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Purification and Characterization of Soluble (Cytosolic) and Bound (Cell Wall) Isoforms of Invertases in Barley (*Hordeum vulgare*) Elongating Stem Tissue[1]

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ABSTRACT

Three different isoforms of invertases have been detected in the developing internodes of barley (*Hordeum vulgare*). Based on substrate specificities, the isoforms have been identified to be invertases (β-fructosidases EC 3.2.1.26). The soluble (cytosolic) invertase isoform can be purified to apparent homogeneity by diethylaminoethyl cellulose, Concanavalin-A Sepharose, organo-mercurial Sepharose, and Sephacryl S-300 chromatography. A bound (cell wall) invertase isoform can be released by 1 molar salt and purified further by the same procedures as above except omitting the organo-mercurial Sepharose affinity chromatography step. A third isoform of invertase, which is apparently tightly associated with the cell wall, cannot be isolated yet. The soluble and bound invertase isoforms were purified by factors of 60- and 7-fold, respectively. The native enzymes have an apparent molecular weight of 120 kilodaltons as estimated by gel filtration. They have been identified to be dimers under denaturing and nondenaturing conditions. The soluble enzyme has a pH optimum of 5.5, K_m of 12 millimolar, and a V_{max} of 80 micromole per minute per milligram of protein compared with cell wall isozyme which has a pH optimum of 4.5, K_m of millimolar, and a V_{max} of 9 micromole per minute per milligram of protein.

Invertase (EE 3.2.1.26) is responsible for the hydrolysis of photosynthate sucrose to D-glucose and D-fructose. The level of this enzyme has been found to be regulated by GAs[2] (4, 5) as well as by gravity (7). In GA_3-treated oat stem segments and gravistimulated leaf-sheath pulvini of barley the invertase activity is induced within 6 h and continues to increase over 48 h.

Although plant invertases have been examined in a number of plants (1–5, 8, 12, 15, 16–18) and yeast (14), little is known about their biochemical structure, the genes responsible for their synthesis, and the mechanism by which hormones regulate their expression. Therefore, investigating the enzyme invertase will contribute significantly to our understanding of the mechanism underlying elongation growth in stems (4, 5) and asymmetric growth in graviresponding organs (roots and shoots) (6).

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[2] Abbreviations: GAs, gibberellins; Con A, concanavalin A; BME, β-mercaptoethanol; DTNB, dithiobis-2-nitrobenzoic acid; NEM, N-ethylmaleimide; SST, sucrose-sucrose transferase.

As a first step of such an investigation, we have attempted to purify the invertases which are predominantly found in intercalary meristem tissue at the bases of elongating barley internodes. In the present investigation, two isoforms of invertase, cytosolic and cell-wall bound, were extensively purified and some of their chemical properties investigated.

MATERIALS AND METHODS

Growth and Harvest of Barley Plants

Barley (*Hordeum vulgare* cv 'Larker') plants were grown to age 42 to 45 d under the following regime: 16 h light at 22.5°C and 8 h dark at 16.5°C. P-1 (next-to-last) internodes, 1 to 2 cm in length, were harvested and kept frozen at −80°C.

Invertase Assay

The enzymatic reaction was carried out in a total volume of 0.5 mL, containing 50 mM sodium acetate buffer (pH 5.5 for the soluble isoform and pH 4.5 for the bound isoform). The reaction mixture was incubated for 30 min at 30°C and terminated by the addition of 0.5 mL of Somogyi reagent (13). The reducing sugars formed were detected by Nelson's reagent (13) and quantitated by measurement of absorbance at 550 nm. One unit of invertase is defined as that amount which will hydrolyze 1 μmol of sucrose in 1 min at 30°C at a specified pH.

Extraction and Purification of Soluble Invertase

Approximately 50 g of p-1 internodal tissue were homogenized with a Waring blender in 300 mL of 10 mM sodium phosphate buffer (pH 6.0) containing 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 1 mM benzamidine, hereafter referred to as buffer A. The homogenate was squeezed through cheesecloth to collect the filtrate. The residue was suspended in half the original volume of buffer A and the whole process repeated two more times. The three filtrates containing the soluble invertases were pooled together and subjected to the purification steps of Krishnan *et al.* (8) with the following modifications.

