellet discarded. The supernatant was then recentrifuged at 15,000*g* for 30 min. This supernatant was adjusted to 15 ml with isolation buffer and used as a source of soluble enzyme. The pellet was suspended in 10 ml of isolation buffer to which 0.1% digitonin had been added and this was used as a source of insoluble enzyme. The above procedures were performed at 4°C.

For the assay of this enzyme, 0.2 ml of crude enzyme preparation were added to 2.7 ml of reaction mixture containing 43 mM KH_2PO_4, 6.5 mM $MgCl_2$, 4.3 mM phenylhydrazine HCl, and 2.5 mM EDTA. The mixture was adjusted to pH 7.1 with NaOH. The reaction was initiated by the addition of 0.1 ml of 20 mM DL-isocitric acid to the reaction mixture which was then placed in a Beckman DB spectrophotometer at 25°C. The linear rate of product formation (absorbance increase at 324 nm cotyledon^{-1} min^{-1}) was utilized as a measure of enzyme activity.

Invertase Extraction and Assay. The following procedure is a

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[2] Present address: Department of Agronomy, The Pennsylvania State University, University Park, PA 16802.

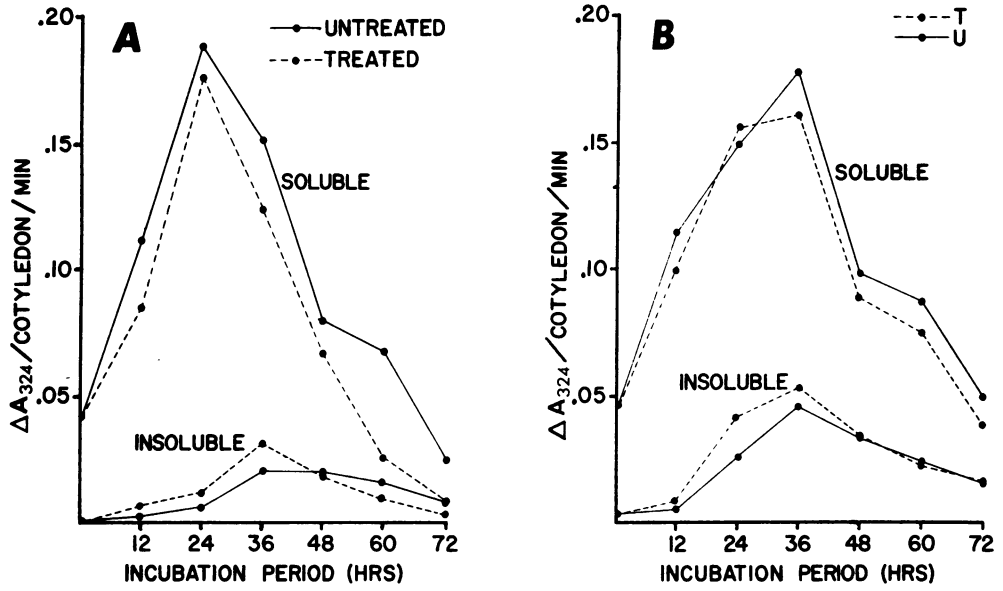

FIG. 1. Influence of kinetin treatment on isocitrate lyase activity in soluble and insoluble fractions. Cotyledons were incubated in (A) light and (B) darkness.

