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Invertases in Oat Seedlings

SEPARATION, PROPERTIES, AND CHANGES IN ACTIVITIES IN SEEDLING SEGMENTS

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ABSTRACT

The soluble invertase activity in etiolated *Avena* seedlings was highest at the apex of the coleoptile and much lower in the primary leaf, mesocotyl, and root. The activity in all parts of the seedling consisted of two invertases (I and II) which were separated by chromatography on diethylaminoethylcellulose. Both enzymes appeared to be acid invertases, but they differed in molecular size, pH optimum, and the kinetic parameters K_m and V_{max} of their action on sucrose, raffinose, and stachyose. Invertase II had low stability at pH 3.5 and below, and exhibited high sensitivity to Hg^{2+}, with complete inhibition by 2 micromolar $HgCl_2$. Segments of coleoptiles incubated in water lost about two-thirds of the total invertase activity after 16 hours. The loss of activity was due primarily to a decrease in the level of invertase II. The loss of invertase was decreased by indoleacetic acid, 2,4-dichlorophenoxyacetic acid, and α-naphthaleneacetic acid but not by β-naphthaleneacetic acid and *p*-chlorophenoxyisobutyric acid. Conditions that inhibited auxin-induced growth of the segments (20 millimolar $CaCl_2$ and 200 millimolar mannitol) also blocked the auxin effect on invertase loss.

Invertase activity is present in many plant tissues including seedlings of beans (4), lentils (15), peas (9), and other species. The results of several studies have suggested that this enzyme is essential for growth by making sugars available for cell expansion (1, 8, 15). Plant invertases are classified as acid or alkaline invertases on the basis of their pH optima (1). Both types of enzymes often occur in the same tissue; the acid invertase is associated with the cell wall whereas the alkaline invertase is located in the cytoplasm (1). We found that acid invertase extracted with 0.5 M NaCl from oat seedlings could be separated into two peaks of activity by chromatography on DEAE-cellulose. This paper describes the procedure for that separation and some of the properties of the invertases. Also, segments of oat seedlings were incubated in water and different aqueous solutions, and changes in invertase activity were examined.

MATERIALS AND METHODS

Seedlings of *Avena sativa* cv. Victory were grown in moist Vermiculite in stainless steel trays in the dark at 22 C. The seedlings were harvested after 5 days when approximately 4 cm tall.

For the preparation of crude invertase, 100 g of whole seedlings was blended with 400 ml cold 0.5 M NaCl in a VirTis homogenizer.[1] All subsequent steps were conducted at about 4 C. The homogenate was stirred for 0.5 h, then centrifuged at 8,000*g* for 20 min. The protein in the supernatant solution was precipitated with ammonium sulfate at 75% of saturation and collected after centrifugation. It was dissolved in 35 ml of 0.15 M NaCl and dialyzed overnight against 4 liters of 0.15 M NaCl.

The reaction mixture for the invertase assay consisted of 0.1 ml enzyme solution, 0.2 ml 0.1 M sodium acetate (pH 5), 0.1 ml 0.15 M NaCl, and 0.1 ml 0.73 M sucrose. The enzyme solution was diluted with 0.15 M NaCl to produce approximately 1-μmol reducing groups. The blank prepared for each sample was like the assay mixture but was heated before the addition of sucrose. The sample and blank tubes were incubated for 30 min at 30 C, and the reaction was terminated by the addition of 0.5 ml 0.5 M dibasic Na-phosphate followed by immersion of the test tubes in boiling water for 3 min. A 0.5-ml aliquot was then analyzed for reducing groups by the arsenomolybdate method of Nelson (10) standardized with glucose. A unit of invertase is defined as that amount which catalyzes the release of 1-μmol reducing groups under the conditions of the assay.

The invertase activity in the sediment obtained upon centrifugation of the 0.5 M NaCl extract of the seedlings was also determined. The sediment was washed with 0.5 M NaCl and then suspended in water with a Polytron homogenizer. A 0.5-ml aliquot of the suspension was added to a reaction mixture consisting of 0.5 ml 0.2 M acetate (pH 4.5), 0.5 ml 0.15 M NaCl, and 0.5 ml 0.73 M sucrose. After 30 min at 30 C, the sample was centrifuged and the supernatant analyzed for reducing groups as described above for the soluble enzyme.

Pectinesterase, polygalacturonase, β-galactosidase, and β-glucosidase were measured as described earlier (13). The levels of these enzymes are presented for comparative purposes. Protein was measured by the biuret method.