While Krishnan *et al.* (8) preceeded to DEAE-cellulose chromatography, we have introduced the following changes in protocol to remove most of the Chl and some other proteins which seem to interfere with DEAE-cellulose chromatogra-

Table I. *Steps in the Purification of the Soluble Isoform of Barley Invertase*

Fraction	Total Protein	Total Activity	Specific Activity*	Purification Factor	Yield
	mg	*units**	*units·mg⁻¹ protein*		%
Homogenate	337	411.3	1.2	1.0	100
DEAE-cellulose	90	284.2	3.2	2.6	69
Con-A sepharose	10	187.5	18.8	15.4	45.6
Organo-mercurial Sepharose	2.5	148.5	59.4	48.6	36.1
Sephacryl S-300	1.8	135	75.0	61.4	32.8

* One unit of invertase activity is defined as the amount of enzyme that will hydrolyze 1 μmol of sucrose per min at 30°C at a pH of 5.5.

Table II. *Steps in the Purification of Cell Wall-Bound Isoform of Barley Invertase*

Fraction	Total Protein	Total Activity	Specific Activity*	Purification Factor	Yield
	mg	*units**	*units·mg⁻¹ protein*		%
Homogenate	46	57	1.239	1	100
DEAE-cellulose	22	35	1.6	1.3	61
Con-A sepharose	5	15	3	2.42	26
Sephacryl S-300	1	9	9	7.0	15.8

* One unit of invertase is defined as that amount of enzyme that will hydrolyze 1 μmol of sucrose per min at 30°C at a pH of 4.5.

phy. Hence, the pooled filtrates were treated by the slow addition of solid PVP to a final concentration of 1.0%, while stirring at 4°C. This suspension was stirred for an additional 30 min at 4°C and centrifuged at 30,000*g* for 20 min.

In the procedure of Krishnan *et al.* (8) the protein solution containing the soluble invertase was concentrated by the addition of solid $(NH_4)_2SO_4$ and dialysed prior to each of their respective purification steps. However, in the present work, the fractions containing the invertase activity were subjected directly to the respective chromatographic steps, except where noted. Finally, since plant invertases have been previously shown to contain sulfhydryl groups (8), and since preliminary studies indicated the presence of sulfhydryl groups in barley invertase, an additional purification step of organo-mercurial Sepharose affinity chromatography was introduced into the purification protocol.

DEAE-Cellulose Chromatography

The supernatant was applied at a flow rate of 5 mL·min^{-1} to a 1.5 × 20 cm column of DEAE-cellulose (Sigma Chemical Co., St. Louis, MO) previously equilibrated with buffer A. The column was washed with 2 to 3 bed volumes of buffer A, and then eluted with a linear gradient of 0 to 0.5 M NaCl (pH 6.0) in a total volume of 500 mL. 3.5 mL fractions were collected and 100 μL aliquots assayed for invertase activity at pH 5.5. Fractions containing invertase activity were pooled and then subjected to lectin (Con A)-Sepharose affinity chromatography.