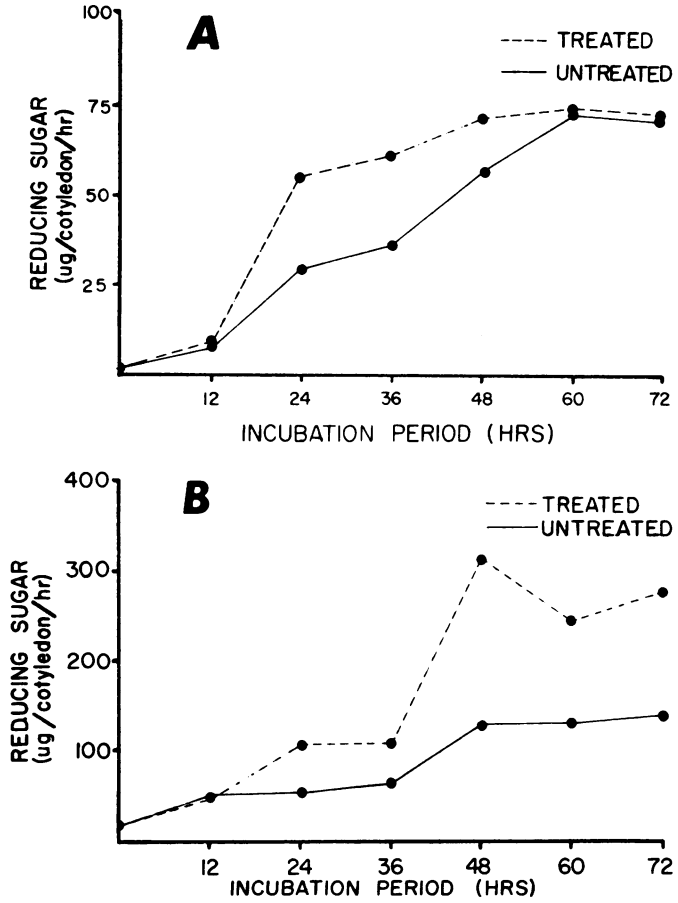

FIG. 2. Influence of kinetin treatment on (A) soluble and (B) insoluble invertase activities in cotyledons incubated in light.

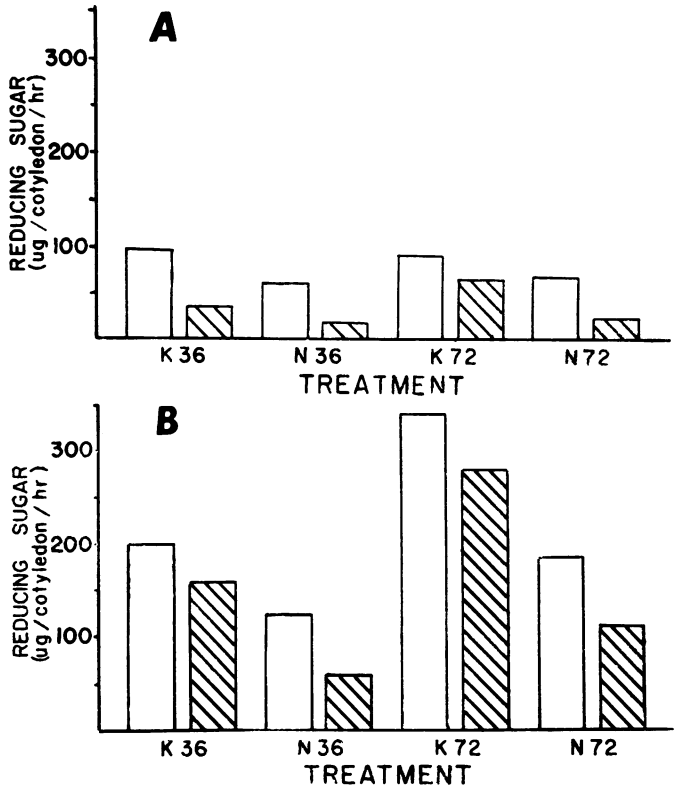

FIG. 3. Comparison of the effects of light and dark incubation on invertase activity in the (A) soluble and (B) insoluble fractions. (K = kinetin treated, N = no kinetin treatment; 36 and 72 represent hours of incubation; unshaded and shaded bars represent light and dark incubation, respectively). In each case, the difference between kinetin and untreated cotyledons was significant at the 5% level according to Fisher's LSD test.

modification of that employed by Tsai *et al.* (14) as modified by Shannon and Dougherty (12). Twenty cotyledons were homogenized in a Teflon pestle tissue homogenizer containing 10 ml of grinding buffer consisting of 10 mM Tris-HCl (pH 7.0). The homogenate was centrifuged at 30,900*g* for 20 min. The pellet was washed by suspension in 10 ml of grinding buffer and centrifugation as above. The pellet was washed a second time as above. All supernatants were combined and saved. The final pellet was suspended in 10 ml of grinding buffer. The supernatant and pellet suspension were dialyzed separately against grinding buffer for 3 h to remove endogenous reducing sugars. The

