

Wheat Invertases¹

CHARACTERIZATION OF CELL WALL-BOUND AND SOLUBLE FORMS

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ABSTRACT

Wheat coleoptiles have two distinct invertases, a soluble and a cell wall-bound form as indicated by results from cytochemical and biochemical studies. These enzyme activities differ in their pH optima, chromatographic behavior on diethylaminoethyl cellulose, kinetic properties, thermal stability, and response to light treatment. The soluble invertase was purified to near homogeneity by diethylaminoethyl-cellulose, concanavalin-A Sepharose, and Sephacryl S-300 chromatography. The overall purification was 175-fold with a recovery of about 26%. The holoenzyme has an apparent molecular weight of 158,000 and subunit molecular weight of 53,000 as estimated by polyacrylamide gel electrophoresis under denaturing conditions. Illumination of wheat seedlings caused an increase in the cell wall, but not the soluble, invertase activity.

MATERIALS AND METHODS

Buffer Solutions. The buffer solutions used were as follows: A, 20 mM sodium phosphate (pH 6.5), 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine; B, 10 mM sodium phosphate (pH 6.5), 1 mM EDTA, and 0.1 mM DTT; C, 100 mM sodium phosphate (pH 6.5), 1 mM MnCl₂, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM DTT, and 0.5 M NaCl; D, 50 mM sodium phosphate (pH 6.5), and 1 mM DTT.

Plant Material. Seeds of *Triticum aestivum* L. cv Yecovo Rojo were sown on two layers of filter paper moistened with distilled H₂O on Petri plates. One set was grown under continuous light and the other placed in total darkness at room temperature. The coleoptiles and roots were harvested after 4 d and analyzed for invertase activity as discussed below.

Invertase Extraction. Five g of tissue were homogenized with a mortar and pestle containing 30 ml of buffer A and then centrifuged at 3000g for 20 min. The resulting pellet was washed by repeated cycles of suspension in 30 ml of buffer A and centrifugation as described above. All supernatant fluids were combined and centrifuged at 31,000g for 20 min, and the clear supernatant fluid was designated as the soluble invertase fraction. The final pellet was resuspended in 30 ml of buffer A containing 1 M NaCl and shaken overnight at 4°C. The resuspended pellet was then centrifuged at 31,000g for 20 min, and the resulting supernatant fluid was designated as the cell wall enzyme fraction. Both the soluble and cell wall enzyme fractions were dialyzed separately against buffer B to remove endogenous reducing sugars.

Invertase Assay. Appropriate amounts of an enzyme preparation were added to a test tube containing 2 ml of 20 mM sucrose in 50 mM sodium acetate buffer, pH 5.5, and incubated at 37°C for 30 min. The reaction was terminated by adding 2 ml of Somagi reagent (17) followed by immersing the reaction mixtures in boiling water for 20 min. The copper reagent of Nelson's arsenomolybdate method (17) was used to measure the amount of reducing sugars produced. One unit of invertase is defined as that amount which will hydrolyze 1 μ mol of sucrose in 1 min.

Purification of Soluble Invertase. All purification steps were performed between 0 and 5°C. A crude soluble fraction was prepared from 190 g of wheat coleoptiles as described earlier. Enzyme grade solid (NH₄)₂SO₄ was added slowly to the crude extract with gentle stirring and brought to 75% saturation. The protein pellet obtained after centrifugation was dissolved in 150 ml of grinding buffer and dialyzed overnight against buffer B.

DEAE-Cellulose Chromatography. The concentrated enzyme solution was applied at a flow rate of 3 ml/min to a column of DEAE-cellulose (5 \times 15.5 cm) previously equilibrated with buffer B. The column was washed with 2 volumes of buffer and then subjected to a 1 L linear gradient, 0 to 0.5 M NaCl, prepared in buffer B. Seven-ml fractions were collected and assayed for invertase activity at pH 5.5. The fractions containing invertase

