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Purification and Some Properties of Cell Wall-bound Invertases from Sugar Beet Seedlings and Aged Slices of Mature Roots

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ABSTRACT

Cell wall-bound invertases (EC 3.2.1.26) from both sugar beet seedlings and aged slices of mature roots were purified to homogeneity separately with CM-cellulose chromatography and Bio-Gel P-150 gel filtrations. The enzymes behaved similarly throughout the purification procedures. The purified enzymes are identical as characterized by specific activity, gel electrophoretic mobility, K_m for sucrose and raffinose (1.33 and 4.0 millimolar, respectively), mobility on Bio-Gel P-150 (molecular weight 28,000), optimum pH (4.6 to 5.0), optimum temperature, and dependence on NaCl concentration for insolubilization by DNA. The results suggest that the enzymes may be encoded for by the same structural gene.

In the developing tissues of plants, high activity of invertase is detected at an early stage in which the rate of growth is high and sucrose content is very low (3, 4, 6, 13, 14). In maturing tissue in which sucrose accumulation is observed, the enzyme activity is hardly detectable. Aging of mature tissue slices under aerobic conditions leads to a remarkable increase in invertase activity (1, 2, 10, 13). In storage tissues of some plants, the development of invertase during aging is found by *de novo* synthesis, and not by activation of the enzyme (2, 11, 16). If invertases in the developing tissue and the enzyme induced by aging of mature tissue (a rather artificial treatment) are identical, presumably there must be a mechanism repressing invertase biosynthesis in mature tissue which is derepressed by aging. Elucidation of such a mechanism may give a clue to some role of invertase in sugar metabolism in storage tissues.

In sugar beet, two invertases, cytoplasmic and cell wall-bound forms, were present in both seedlings and aged slices of mature roots (6, 17). We have studied the cell wall-bound invertases in seedlings and aged slices (7-9).

To find out if these enzymes are the same, we purified them to homogeneity and determined some of their characteristics. Some evidence for this identity has been reported (6).

MATERIALS AND METHODS

Seedlings. Sugar beet (*Beta vulgaris* L.) seeds were germinated in moist Vermiculite at 26 C in the dark and 4-day-old seedlings were harvested.

Aging of Mature Roots. Mature sugar beets were harvested at the Experimental Farm at Obihiro University in late autumn and kept cool for about 2 months. The roots were washed thoroughly with running tap water, surface-sterilized with 0.1% NaOCl, and washed with water to remove the reagent. The roots were then cut into 2-mm thick slices about 3 cm^2, which were aged by placing them on filter paper moistened with distilled H_2O at 29 C for 18 h in the dark.

Preparation of Cell Wall. Seedlings (2.4 kg) or aged slices (5 kg) were homogenized with two volumes of distilled H_2O in a Waring Blendor and a Polytron (Kimematica). The cell wall fraction was collected by passing the homogenate through two layers of cheesecloth, washed with distilled H_2O, and suspended in 0.1% (w/v) sodium deoxycholate. The suspension was allowed to stand at 26 C for 2 h, and the cell wall fraction was washed thoroughly with distilled H_2O to remove deoxycholate. The resulting cell wall fraction was used for extraction of bound invertase.

Invertase Assay. The reaction mixture consisted of 30 mM sucrose, 20 mM citrate buffer (pH 5.0), and 300 μl of enzyme solution in a final volume of 1 ml. The mixture was incubated at 37 C for 10 min. The amount of reducing sugar liberated was determined according to the method of Somogyi-Nelson (15). One unit of invertase activity was defined as the amount of enzyme which catalyzed the production of 1 μmol of reducing sugar as glucose per min at 37 C.

Protein Estimation. Protein concentration was estimated by the method of Lowry *et al.* (5), using BSA as a standard. The protein concentration in the eluates from chromatography columns was determined by A at 280 or 230 nm.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed by the method of Reisfeld using pH 4.3 gel (15%) (12). The buffer used was β-alanine acetate (pH 4.5). The electrophoresis was carried out for 120 min at 5 mamp/tube. The gel was stained with Coomassie brilliant blue G-250.

Mol Wt Determination. The mol wt of the enzyme was determined by gel filtration on a Bio-Gel P-150 column (1.9 × 61 cm), previously equilibrated with 2 M NaCl. The markers used were BSA (mol wt 68,000), ovalbumin (mol wt 45,000), chymotrypsinogen (mol wt 25,000), and Cyt c (mol wt 12,400).