Isoelectric focusing was performed with a Desaga/Brinkmann TLE double chamber according to the manufacturers instructions. A glass plate (20 × 20 cm) was coated with a suspension of 7 g Sephadex G-75 superfine in 100 ml of 2% pH 2–10 pH isolytes (Brinkmann Instruments). The layer was dried at room temperature until fine cracks appeared along the edges. The enzyme solutions were concentrated to about 3 ml by ultrafiltration with an Amicon model 52 cell and a PM-10 membrane and dialyzed against 2% glycine (pH 6.5). Sephadex G-75 was added to the solution to produce a fairly liquid suspension. A trough was cut out of the gel layer in the middle of the plate and filled with the enzyme suspension. Focusing was conducted at 4 C at 200 v for 12 h and then at 800 v for 2 h. One-cm strips of the gel were cut parallel to the electrodes, suspended in a small volume of 0.15 M NaCl, and assayed for invertase.

The studies on changes in invertase in oat seedling segments were conducted with 1-cm segments cut from seedlings approximately 4 cm tall after removing the top 3 mm. The leaves were not removed from the coleoptiles. The segments were stirred in deionized H_2O for 1 h before extraction or incubation. Each experiment consisted of six samples of 10-g oat segments/200 ml

[1] Mention of trademark of proprietary product does not constitute a guarantee or warranty of product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may be suitable.

solution, and the entire experiment was replicated several times on different days. Extracts of the segments were prepared and assayed for invertase according to the standard procedures. The standard deviation among samples of segments incubated in water was 11%, which may be taken as the error in these measurements. The results of representative experiments are presented here.

RESULTS

Separation and Purification of the Two Invertases. The crude enzyme solution had an invertase activity of 510 units/ml, which corresponds to 180 units/g oat seedlings (fresh weight basis), and a protein content of 8.7 mg/ml. The enzyme solution remaining after the initial assays were performed (34 ml) was concentrated to 10 ml by ultrafiltration with an Amicon model 202 cell and a PM-10 membrane. The concentrated solution was dialyzed against 10 mM Tris (pH 7), and applied to a column of DEAE-cellulose (2.5 × 40 cm) previously equilibrated with 10 mM Tris (pH 7). Elution was then performed with 1 liter of a linear gradient of 10 mM to 0.50 M NaCl. A plot of invertase activity in the eluate *versus* fraction number showed a small peak eluted by approximately 50 mM NaCl and a larger peak eluted by 0.25 M NaCl (Fig. 1). The two enzymes were designated invertases I and II in the order of their elution off the DEAE-cellulose column. The fractions corresponding to each peak were combined, concentrated to 5 ml by ultrafiltration, and dialyzed against 0.15 M NaCl.

The invertases were then chromatographed on a column of Sephadex G-100 (2.5 × 95 cm) equilibrated with 0.15 M NaCl. The elution curves for the two enzymes are presented in Figure 2. The fractions with the highest activity were combined and concentrated to 5 ml by ultrafiltration. The specific activities of the purified enzymes were 180 and 3,670 units/mg protein for invertases I and II, respectively.

Effect of pH and Temperature. The activities of the purified invertases were determined with reaction mixtures maintained at pH values 3–7 by 0.08 M citrate-phosphate buffers. The pH optima for invertases I and II were approximately 4.3 and 5.0, respectively (Fig. 3). The sharp decrease in activity of invertase II as the pH was lowered below 4.5 reflected the instability of this enzyme at low pH. This was demonstrated by adjusting solutions of invertase II to various pH values and maintaining them at 0 C for 30 min before assaying at pH 5 and 30 C. The enzyme was stable down to pH 3.6 but was rapidly inactivated as the acidity increased, with a total loss of activity at pH 3. At 30, the inactivation of invertase