Invertases (β -D-fructofuranoside fructohydrolase, EC 3.2.1.26) have been isolated from several plant tissues, especially those engaged in active growth and development (1). This enzyme hydrolyzes sucrose and related sugars thereby providing hexoses which can be utilized for the energy and carbon requirements of the cell. In terminally differentiated tissue, invertase levels are markedly influenced by hormones (8-10), wounding (16), and light (8, 25). In several instances of infection by biotrophic pathogens (2, 3, 14), elevated invertase levels have been correlated with the accumulation of reducing sugars in diseased leaf tissue (14). Our own preliminary studies show a 3- to 4-fold increase in total invertase activity when wheat leaves are infected by the rust fungus, *Puccinia striiformis*. It is not clear, however, whether the host plant and/or the pathogen is responsible for the elevated levels of invertase activity (2, 3). As the first step in understanding the molecular basis of metabolic changes associated with diseased tissue, we have initiated a study on the wheat invertases, an enzyme extensively analyzed but not well characterized from higher plants. Here, we report on the presence of two invertase activities, a soluble and a cell-wall bound form which can be further distinguished by several biochemical criteria. The soluble form was extensively purified and its properties studied. This purified invertase will be useful in the generation of antibodies which in turn can be employed to determine the organismic origin of elevated invertase levels in diseased wheat tissue.

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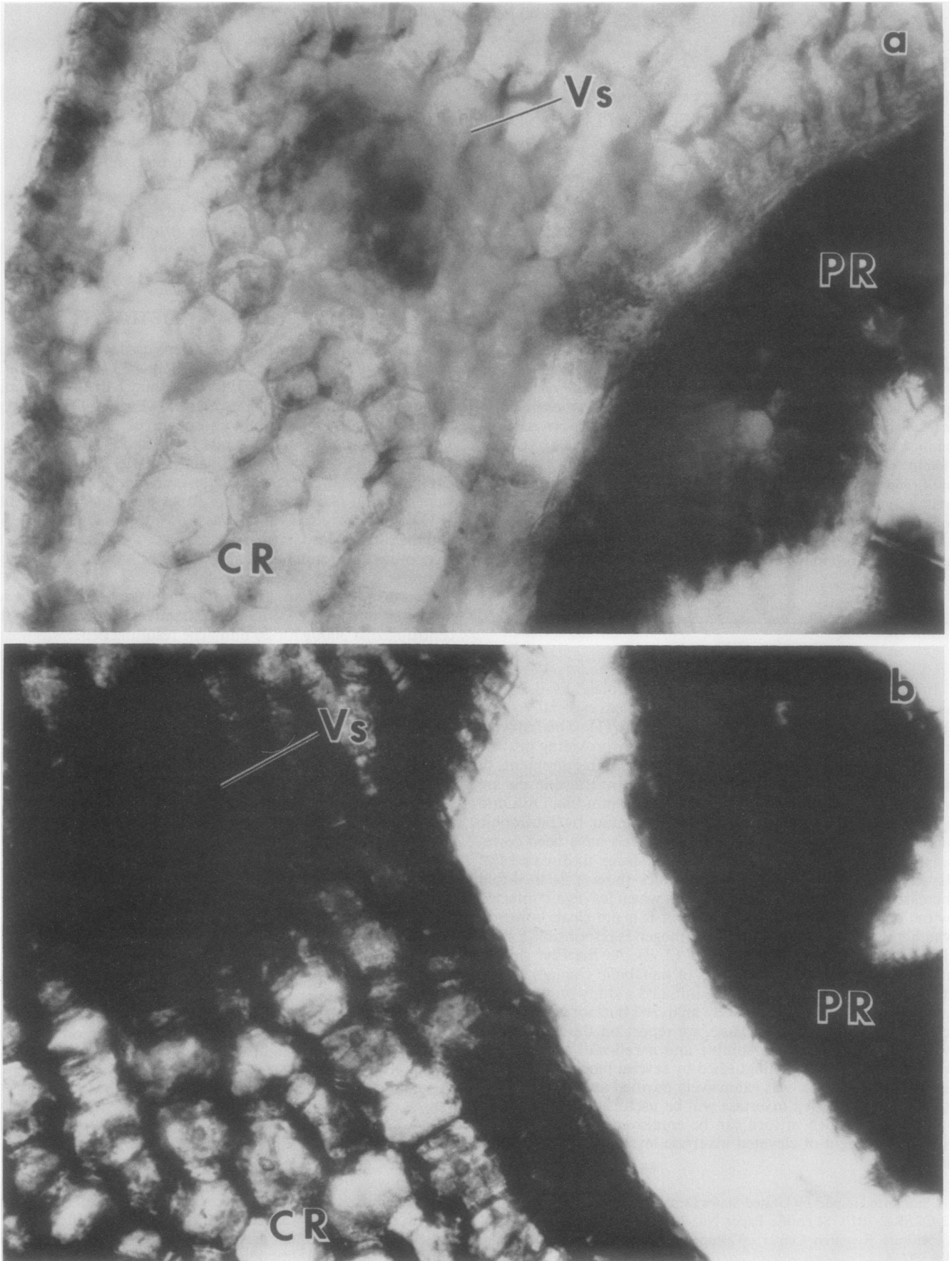