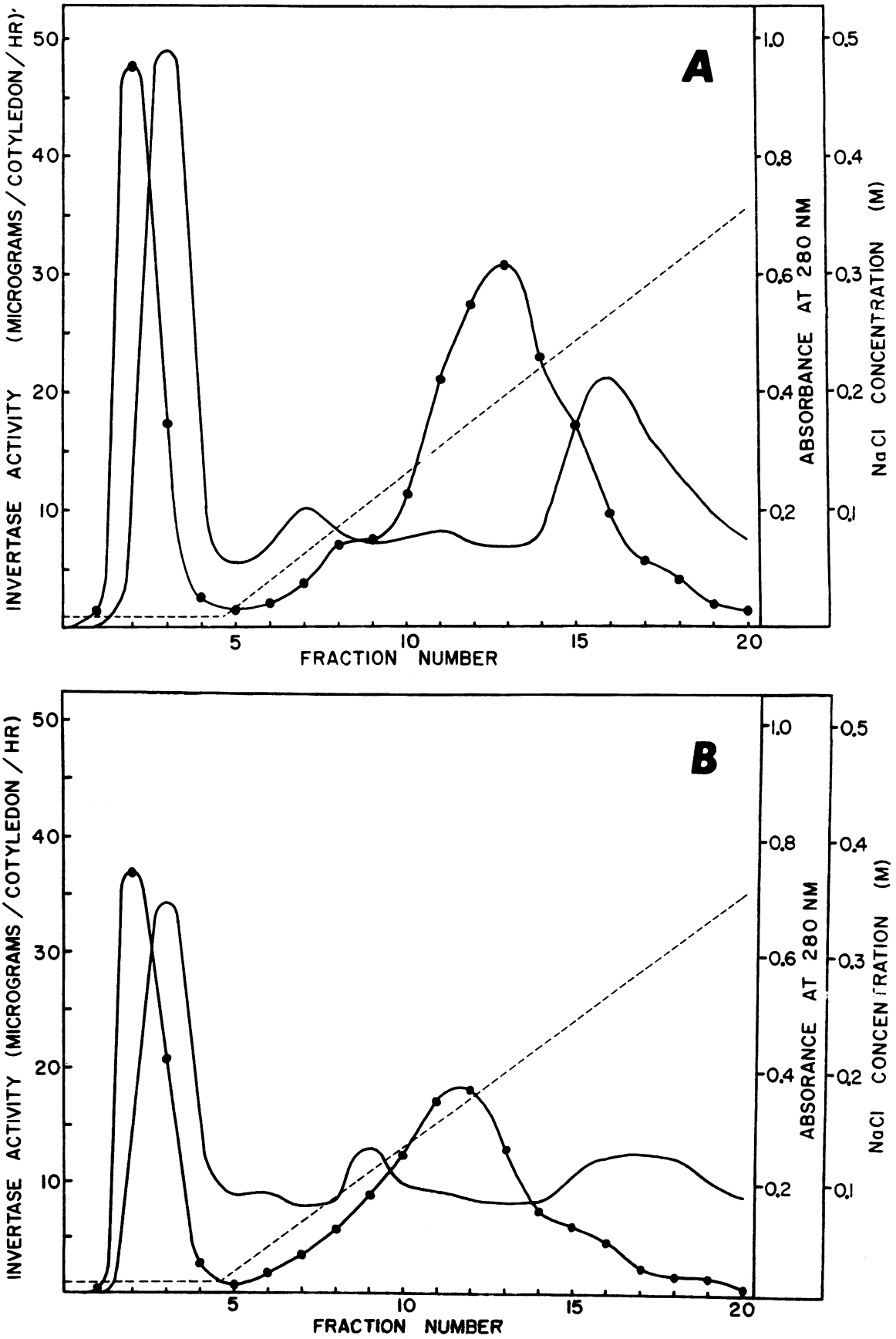

FIG. 4. Chromatography on a DEAE-cellulose column of 300 mM NaCl-extracted invertases from cotyledons incubated in light for 72 h with (A) and without (B) kinetin treatment. Elution was conducted with NaCl (----) and the fractions were assayed for invertase activity (●——●) and protein (——).