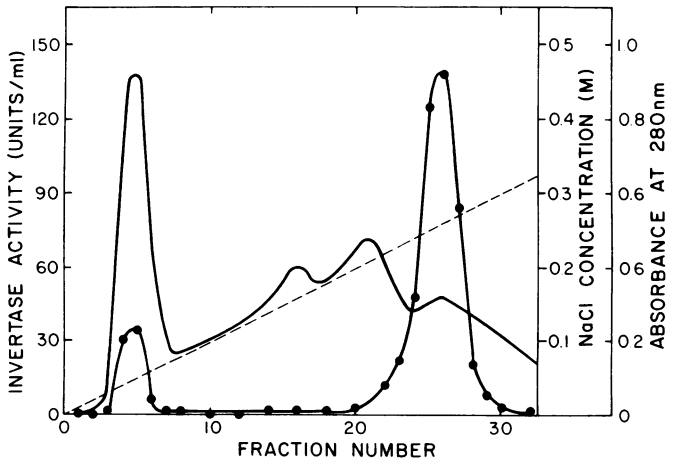

FIG. 1. Chromatography of crude invertase prepared from oat seedlings. Enzyme solution (10 ml) was applied to a column of DEAE-cellulose (2.5 × 40 cm) in 10 mM Tris (pH 6.5). Elution was conducted with 1 liter of linear gradient of 0 to 0.5 M NaCl (– – –), and 20-ml fractions were collected. Fractions were assayed for invertase (●——●) and protein (——).

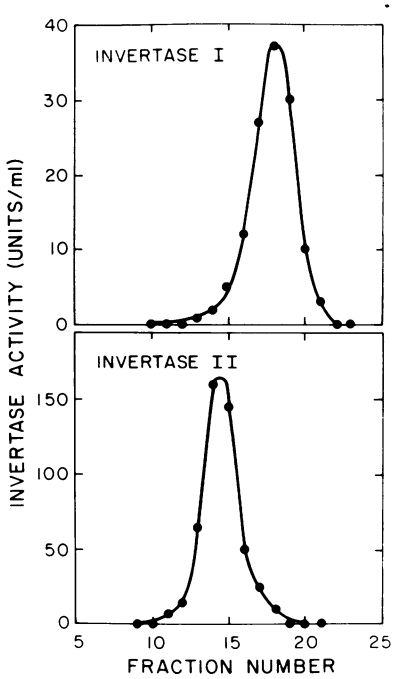

FIG. 2. Chromatography of oat invertases on Sephadex G-100. Enzyme concentrates (10 ml each) were applied to a column of Sephadex G-100 (2.5 × 95 cm) in 0.15 M NaCl. Eluent was 0.15 M NaCl, and 10-ml fractions were collected. Fractions were assayed for invertase according to the standard procedure.

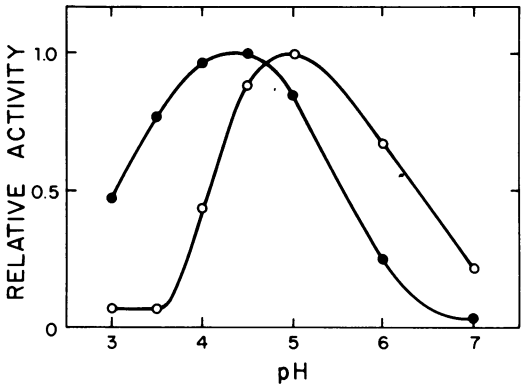

FIG. 3. Effect of pH on oat invertases. Reaction conditions were standard except that the buffer solution was 0.2 M citrate-phosphate at the pH values indicated. (○——○): Invertase I; (●——●): invertase II.

II was complete in less than 5 min at pH 3. The loss of invertase II at pH 3 could not be prevented by addition of BSA (2 mg/ml) or mercaptoethanol (2 mM) to the reaction mixture. At pH 5 and higher, invertase II was stable for at least 30 min at 30 C. Invertase I was much more stable than invertase II at all conditions tested. It retained its activity at pH 3 and 0 C for 30 min, and required heating to 47 C for 5 min at pH 4.5 for it to be 50% inactivated. A sample of invertase I was refrigerated for 4 weeks without appreciable loss of activity.

Substrate Specificity and Concentration. Values of K_m and V_{max} (relative maximum velocity) for the oat invertases acting on the substrates sucrose, raffinose, and stachyose were determined (Table I). The kinetic studies were conducted at pH 4.5 and 5.0 for invertases I and II, respectively. The reaction rates were measured at substrate concentrations of 0.58 to 146 mM sucrose, 1.7 to 34 mM raffinose, and 2.7 to 27 mM stachyose. The double reciprocal plots were linear for these substrate concentrations. Invertase I was characterized by lower K_m values than those of

Table I. *Michaelis Constants and Relative Maximum Velocities for Oat Invertases and Three Substrates*