FIG. 1. Cytochemical localization of invertase activity in sectioned wheat coleoptiles. Sucrose was omitted (a) or included (b) in the reaction mixture. Invertase activity is localized predominantly in cells of the vascular tissue (Vs) and primary leaf (PR), while lower levels are detected in the cortex (CR).

activities were pooled and precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 70%. The resulting protein was collected by centrifugation, resuspended in 9 ml of 10 mM sodium phosphate, pH 6.5, and 1 mM DTT, and dialyzed overnight against buffer C.

Concanavalin A-Sepharose Affinity Chromatography. A Con-A³ Sepharose (Sigma Chemicals) column (1 × 16 cm) was equilibrated with buffer C. After sample application, the column was washed extensively with buffer C and then subjected to a linear gradient of 0 to 250 mM α -methyl manoside in buffer C. Fractions containing invertase activity were pooled and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation as above.

Sephacryl S-300 Gel Filtration. The concentrated enzyme solution was resuspended in buffer D and applied to a Sephacryl S-300 column (2 × 93 cm). The column was run at 2 cm/h and 2.5-ml fractions were collected. Fractions containing the invertase activity peak were pooled and concentrated by ultrafiltration using an Amicon model 52 cell with a PM-10 membrane. The Sephacryl S-300 column was calibrated by using thyroglobulin (669,000), ferritin (440,000), catalase (232,000) and aldolase (158,000) as mol wt standards. The void volume of the column was determined by using blue dextran 2000.

PAGE. SDS-PAGE was performed using the system of Laemmli (11) Proteins were separated on a 12% resolving gel (1 mm thick) at a constant power of 5 w. Mol wt standards lysozyme (14,300), β -lactoglobulin (18,400), α -chymotrypsinogen (25,700), ovalbumin (43,000), BSA (68,000), phosphorylase b (97,400), and myosin (H chain; 200,000) were run on adjacent lanes of the gel.

Cytochemical Localization of Invertase Activity. Free-hand sections of 4-d-old dark-grown coleoptiles were used for localization of enzyme activity. Invertase activity in the tissue sections were detected using a coupled glucose oxidation with nitroblue tetrazolium reaction (13).

Protein Determination. Protein was determined by the method of Lowry *et al* (15), using BSA as standard.

RESULTS

Cytochemical Localization of Invertase Activities. Free hand sections of wheat coleoptiles were analyzed for invertase activities by using a coupled glucose oxidation and nitroblue tetrazolium assay. Invertase activity, as indicated by the deposition of the insoluble formazan product, is clearly evident in all cells of the coleoptile (Fig. 1). Although the exact intracellular location cannot be determined with this procedure, invertase activity is associated with both the cell wall and cytoplasmic regions (Fig. 1B). Compared to the bulk parenchyma cells, relatively higher levels of invertase activity are found associated with the vascular bundles, supporting the role of this enzyme in sucrose translocation and utilization.

Presence of Two Types of Invertase in Wheat Coleoptiles. The soluble and cell wall-bound invertase activities were isolated and biochemically characterized. The soluble enzyme activity was readily extracted with 20 mM sodium phosphate (pH 6.5), whereas the cell wall activity remained associated with a 3000g pellet fraction. Treatment of the 3000g pellet fraction, however, with either 1 M NaCl or 1 M sodium phosphate buffer (pH 6.5) released more than 90% of the enzyme activity indicating that this form is bound to a particulate fraction presumably by ionic interactions. Both types of enzymes were found to be acidic in nature. The soluble form has a pH optimum of 5.5, while the cell wall form exhibits maximum activity at pH 4.5 (Fig. 2). The two forms also differ in their chromatographic behavior on

DEAE-cellulose as shown in Figure 3. The purified soluble enzyme elutes with approximately 200 mM NaCl (Fig. 3A) while the cell wall enzyme elutes with a higher salt concentration of about 350 mM (Fig. 3B).