RESULTS AND DISCUSSION

PURIFICATION OF BOUND INVERTASE FROM SEEDLINGS AND AGED SLICES OF MATURE ROOTS

The purification procedures used with both materials were substantially the same.

Extraction. The cell wall preparation obtained from the seedlings (2.4 kg) or the aged slices of roots (5.0 kg) was suspended in 2.4 and 5.0 liters, respectively, of 1.0 M NaCl to release bound enzyme. After standing at 26 C for 15 h, the suspension was passed through two layers of cheesecloth, and then filtered again to remove solids.

Dialysis. The filtrate was dialyzed overnight against deionized H_2O resulting in a precipitate. The precipitate was collected by centrifugation, dissolved in a small amount of 2.0 M NaCl, and the solution centrifuged to remove insoluble matter.

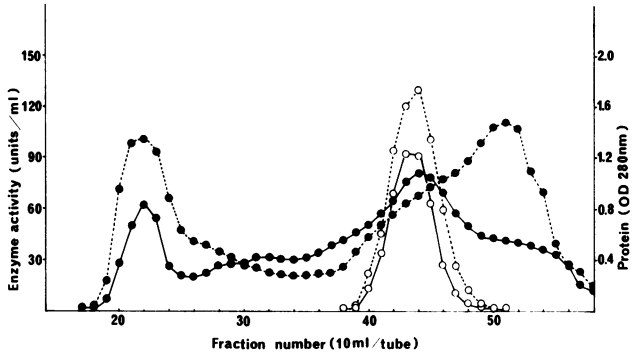

FIG. 1. Gel filtration of invertases from seedlings and aged slices of roots on Sepharose 6B. (○); Enzyme activity; (●), OD 280 nm; (——), seedlings; (- - -), aged slices.

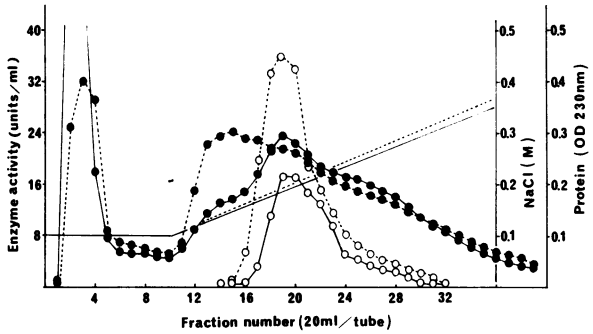

FIG. 2. CM-cellulose chromatography of the invertases. (○), enzyme activity; (●); OD 230 nm; (——, -----) NaCl (M). (——), seedlings; (-----), aged slices.

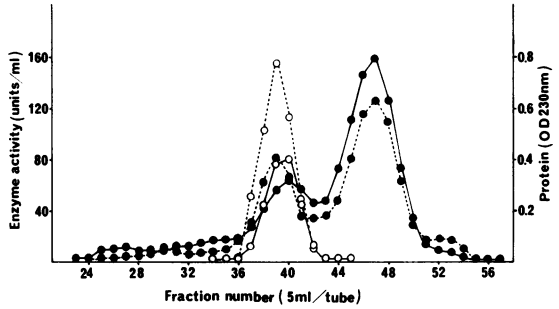

FIG. 3. First Bio-Gel P-150 gel filtration of the invertases. Symbols as in Figure 2.

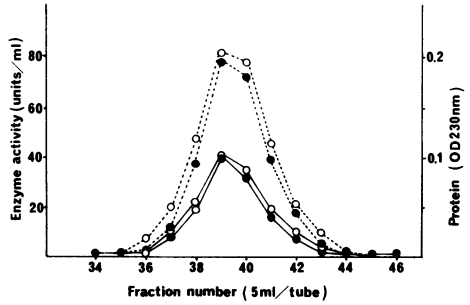

FIG. 4. Second Bio-Gel P-150 gel filtration of the invertases. Symbols as in Figure 2.