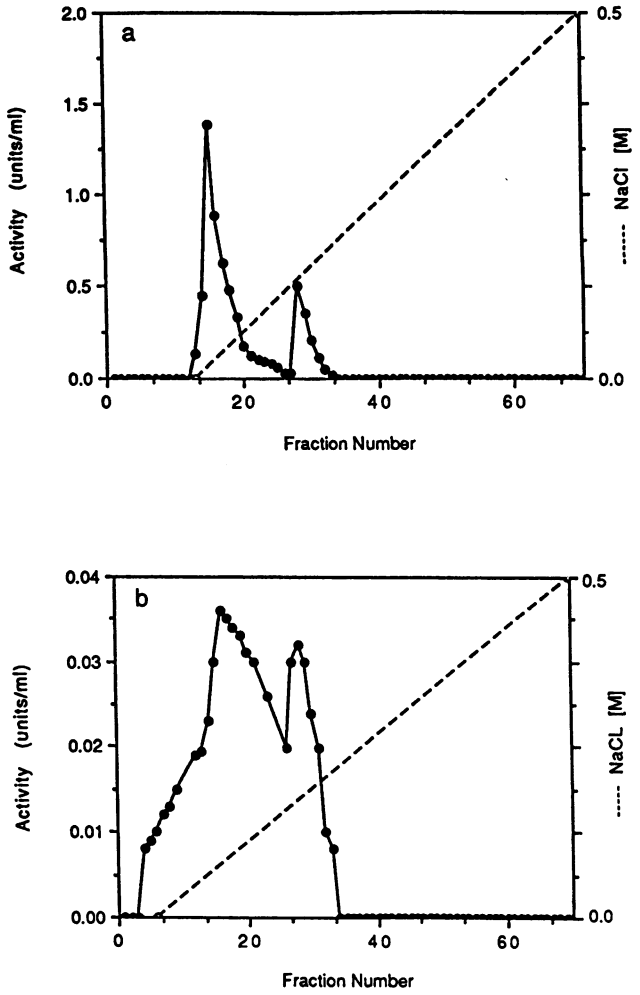

Figure 1. DEAE-cellulose anion exchange chromatography of soluble (a) and cell wall (b) isoforms of barley invertase; 3.5 mL was collected per tube from a linear gradient of 0 to 0.5 M NaCl in a total volume of 500 mL.

Con A-Sepharose Affinity Chromatography

A 1 × 16 cm chromatography column was packed with Con A-Sepharose (Sigma Chemical Co., St. Louis, MO) and equilibrated with sodium phosphate buffer (pH 6.0) containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.5 M NaCl. Fractions containing invertase activity from the previous step

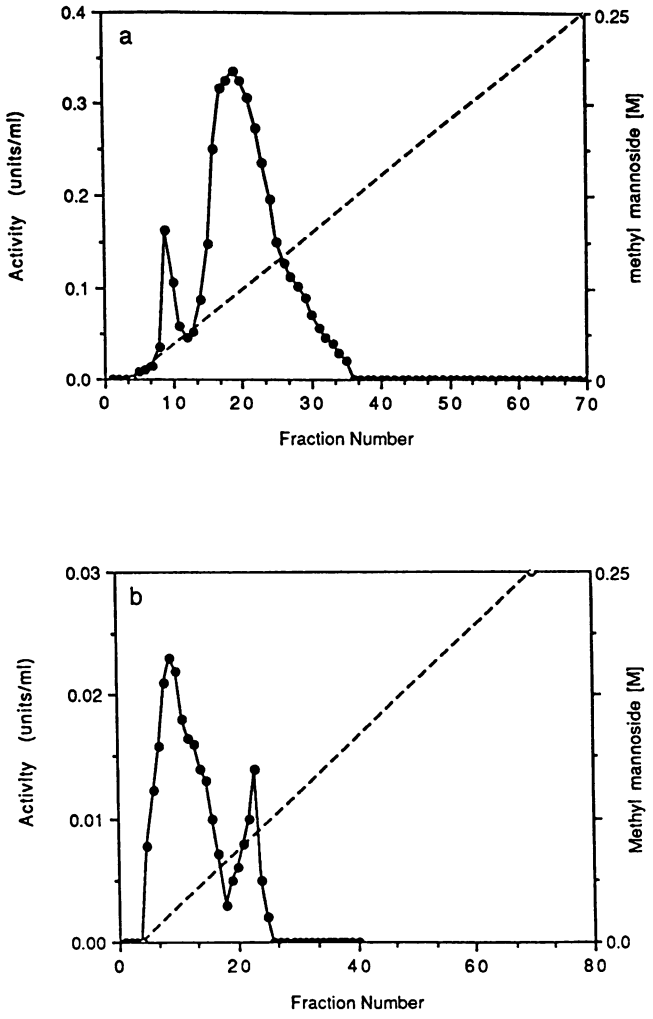

Figure 2. Con A Sepharose affinity chromatography of soluble (a) and cell wall (b) invertase isozymes of barley; 3.5 mL was collected per tube from a linear gradient of 0 to 0.25 M methyl mannoside in a total volume of 250 mL.

were pooled and applied to the affinity column at a flow rate of 1 mL·min^{-1}. The column was then washed extensively with the same buffer and eluted with a linear gradient between 0 and 250 mM α-methyl mannoside in a total volume of 250 mL. Fractions containing invertase activity were pooled and subjected to organo-mercurial Sepharose affinity chromatography.