The soluble and cell wall-bound enzymes also differ in their thermal stability. These enzyme activities were preincubated from 2 to 30 min at 50°C and then assayed with sucrose at 30°C. The purified soluble enzyme was more stable than the cell wall-

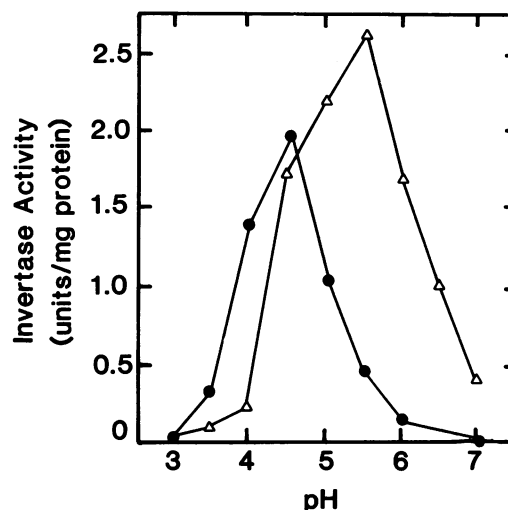


FIG. 2. Effect of pH on the activity of soluble (Δ) and cell wall-bound (\bullet) invertases.

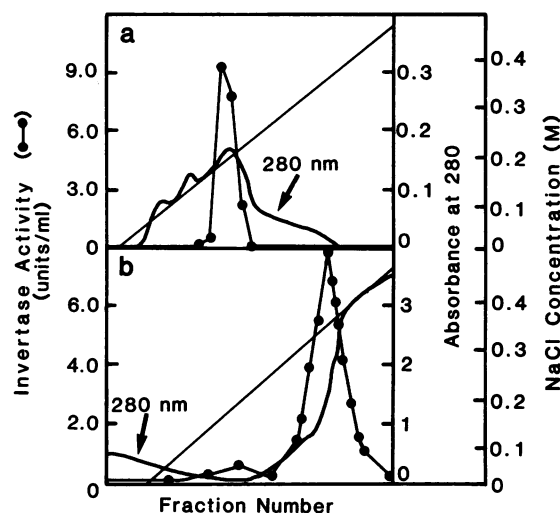


FIG. 3. Elution profile of soluble (a) and cell wall-bound (b) invertases obtained by DEAE-cellulose chromatography. Invertase activity was eluted from the column using a 0 to 0.5 M NaCl gradient.

Table 1. Effect of Light and Dark Treatment on Invertase Activity
One unit of invertase is defined as enzyme activity that will hydrolyze 1 μ mol sucrose/min.

Treatment	Invertase Activity	
	Soluble enzyme	Cell wall enzyme
	<i>units/mg protein</i>	
Dark-grown coleoptiles	2.02 \pm 0.11	0.95 \pm 0.04
Light-grown coleoptiles	1.80 \pm 0.15	1.52 \pm 0.06
Dark-grown roots	1.51 \pm 0.09	0.78 \pm 0.07
Light-grown roots	1.17 \pm 0.10	1.45 \pm 0.21

³ Abbreviations: Con-A, concanavalin A; DTNB, dithiobis 2-nitrobenzoic acid; PCMB, *p*-chloromercuribenzoate; PCMS, *p*-chloromercuriphenyl sulfonic acid; NEM, *N*-ethylmaleimide.

Table II. Purification of Invertase

One unit of invertase is defined as enzyme activity that will hydrolyze 1 μ mol sucrose/min.

Step	Total Protein mg	Total Activity units	Specific Activity units/mg protein	Yield %	Purification Factor -fold
(NH ₄) ₂ SO ₄ precipitation, 75% saturation	1114.8	8315	7.5	100	1.0
DEAE-cellulose	336.1	5987	17.8	72	2.3
Con-A	4.73	4211	890.3	50.6	119.3
Sephacryl S-300	1.68	2192	1305.0	26.4	174.9