Sepharose 6B-Gel Filtration. The supernatant obtained was applied to a Sepharose 6B column (2.5 × 60 cm), equilibrated with 2.0 M NaCl, and the enzyme eluted with the same solution (flow rate about 35 ml/h) (Fig. 1). The active fractions were pooled and dialyzed against 0.1 M acetate buffer (pH 5.6) containing 0.1 M NaCl.

CM-Cellulose Column Chromatography. The dialyzed enzyme solution was applied to a CM-cellulose column (1.5 × 24 cm) equilibrated with 0.1 M acetate buffer (pH 5.6) containing 0.1 M

Table I. *Purification of Bound Invertases From Seedlings and Aged Root Slices*

Step	Volume	Protein	Activity	Specific Activity	Recovery
	ml	*mg*	*units*	*units/mg*	*%*
Bound Invertase from Seedlings.					
Crude extract	2,400	1,008	2,640	2.6	100
Ppt after dialysis	33	108	1,795	16.6	68
Sepharose 6B	40	40	1,504	37.6	57
CM-cellulose	120	6.7	502	74.7	19
First Bio-Gel P-150	20	0.78	290	371.8	10.9
Second Bio-Gel P-150	20	0.32	182	568.6	6.7
Bound Invertase from Aged Root Slices					
Crude extract	5,000	1,280	4,800	3.8	100
Ppt after dialysis	40	96	2,688	28.0	56
Sepharose 6B	54	63	2,208	35.0	46
CM-Cellulose	120	7.9	1,200	151.8	25
First Bio-Gel P-150	20	1.28	620	484.4	13
Second Bio-Gel P-150	20	0.63	380	603.2	7.9

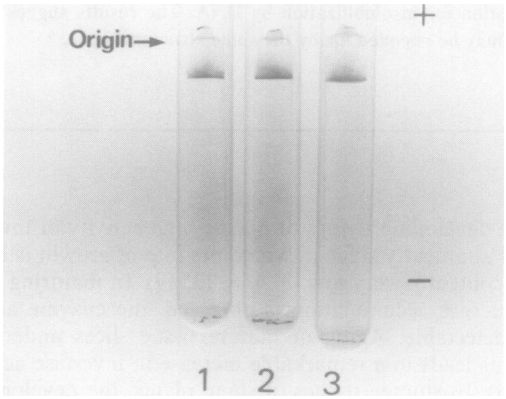

FIG. 5. Disc gel electrophoresis of invertases from seedlings and aged slices of roots, 1, Invertase from seedlings (40 μg); 2, a mixture of the two enzymes (each 20 μg); 3, invertase from aged slices of roots (40 μg). Electrophoresis was carried out as described.

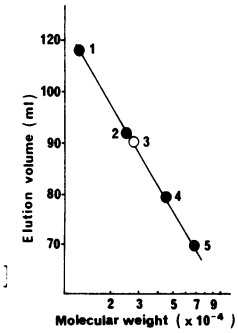

FIG. 6. Estimation of the mol wt of the purified invertases from seedlings and aged slices of roots, 1, Cyt *c*; 2, chymotrysinogen; 3, ovalbumin; 4, BSA.

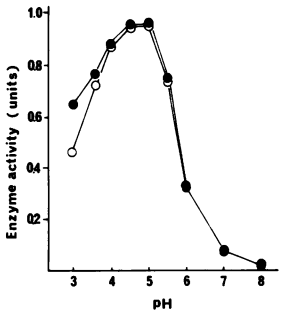

FIG. 7. pH dependence of invertase activities. (●), seedlings; (○), aged slices.

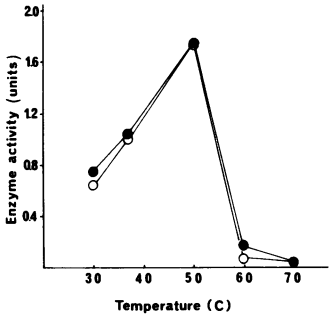

FIG. 8. Temperature dependence of invertase activities. Symbols as in Figure 7.

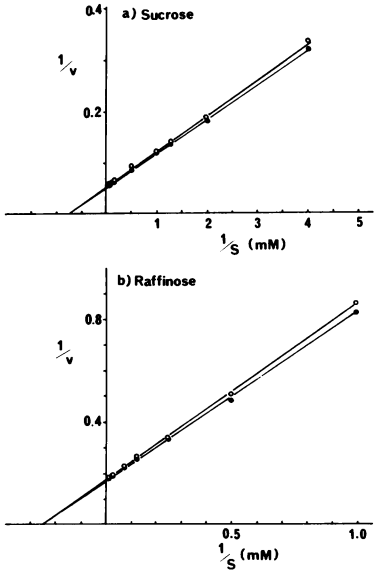

FIG. 9. Effect of substrate concentration on invertase activities. Symbols as in Figure 7.