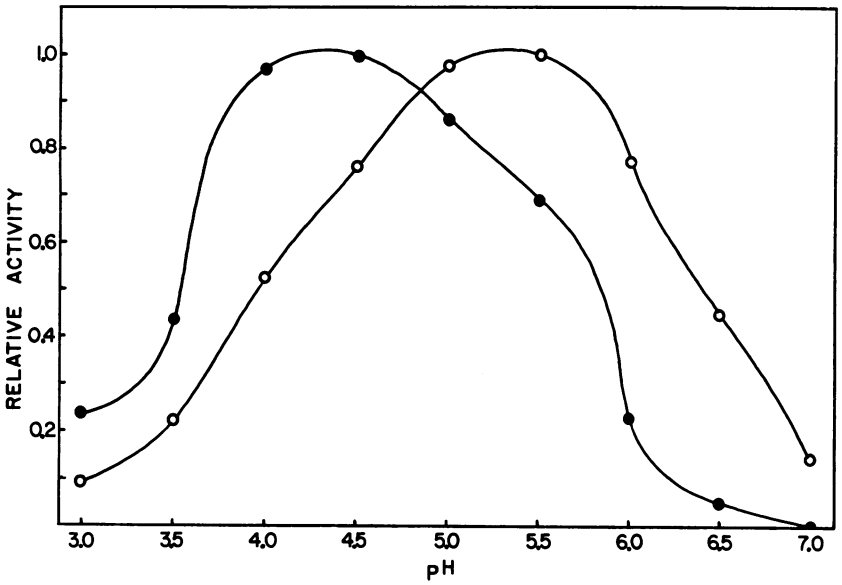

FIG. 5. Effect of pH on invertases types I (O——O) and II (●——●). Reaction conditions were standard except that the buffer solution was 0.2 M citrate-phosphate at the pH values indicated.

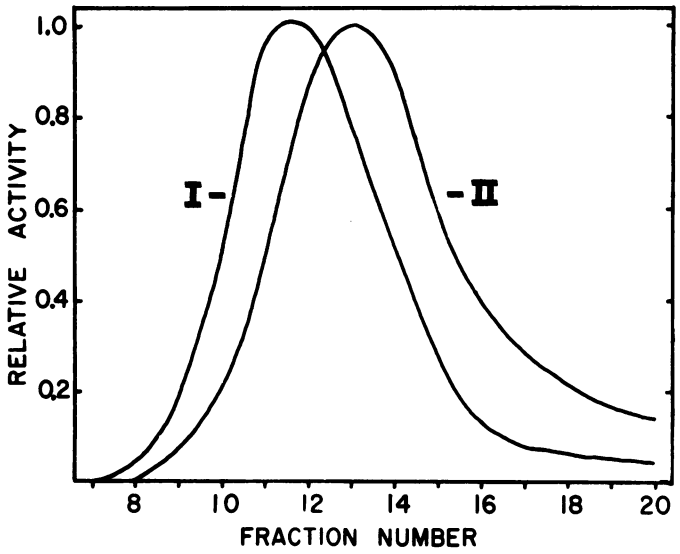

FIG. 6. Chromatography of invertases type I and II on a Sephadex G-100 column. Type I eluted with a peak in fraction number 11 and type II eluted with a peak in fraction number 13. The collection of fractions was begun at V_o.

supernatant was adjusted to 20 ml with grinding buffer and used as a source of soluble enzyme. The pellet was adjusted to 40 ml with grinding buffer and used as a source of insoluble enzyme. The above procedure was carried out at 4°C.