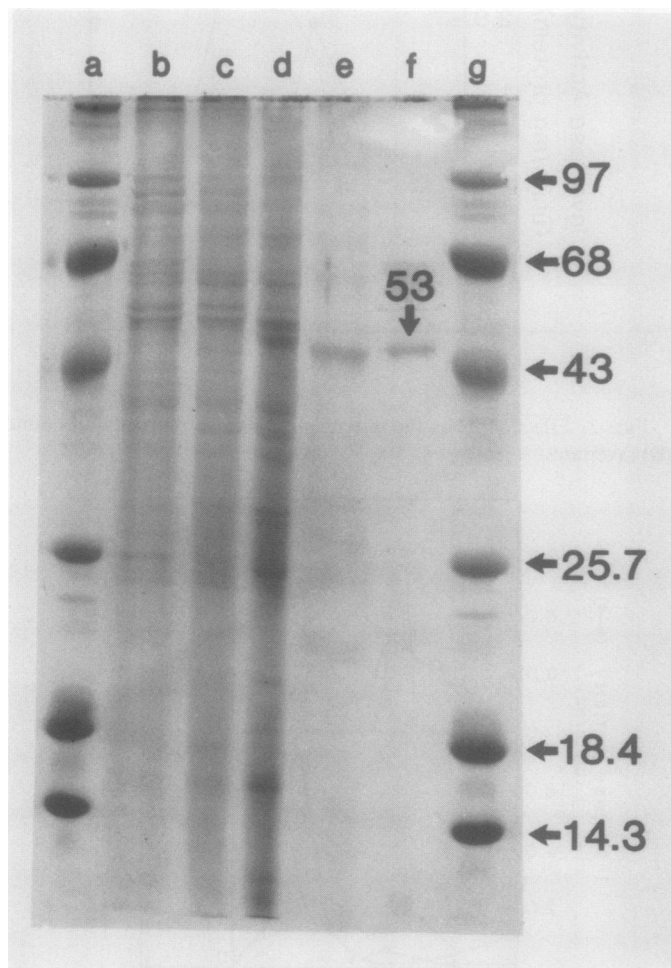


FIG. 4. SDS-PAGE of protein fractions obtained during the purification of soluble invertase. Lanes a and g, mol wt protein standards; lane b, crude extract; lane c, 75% (NH₄)₂SO₄ pellet; lane d, DEAE-cellulose fraction; lane e, Con-A Sepharose fraction; lane f, Sephacryl S-300 fraction.

bound form and retained 60% of its maximal activity after 4 min at 50°C, whereas the cell wall enzyme, obtained after DEAE-cellulose chromatography, retained only 20% under the same conditions. Similar differences in heat lability were observed when the two enzyme activities were preincubated for 5 min at temperatures ranging from 37° to 70°C (results not shown).

Table I depicts the invertase activities of 4-d-old wheat seedlings grown under continuous light or complete darkness. Light-grown coleoptiles and roots exhibited increased levels (60–90%) of cell wall enzyme activity as compared to that observed in dark-grown seedlings. In light-grown tissue, the levels of the soluble enzyme were slightly less than the levels found in the

dark-grown tissue. Hence, illumination has a pronounced influence on the distribution of the cell wall-bound and soluble forms and lesser effect on the total invertase activity detected in these tissues. Inasmuch as the soluble enzyme fraction represents a major portion of the total enzyme activity, it was extensively purified and characterized as discussed below.

Purification and Properties of the Soluble Invertase. Table II presents a purification summary of the soluble invertase activity from wheat coleoptiles. The overall purification was 175-fold with a recovery of about 26% from the starting material. A major step in the purification scheme was the utilization of Con-A-Sepharose affinity chromatography which alone resulted in about 100-fold purification. The invertase activity, which was initially retained on the Con-A column, was eluted as a single peak with approximately 100 mM α -methylmannoside. The resulting invertase peak was precipitated by (NH₄)₂SO₄ and further purified by gel filtration on a Sephacryl S-300 column, previously calibrated with mol wt protein standards. The enzyme eluted in a column volume corresponding to a mol wt of 158,000. Analysis of this protein peak by SDS-PAGE indicated a major polypeptide band at 53,000 D with several faint bands of larger and smaller mol wt (Fig. 4).