NaCl. The column was first washed with the same buffer to remove the unadsorbed protein, and then eluted with a linear gradient from 0 to 0.5 M NaCl in 500 ml of buffer (flow rate 125 ml/h). Enzyme activity from both plant materials emerged at a NaCl concentration of about 0.18 M (Fig. 2).

First Bio-Gel P-150 Gel Filtration. The active fractions were concentrated to 7 ml in a collodion bag and dialyzed against 2.0 M NaCl solution. The dialyzed solution was applied to a Bio-Gel P-150 column (2.5 × 78 cm) previously equilibrated with 2.0 M NaCl and the enzyme was eluted with the same solution (flow rate about 15 ml/h) (Fig. 3).

Second Bio-Gel P-150 Gel Filtration. The active fractions were concentrated to 5 ml in a collodion bag and applied to the same

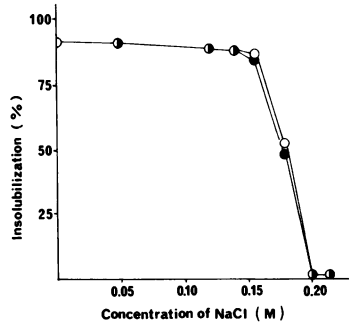

FIG. 10. Effect of NaCl concentration of insolubilization of the purified invertases in the presence of calf thymus DNA. Each mixture of 3 ml of purified enzyme solution and 2 ml of 1 mg calf thymus DNA was dialyzed overnight against 1 liter of various concentrations of NaCl. The activities in the supernatant and precipitate fractions after centrifugation of the dialysate were estimated. The percentage of insolubilization of the enzyme was represented by the ratio of activities in the precipitate fraction and the sum of the two fractions. (○); Invertase from seedlings; (●), invertase from aged slices.

column of Bio-Gel P-150, as described above. The enzyme was eluted under the same concentration of NaCl as above (Fig. 4). The peak showing enzymic activity was nearly symmetrical and paralleled the protein peak. The active fractions (Nos. 38–41) were pooled and used throughout the work. The specific activities of bound invertases of seedling and aged root slices were 586 and 603, respectively. The behaviors of both enzymes throughout purification procedures were quite similar.

The summaries of the purification procedures for both bound invertases are shown in Table I.

PROPERTIES OF THE TWO PURIFIED ENZYMES

Disc Electrophoresis. Both purified enzymes moved towards the cathode essentially as a single band on disc gel electrophoresis, and their mobilities were the same. The movement of the mixture of both enzymes also gave a single band (Fig. 5).

Mol Wt. The mixture of both purified enzymes eluted as a single peak from a Bio-Gel P-150 column with a mol wt of 28,000 (Fig. 6).

Optimum pH and Temperature. Activity pH curves of the two enzymes were similar in a pH range of 3.5–5.0 (Fig. 7). Optimum temperature for the reaction of both enzymes was 50 C (Fig. 8).

Effect of Substrate Concentration. The K_m values of the two enzymes for a given substrate were the same, 1.33 mM for sucrose and 4.0 mM for raffinose, respectively (Fig. 9). Based on V_{max} of the two enzymes, the ratio of turnover rate of sucrose to raffinose is 1:0.35.

Effect of DNA on Insolubilization of the Two Bound Invertases. It was previously reported that bound invertase of sugar beet seedlings was insolubilized by DNAs of sugar beet seedlings or other sources such as calf thymus, and that insolubilization depended on the NaCl concentration (7). The effect of NaCl concentration on insolubilization of purified invertases was then tested by using calf thymus DNA. Both enzymes were insolubilized at NaCl concentrations below 0.16 M (Fig. 10).

The present data indicate that bound invertases from the two materials which were quite different in growth stage were the same enzyme and may be coded for by the same structural gene. The availability of pure enzyme will allow us to investigate the role of this enzyme in sucrose metabolism.

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