Organo-Mercurial Sepharose Affinity Chromatography

Approximately 3 mL of an organo-mercurial derivative of Bio-gel (Sepharose) (Bio-Rad Laboratories, Richmond, CA) was washed and equilibrated with 20 mM sodium phosphate buffer, containing 0.1 mM PMSF, and 1 mM benzamidine in a 0.8 × 2 cm column. Pooled fractions from the previous chromatography step were applied to the organo-mercurial Bio-gel affinity column and washed extensively with the equilibrating buffer. The invertase on the column was then eluted by resuspending the gel within the column with the same buffer, containing 10 mM DTT, and shaking gently overnight

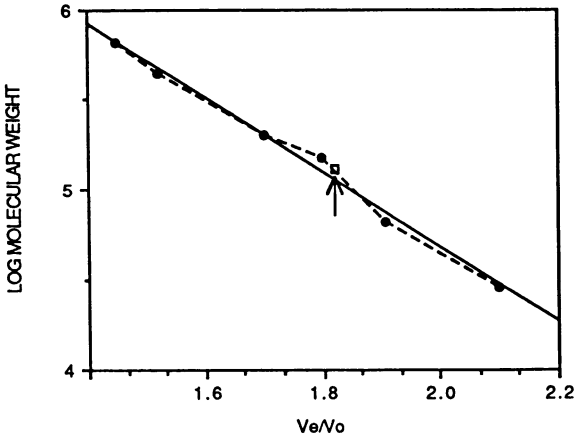

Figure 3. Elution profile of standard mol wt markers chromatographed on a Sephacryl S-300 column. Standard mol wt markers include: thyroglobuline, bovine (669 kD); apoferritin, horse speen (443 kD); β-amylase, sweet potato (200 kD); alcohol dehydrogenase, yeast (150 kD); albumin, bovine serum (66 kD); carbonic anhydrase, bovine erythrocytes (29 kD). Void volume was determined with Blue Dextran. Arrow indicates Ve/Vo values obtained for soluble and cell wall bound invertases. All gel filtration mol wt markers were obtained from Sigma Chemical Co. (St. Louis, MO).

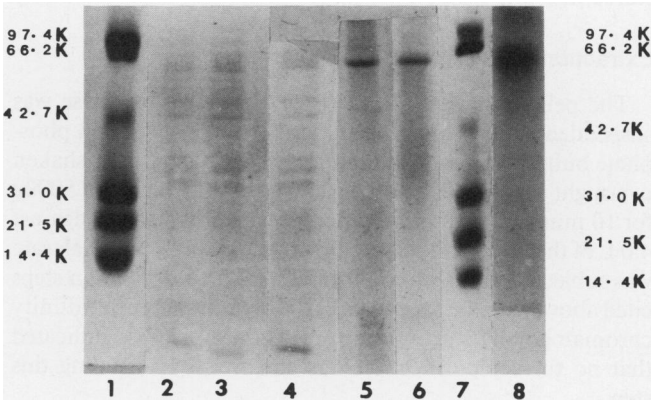

Figure 4. SDS-PAGE of protein fractions obtained at each stage of purification of (a) soluble barley invertase: lane 2, crude extract; lane 3, DEAE-eluate; lane 4, Concanavalin-Sepharose eluate; lane 5, Organo-mercurial Sepharose eluate; lane 6, Sephacryl S-300 fraction; (b) cell wall invertase: lane 8, Sephacryl S-300 fraction. Lanes 1 and 7 indicate standard protein mol wt markers.

at 4°C. The next morning, the eluate was collected by allowing the gel to repack within the same column under gravity. Then, the gel was washed once with a minimal volume of the elution buffer and the two eluates were pooled. The enzyme was then concentrated by adding solid $(NH_4)_2SO_4$ while stirring, to 75% saturation. The resulting precipitate was collected by centrifugation.

Sephacryl S-300 Gel Filtration

The concentration enzyme was resuspended in a total volume of 3 mL of 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM DTT and applied to a 2 × 95 cm column of Sephacryl S-300 previously equilibrated with the same