Substrate	Invertase I		Invertase II	
	K_m	V_{max}	K_m	V_{max}
	mM		mM	
Sucrose	2.4	1.00	6.7	1.00
Raffinose	2.9	0.45	17	0.17
Stachyose	14	0.33	25	0.06

Table II. *Inhibitors of Oat Invertases*

Inhibitor	Inhibitor Concentration	Inhibition	
		Invertase I	Invertase II
	μM	%	
Aniline	10,000	45	0
Pyridoxal	1,000	65	25
Pyridoxine	2,500	70	20
Pyridoxamine	10,000	28	65
p-Chloromercuribenzoate	2	21	79
$HgCl_2$	2	55	100
$HgCl_2$	0.2	4	42
$AgNO_3$	20	0	44
I_2	4	9	100

invertase II and by similar values of K_m for the substrates sucrose and raffinose. The enzymes further differed by the rates at which V_{max} decreased as the size of the substrate increased from sucrose to stachyose, with a much smaller decrease for invertase I than invertase II. Neither enzyme hydrolyzed 0.5% inulin to a measurable extent during the 30-min incubation period.

Inhibitors of the Invertases. A variety of reagents known to inhibit other invertases were tested with the oat enzymes (Table II). Aniline, an inhibitor of yeast invertase, was relatively ineffective on the oat invertases. The magnitude and order of effectiveness of pyridoxal, pyridoxine, and pyridoxamine were the same as reported for potato invertase (12). Aniline, pyridoxal, and pyridoxine were more effective inhibitors of invertase I than invertase II. In contrast, pyridoxamine, *p*-chloromercuribenzoate, Hg^{2+}, Ag^{+}, and I_2 were much more effective on invertase II than on invertase I. The thiol-alkylating agents iodoacetate and iodoacetamide and the thiol-oxidizing agents iodosobenzoate and oxidized glutathione at a concentration of 400 μM did not inhibit the two oat enzymes. The following cations had no effect on the enzyme at a concentration of 2 mM: Mn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Th^{4+}. Potato invertase inhibitor (11) was also ineffective on the oat invertases when tested at a concentration that completely inhibited a comparable level of potato invertase.

Isoelectric Points. Isoelectric focusing of the purified invertases yielded single bands of activity for each enzyme. Invertase I was found in a band between 4 and 6 cm from the origin on the cathode side, and the peak corresponded to an isoelectric point of 8.6. Invertase II was found in a band between 3 and 5 cm from the origin on the anode side, and the peak corresponded to an isoelectric point of 4.4. The amounts of invertases I and II recovered were 78 and 66%, respectively.

Molecular Weights. A column of Sephadex G-100 (2.5 × 90 cm) in 0.15 M NaCl was calibrated with standard proteins (Cyt *c*, chymotrypsinogen A, ovalbumin, BSA monomer and dimer). From the elution volumes of the standards, the mol wt of invertases I and II were calculated to be 59,000 and 108,000, respectively.

Distribution of the Invertases in Oat Seedlings. The shoots of oat seedlings were cut at the nodes and separated into coleoptiles and mesocotyls. Each coleoptile was broken 1 cm below the apical tip by bending the coleoptile, and the segment was carefully pulled off without breaking the primary leaf. The leaf was then gently pulled from the remaining coleoptile. Two additional 1-cm segments were cut from the leaf-free coleoptile. A sample of the roots was obtained by shaking off the Vermiculite and rinsing with water. Equal weights (5.2 g) of the six samples of tissue were extracted and assayed for invertase according to the standard procedure. The highest invertase was found in the apical segment of the coleoptile (Table III), and the level of activity decreased sharply with distance from the apex. The total invertase activity was lowest in the leaf and root.

The extracts were then analyzed for the component invertases by chromatography on DEAE-cellulose. All of the samples contained two invertases corresponding to invertases I and II and their levels are summarized in Table III.

Oat seeds were also analyzed for invertase. The meal prepared with a Wiley mill from 25 g oats was extracted with 100 ml of 0.5 M NaCl according to the procedure for seedlings. The total invertase measured in the extract corresponded to only 4 units/g of oat seeds.