Substrate Specificity. The purified soluble enzyme readily hydrolyzes sucrose and raffinose but not melibiose and melizitose to a measurable extent in a 30-min incubation period. Therefore, based on substrate specificity this enzyme is a β -fructofuranosidase. The enzyme has a K_m of 3.5 mM and V_{max} of 307.7 μ mol/min for sucrose and a K_m of 19.6 mM and V_{max} of 162.6 μ mol/min for raffinose as a substrate. In contrast, the cell wall-bound enzyme has an apparent K_m of 1.7 mM for sucrose.

Inhibitors of the Invertase Activity. Several reagents known to inhibit plant invertases (20, 23) were tested against the purified soluble invertase from wheat coleoptiles. AgNO₃ and I₂ at 4 μ M concentration and HgCl₂ at 2 μ M concentration completely abolished invertase activity. Pyridoxal (1 mM), pyridoxine (2.5 mM), and pyridoxamine (10 mM) also inhibited invertase activity by 15, 24, and 85%, respectively. DTNB, PCMB, PCMS, and NEM, which react with sulfhydryl groups, were also employed in these studies. PCMB and PCMS (100 μ M) completely inactivated the enzyme while at the same concentration DTNB and NEM had no effect on enzyme activity under the same assay conditions. The activity of the soluble invertase was unaffected by CaCl₂, MgCl₂, CoCl₂, EDTA, or cysteine.

DISCUSSION

The results of our study indicate that there are two forms of invertase activity in wheat coleoptiles. These two forms can be distinguished by their pH optima, chromatographic behavior on DEAE-cellulose, intracellular location, K_m values, thermal stability, and in their response to light treatment. Both activities are acid invertases in that the crude and purified preparations of the soluble and cell wall-bound forms exhibit pH optima of 5.5 and 4.5, respectively. In this respect, invertase from wheat coleoptiles differ from onion (12), sugar cane (7, 22), and carrot (21) which

possess both acid and neutral invertases. The cell wall enzyme appears to be bound to the particulate fraction due to ionic interactions since it is readily solubilized with high salt solutions. Radish seedlings contain, in addition to the ionically bound form, another invertase activity presumably covalently bound to the cell walls (5). Immunochemical studies, however, indicate that the soluble, ionically bound, and covalently bound invertase forms are antigenically similar and showed close structural relationships (5). Thus, it is not clear whether these different invertase forms found in plant cells are products of multiple genes or a single gene as observed for yeast invertases (19).

We also find in this study that the levels of the cell wall-bound invertase activity are enhanced by light treatment. Similar observations have been recorded for radish hypocotyls (26, 27). It has been suggested that light via phytochrome induces a transfer of the radish enzyme from the cytosol to the cell wall. Therefore, a possible involvement of phytochrome in the increase of the cell wall invertase activity in wheat coleoptiles is not excluded.

The soluble invertase activity was purified 175-fold, representing more than 70% of the final protein. The purified enzyme is probably a glycoprotein based on its ability to bind to the lectin Con-A. This is consistent with other studies (4, 18, 23) where it has been shown that invertase is a glycoprotein. The precise role of the carbohydrate moiety of the enzyme is not well understood since it is not necessary for enzymic activity or stability (4). It has been proposed, for radish seedlings, that changes in the carbohydrate moiety may result in charge modification of the enzyme and thereby affect the wall-binding capacity of the secreted forms (6).

The soluble invertase form is completely inactivated when preincubated for 5 min with 100 μ M PCMB and 100 μ M PCMS. This result is consistent with studies of lily pollen invertases where the involvement of a sulfhydryl group in the enzyme's catalytic site has been suggested (23). However, 100 μ M DTNB and 100 μ M NEM, reagents known to react with sulfhydryl groups, failed to inhibit the wheat invertase activity to any measurable extent. This suggests that either sulfhydryl groups do not participate directly in the catalytic process or these groups were inaccessible to DTNB and NEM under our assay conditions.

The subunit mol wt of the soluble enzyme was estimated by SDS-PAGE to be about 53,000. Results obtained by gel filtration showed an apparent mol wt of 158,000, indicating that wheat invertase is a multimeric protein. There is a considerable variability in the mol wt of plant invertases, and they range from 48,500 (4) to 450,000 (23). It should be pointed out, however, that the glycosylated nature of this enzyme may lead to a lack of precision in estimating the mol wt. For example, ovomucoid, a glycoprotein with 27% carbohydrate content and a molecular weight of 27,000, when subjected to gel filtration on Sephadex reveals a mol wt of 38,000 to 45,000 (24).

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