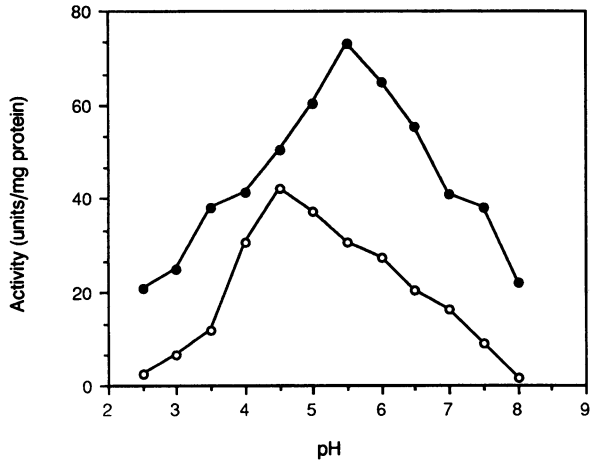

Figure 5. Effect of pH on the activity of soluble (●) and cell wall-bound (○) invertases. Note the cell wall invertase activity has been multiplied by a factor of 5 in order to distinguish the pH optimum.

buffer. The column was then eluted with the above buffer at a flow rate of 2 $cm \cdot h^{-1}$ and 2 mL fractions were collected. Fractions containing invertase activity were pooled and concentrated by dialyzing against 40% glycerol for 4 to 6 h and stored in 1 mL aliquots at −80°C.

Extraction of Cell Wall-Bound Invertase

The pellet obtained after extracting soluble invertase was suspended in approximately 300 mL of 20 mM sodium phosphate buffer (pH 6.0) containing 1 M NaCl and gently shaken overnight at 4°C. The suspension was centrifuged at 5000*g* for 10 min and the supernatant collected and dialyzed against 4.0 L of the same buffer for 6 h with one change. The dialysate was subjected to the same chromatographic purification steps cited above with the exception of the organo-mercurial affinity chromatography step, because preliminary studies indicated that no further purification was achieved by including this step.

SDS-PAGE

Vertical slab gel electrophoresis of protein samples obtained at the various stages of purification was carried out according to the method of Laemmli (9).

Protein Estimation

Total protein concentration was determined according to the procedures of Lowry *et al.* (11) using BSA fraction V as the standard.

RESULTS

Purification and Properties of Soluble (cytosolic) and Bound (cell-wall) Invertases

Tables I and II summarize the steps used to purify the predominant soluble and cell wall-bound isoforms of invertase in barley internodal intercalary meristem tissue. The overall purification of the soluble isoform is approximately 60-fold in comparison to the cell wall-bound isoform, which is only about 7-fold. The soluble invertase isozyme can be purified to apparent homogeneity by employing all the purification steps shown in Table I. The cell wall invertase can be released with 1 M salt (NaCl) solution and purified further by employing the same purification procedure used by cytosolic invertase except for omitting the organo-mercurial Sepharose affinity chromatography step. Considerable invertase activity could still be detected in the remaining pellet after solubilization with 1 M salt.

Two peaks for the soluble and cell wall invertase isoforms were observed by DEAE cellulose anion-exchange chromatography (Fig. 1). Similarly, two peaks were again observed of both forms when the eluate from the anion exchange column was subjected to Con A Sepharose affinity chromatography (Fig. 2). However, when pooled fractions of the two peaks of each isoform from the Con A Sepharose column were subjected to gel filtration on Sephacryl S-300, the invertase activity eluted as a single peak. However, with the same gel filtration column, the cell wall invertase isoform eluted as a single peak but at a slightly higher elution volume than the soluble isoform. Both invertase isoforms eluted from the S-300 column corresponding to an apparent mol wt of 120 kD when compared with the elution profile of standard mol wt markers (Fig. 3). SDS-PAGE analysis revealed a major protein band with a mol wt of approximately 60 kD for both the soluble and bound invertase isoforms (Fig. 4). A single protein band was observed for both purified isoforms when analyzed further by nondenaturing PAGE (data not shown).

pH and Substrate Specificity

The soluble invertase isozyme exhibits a pH optimum of about 5.5, while the cell wall invertase isoform has maximum activity at about 4.5 (Fig. 5). The kinetic constants for the purified soluble (cytosol) isozyme are a K_m of 12 mM and a V_{max} of 80 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ with sucrose as substrate. In contrast, the cell wall enzyme has an apparent K_m of 5 mM and a V_{max} of 9 $\mu mol \cdot min^{-1} \cdot protein^{-1}$. With raffinose as substrate, the soluble isoform has a high K_m value of 182 mM and a low V_{max} of 3 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$, while the cell wall isoform shows a K_m of 195 mM and a V_{max} of 0.5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$. No hydrolytic activity was detected with melibiose or melizitose as substrates.