Changes in Invertase in Incubated Coleoptile Segments. A sample of coleoptile segments was stirred in water for 1 h and divided into three parts of 10 g each. One part was extracted immediately with 0.5 M NaCl. Another part was added to 200 ml water in a 500-ml Erlenmeyer flask, and the last portion was added to 200 ml of water containing 11.4 μM IAA. After 16 h in a water bath at 25 C with shaking at an adequate rate to keep them in suspension, the segments were rinsed with water and extracted with 0.5 M NaCl. The three extracts were assayed for total invertase activity and for the individual enzymes by chromatography on DEAE-cellulose. Representative results obtained in these studies are shown in Table IV. The total invertase activity decreased to about one-third of the initial level in the segments incubated in water. The addition of IAA to the water reduced the loss of invertase to over 60% of the initial level. The decrease in total invertase was due entirely to a decrease in the level of invertase II. The amount of invertase I in the segments did not change during incubation. Pectinesterase, polygalacturonase, and β-galactosidase also remained constant during the incubation, but

Table III. *Distribution of Invertases in Oat Seedlings*

Seedling Part	Activity		
	Total invertase	Invertase I	Invertase II
	units/g		
Leaf	43	20	18
Coleoptile, 1st cm	267	47	205
Coleoptile, 2nd cm	186	38	136
Coleoptile, 3rd cm	98	21	70
Mesocotyl	78	18	52
Roots	18	4	12

Table IV. *Invertase Activity in Coleoptile Segments Incubated in Water ± IAA for 16 h*

Sample	Weight	Invertase Activity		
		Total	Invertase I	Invertase II
	g	units		
No incubation	10.0	1980	340	1610
Incubated −IAA	12.2	680	320	340
Incubated +IAA	14.9	1240	350	830

Table V. *Effect of IAA Concentration on Loss of Invertase in Coleoptile Segments Incubated in Water for 16 h*

IAA Concentration	Final Weight	Total Invertase
μM	g	units
0	11.6	640
0.1	12.3	790
0.5	13.2	980
2.3	14.3	1,030
11.4	14.2	1,060
22.8	13.5	910

Table VI. *Effects of Growth-promoting and Growth-inhibiting Substances on Invertase in Coleoptile Segments*

Addition	Final Weight	Total Invertase
	g	units
None	11.9	690
11.4 μM IAA	14.6	1260
10.7 μM α-NAA	14.4	1190
10.6 μM IPA	13.9	1080
9.0 μM 2,4-D	13.7	1390
11.4 μM IAA + 20 mM $CaCl_2$	10.3	650
11.4 μM IAA + 200 mM mannitol	10.6	620

β-glucosidase increased about 20% in the presence of IAA (data not shown).

The optimum concentration of IAA for decreasing the loss of invertase in incubated coleoptile segments was between 2.3 and 11.4 μM (Table V). Other growth-promoting substances and inactive analogs were evaluated for effectiveness in preventing the loss of invertase (Table VI). α-NAA[2] and 2,4-D were approximately as effective as IAA, and IPA was somewhat less effective than the other auxins. In contrast, β-NAA, *p*-chlorophenoxyisobutyric acid, and fusicoccin did not affect the loss of invertase (data not shown).

The loss of invertase in segments incubated in water was not prevented by either 35 μM cycloheximide or 6 μM actinomycin D (data not shown). Carbonyl cyanide *m*-chlorophenyl hydrazone (12 μM) appeared to stabilize the invertase, but this reagent killed the segments, judging from their appearance and the abnormal level of carbohydrates released into the solution. The changes in killed segments were determined by freezing and thawing a sample of the segments before incubation in water. Even though some of the activity may have been solubilized by the freezing and thawing, 73% of the initial activity was still associated with the segments after 16-h incubation (data not shown).

The effects of growth-inhibiting concentrations of $CaCl_2$ and mannitol on invertase in incubated coleoptile segments were also determined (Table VI). $CaCl_2$ (20 mM) and mannitol (200 mM) reduced cell enlargement to almost zero with small effects on invertase in segments incubated in the absence of IAA. Both reagents inhibited auxin-stimulated growth of the segments and abolished the effect of IAA on preventing the loss of invertase activity. Similar results were obtained with 50 mM galactose in the absence and presence of IAA (data not shown).

The effect of pH on the loss of invertase activity was determined next. Oat segments were incubated at different pH values in 5 mM sodium citrate rather than water, and to prevent changes due to pH after segment homogenization, 0.1 M citrate-phosphate buffer (pH 5.5) containing 0.4 M NaCl rather than 0.5 M NaCl was used for extracting the samples. Almost all of the activity was lost in segments incubated at pH 3 for 16 h (data not shown). Those segments actually lost weight, and the solution contained a high concentration of soluble carbohydrates, as measured by the anthrone method (5). The extent of the loss in activity decreased as the pH of the incubating solution was raised. Much less activity was lost by incubation in citrate at pH 6 than in water. Similar results were obtained with a series of oat segments incubated in 2.5 and 5 mM potassium maleate solutions over the pH range of 3–6. The rate of invertase loss at pH 3 was rapid, with only 22% of the activity left after 2 h. In contrast, segments incubated for 2 h at pH 4 contained 73% of the initial activity, and segments incubated in water contained 91%.