To assay the invertase, 1.0 ml of enzyme preparation, 1.0 ml of 50 mM sucrose and 2.0 ml of assay buffer were combined and incubated at 37°C for 1 h. At the end of the incubation time, the reaction was terminated with the copper reagent of Nelson's Arsenomolybdate Method which was used to measure the release of reducing sugars from sucrose substrate (5). Unless otherwise noted, the assay buffer contained 200 mM sodium acetate adjusted to pH 5.5 for the soluble and pH 4.5 for the insoluble forms.

Separation of Salt-Extracted Invertases by DEAE-Cellulose Chromatography. Twenty-five cotyledons were homogenized in 15 ml of extraction buffer containing 10 mM Tris-HCl and 300 mM NaCl, and then centrifuged at 30,900*g* for 20 min. The high ionic strength of the media allowed the removal of invertase which was previously described as insoluble in 10 mM Tris-HCl. The pellet was discarded. Invertase was precipitated from the supernatant by the addition of ammonium sulfate to 75% saturation and centrifugation at 30,900*g* for 10 min. The pellet was resuspended in 1.5 ml of extraction buffer and dialyzed overnight against extraction buffer. The above procedure was performed at 4°C.

The dialyzed suspension was applied to a DEAE-cellulose column (0.9 × 10 cm) equilibrated with 10 mM Tris-HCl and 10 mM NaCl. After the suspension had entered the media, the collection of 2 ml eluent fractions was begun. The protein was then eluted from the column by a 75-ml linear gradient of 10 mM Tris-HCl and 10 to 500 mM NaCl. Two-ml fractions of column eluent (pH was approximately 6.0) were collected and assayed for invertase activity of pH 5.0. The fractions corresponding to each invertase activity peak were pooled and used as an enzyme source for pH optimum, kinetic constant, and mol wt determinations. For the determination of salt concentration in the eluent, a 1.5-ml blank containing 300 mM NaCl only was chromatographed as above. Each eluent fraction was evaporated. The salt concentrations were then calculated from the weights of the salt present in each fraction. The column chromatography was a modification of the method of Pressey and Avants (10) and was performed at room temperature.

Mol Wt Determination. One-ml aliquots of the pooled fractions obtained by DEAE-cellulose chromatography were applied to a Sephadex G-100 (Pharmacia) column (1.1 × 60 cm). The column V_t was 54 ml and the V_o was 19 ml as determined with blue dextran. The enzyme was eluted from the column with 300 mM NaCl. Beginning when the V_o eluted, fractions of 1.0 ml were collected and assayed for invertase by the standard method at pH 5.0. The K_{av} values of the invertase fractions and of standard proteins were used to calculate the invertase mol wt. The column was calibrated for mol wt determination with the standard proteins: bactracin, Cyt *c*, chymotrypsinogen A, and BSA (Mann Research Laboratories, Inc.).

RESULTS

Isocitrate Lyase. Isocitrate lyase activity in light-incubated cotyledons was low at the time of excision, increased 4-fold at 24 h and then declined to low levels at 75 h (Fig. 1A). Most of

the enzyme activity was present in the soluble fraction. The activities of enzyme extracted from kinetin-treated and untreated cotyledons were quite similar. This was true for both the soluble and insoluble fraction. A similar trend (Fig. 1B) was observed with dark incubation except maximum activity occurred at 36 h. Thus, it appears that kinetin and/or light treatment has little effect on isocitrate lyase activity in detached radish cotyledons.

Invertases. An examination of invertase levels in kinetin-treated cotyledons throughout the 72-h time course revealed that the invertase fractions (one soluble [Fig. 2A] and another insoluble [bound to cell fragments after 10 mM Tris-HCl homogenization; Fig. 2B]) increased from near zero at the time of excision to higher levels. Throughout the time course, the insoluble fraction exhibited greater invertase activity than the soluble fraction.

A comparison of soluble invertase levels in kinetin treated *versus* untreated cotyledons (Fig. 2A) illustrates that, from 24 to 48 h, the treated tissues contain more invertase activity compared to untreated tissues. But at 60 to 75 h, the levels are about equal. Although kinetin stimulated insoluble invertase about 2-fold at all times after 12 h, it is especially notable after 36 h (Fig. 2B). The kinetin stimulation is still noted at 60 to 72 h in contrast to the soluble fraction.