[2] Abbreviations: NAA: naphthaleneacetic acid; IPA: indolepropionic acid.

DISCUSSION

We have demonstrated that the soluble invertase activity in oat seedlings is due to two enzymes. Both enzymes appeared to be acid invertases, with pH optima between 4 and 5, but they differ in a number of important properties. The marked differences in ionic properties and mol wt allowed separation of the enzymes by ion exchange chromatography, gel filtration, or isoelectric focusing. Both enzymes hydrolyzed sucrose, raffinose, and stachyose, but the rate of cleavage was more dependent on the substrate size for invertase II than invertase I. Invertase I was more effectively inhibited by aniline, pyridoxine, and pyridoxal, whereas invertase II was more effectively inhibited by *p*-chloromercuribenzoate and Hg^{2+}. The effectiveness of Hg^{2+} on invertase II is especially noteworthy, with complete inhibition by approximately 2 μM $HgCl_2$.

Jones and Kaufman (7) found two soluble invertases in stem segments from oat plants grown in the greenhouse for 40 to 45 days. They separated the invertases by chromatography on DEAE-cellulose and Sephadex G-200 columns. The oat stem enzymes were found to be acid invertases with pH optima near 5, but the oat stem and oat seedling invertases differed in several ways. The mol wt of one of the stem invertases was estimated to be about 300,000, whereas the larger seedling invertase had a mol wt of only 108,000. Both of the stem invertases were relatively stable at pH 3, in contrast to the instability of seedling invertase II at low pH. Furthermore, the oat stem invertases were characterized by considerably higher K_m values than those we determined for the seedling enzymes.

Segments of oat seedlings incubated in water lost about two-thirds of their total invertase after 16 h. This loss of total activity was due primarily to a decrease in invertase II, with only about 20% of initial level remaining after the incubation period. The low stability of invertase II at pH 4 and lower suggests that the loss of invertase may be partly attributed to physical inactivation of this enzyme. This is supported by the observation that inactivation was less when segments were incubated in solutions buffered at pH 5 and 6 than in water. Oat segments incubated in water are known to undergo acidification (3) and this could explain the loss of activity in unbuffered solutions. On the other hand, inactivation was retarded by IAA, which enhances the acidification of seedling segments (2).

The loss of invertase in incubated segments was decreased not only by IAA but also by other growth-promoting substances. But the growth hormones were ineffective in reducing the loss of invertase in the presence of 20 mM $CaCl_2$ or 200 mM mannitol which inhibit auxin-induced growth of the segments. Therefore, it appears that the retention of invertase in the segments is related to their growth rather than to a direct effect by auxin.

In oat seedlings the distribution of invertase activity, highest in the area near the apical region, indicates that invertase is associated with rapidly elongating cells in the intact plant. Similar distribution patterns of higher activity in the region of elongation have been described for pea seedlings (9) and for roots of pea (16), broad bean (14), and corn (6). Without knowing the intracellular location of the two oat invertases, we are unable to speculate on their roles. Presumably they are involved in translocation and utilization of sucrose from the reservoir of carbohydrate in the seed.

The situation appears to change when segments cut below the

apical tips of the seedlings are incubated in water. The transformations of sucrose probably consist of intracellular movement and utilization, and it is not unexpected that the composition of invertase changes. The decline of invertase II, even in the presence of IAA, indicates that this enzyme has a limited role in excised segments of oat seedlings. In contrast to our observation of invertase loss during incubation, Kaufman *et al.* (8) reported that invertase activity increased in segments cut from 45-day-old oat plants. They found that both gibberellic acid and sucrose enhanced the increase in invertase. Seitz and Lang (15) also reported a correlation between invertase and elongation of epicotyls of lentil seedlings incubated in Hoagland nutrient solution. However, they incubated essentially intact plants, with only the seed coat removed and the radicle shortened. Gibberellic acid enhanced the activity of invertase but sucrose had no effect on the lentil system.

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