For both the soluble (Fig. 3A) and insoluble fractions (Fig. 3B) after 36 and after 72 h of incubation, greater invertase activity was observed in light- than in dark-incubated tissues. Also at 36 and 72 h, kinetin stimulation of both fractions was observed in light- and in dark-incubated cotyledons.

When 300 mM NaCl extracts were applied to a DEAE-cellulose column, one peak of activity eluted with the void volume (designated as invertase type I) and another peak eluted with approximately 180 mM NaCl (designated as invertase type II). This trend was observed with both kinetin-treated (Fig. 4A) and untreated tissues (Fig. 4B) at 72 h of incubation. When the 10 mM Tris-HCl-soluble invertase was applied to DEAE-cellulose columns, it eluted with the void volume. Also, invertase which was released from the 10 mM Tris-HCl-extracted pellets by homogenizing in 300 mM NaCl was applied to the DEAE-cellulose columns. This invertase was initially retained on the column but later eluted with approximately 180 mM NaCl. Thus, it was concluded that invertase type I and type II correspond to the 10 mM Tris-HCl-soluble and -insoluble fractions, respectively.

Invertases type I and II displayed distinctly different patterns of relative activity between pH 3.0 and 7.0. The pH optima occurred at approximately 5.3 and 4.3, respectively (Fig. 5).

When chromatographed on Sephadex G-100, type I had a smaller elution volume than type II (Fig. 6). The mol wt as determined by this gel filtration were 65,000 and 57,000 D for types I and II, respectively.

From a double reciprocal plot of relative activity *versus* substrate concentration, the Michaelis constants were determined to be 4.96 and 1.23 mM sucrose for invertases I and II, respectively.

DISCUSSION

Cytokinins reportedly increased the activity of isocitrate lyase in cotyledons of peanuts and squash (9) as well as in sunflower (13). It was suggested (9, 13) that endogenous cytokinins produced in the roots of intact seedlings or exogenously applied to excised cotyledons are involved in the regulation of isocitrate lyase activity in the cotyledons. In contrast, we have shown (Fig. 1) that in excised radish cotyledons, kinetin treatment and/or light incubation have little or no effect on the activity of this enzyme. This substantiates similar findings by Aull (1). Thus, it is not likely that the cytokinin-induced accumulation of reducing sugars is a direct result of increased lipid conversion via the conventional glyoxosomal pathway.

We have demonstrated that in excised radish cotyledons, treatment with kinetin causes increased levels of invertase activity in both the soluble and insoluble fractions. Other workers have observed both soluble and insoluble forms of invertase in corn (12) and in oats (10). The soluble and insoluble forms have been designated as invertase types I and II, respectively.

Both types appear to be acid invertases with pH optima of 5.3 for type I and 4.3 for type II. However, these two types also differed greatly in other important properties. Type II was retained on anion exchange resin while type I was not. The Michaelis constant of type I for sucrose was approximately 4-fold greater than type II. Also, these two types differed in their mobility on a gel filtration column. Their mol wt were determined to be 65,000 and 57,000 D for types I and II, respectively. Thus, it is suggested that in this system, kinetin promotes the activity of two distinctly different invertase forms. It is noted that the development of invertase activity closely resembles the time course of reducing sugar accumulation and subsequent cotyledon expansion (3, 6).

We observed that the differences in invertase levels between kinetin-treated and untreated cotyledons did not appear until after 12 h of incubation. So, perhaps the increased activity was due to *de novo* synthesis of the enzyme. Rijven and Parkash (11) found that when fenugreek cotyledons are incubated in darkness and in the presence of cytokinin, sucrose exerts a powerful stimulation of growth but does so only after the first 24 h of incubation. This evidence supports our suggestion that the hormonal promotion of reducing sugar accumulation and subsequent expansion of light-incubated detached radish cotyledons is associated with increased sucrose hydrolysis by invertase.

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