Inhibitors of Invertase Activity

$HgCl_2$, known to act as a sulfhydryl inhibitor of plant invertases (6, 11, 12), and hence, was tested separately against the purified soluble and cell-wall bound isozymes. The activities of both the isozymes were completely inhibited with 1 μM $HgCl_2$. These enzyme activities were also inhibited by 1 mM pyridoxal phosphate, a compound known to react with invertases (8) and inhibit their hydrolytic activity. Activities of the two invertase isoforms were unaffected by Ca^{2+}, Mg^{2+}, Mn^{2+}, or EDTA, at similar concentrations of up to 1.0 mM.

DISCUSSION

The predominant soluble and cell wall isoforms of invertase in barley were purified by 60- and 7-fold, respectively. Inver-

tase activity detected in the cell wall pellet even after solubilization with high salt suggests that either the release of cell wall bound invertase was incomplete or, perhaps, the existence of yet another isoform of invertase more tightly associated with the cell wall. In order to confirm one or the other of these possibilities, it may be necessary to extract the cell wall isozymes with high concentrations of salts such as 4 M NaCl, 3 M LiCl, 1 M $CaCl_2$, or with 1 to 2 M urea (NC Carpita, personal communication).

The two invertase isoforms, namely the cytosolic and the cell wall, exhibited similar chromatographic behavior on a DEAE cellulose anion exchange column, in contrast with the behavior of the cell wall invertases of wheat (8), where the cell wall isoform of the enzyme elutes at a slightly higher salt concentration than the soluble isoforms. Unlike the cytosolic invertase isozyme of barley, the cell wall isoform was purified to apparent homogeneity by omitting the step of organo-mercurial Sepharose affinity chromatography. No major differences were observed in the purity or specific activity of the cell wall isoform before or after organo-mercurial affinity chromatography. When the organo-mercurial affinity column was employed to purify the cytosolic isoform, most of the activity was still retained on the column even after elution with 10 mM DTT or 20 mM BME. Complete elution of the soluble enzyme was achieved by gentle shaking of the affinity gel with 10 mM DTT or 20 mM BME for 2 to 3 h at 4°C.

Based on substrate specifities, the purified enzyme isoforms have been identified to be invertases (β-fructosidases EC 3.2.1.26). The soluble and cell wall isoforms displayed higher K_m values toward raffinose as compared to sucrose as substrate. Although the K_m values observed here were high, a similar observation of higher K_m values have been reported for wheat invertases with raffinose as substrate (8). Similarly, the cell wall invertase isoforms of barley exhibited lower K_m values with sucrose as substrate, which is consistent with the results reported for cell wall invertase of wheat (8).

By gel filtration, the native invertase isozyme proteins of barley have been determined to have an apparent mol wt of 120 kD. However, the apparent mol wt of these isozymes have been estimated to be approximately only 60 kD by SDS-PAGE. This information indicates that each isoform of the enzyme, *i.e.* cytosolic and cell wall-bound, exists in a dimeric form. This is in agreement with the work from other laboratories where the plant invertases have been reported to exist in multimenic forms, with apparent mol wt ranging from 48.5 to 450 kD (2, 14).

The two invertase isoforms identified in barley are acid invertases because of their optimal enzymatic activity in the acidic range (pH 4.5–5.5). In this respect they differ from onion (10), sugarcane (4, 18) and carrot (16) which possess both acid and neutral invertases. Also, the invertase isozyme in barley have been identified to be glycoproteins based on their ability to bind to Con A-Sepharose. Other investigators, using similar and other methods, such as periodic-acid Schiff reagent, have reported the plant invertases to be glycoproteins (2, 8, 15, 18). The multiple peaks, observed for the soluble and cell wall bound forms of invertase with the anion exchange and Con A Sepharose affinity chromatography could be invertase isozymes. On the other hand, the peaks could be indicative of a mixture of invertase and SST. However, this speculation needs further investigation. SST converts two molecules of sucrose into a molecule of trisaccharide and glucose. Hence, the possibility that our purified soluble and cell wall invertases were SST was ruled out by analyzing for the monosaccharide, fructose, formed by the hydrolytic action of invertase.

The two invertase isoforms detected in barley were found to be thiol-containing enzymes, since they bind to organo-mercurial Sepharose, and in addition, these isoforms were completely inactivated in the presence of $HgCl_2$, suggesting the importance of thiol groups in the catalytic activity of the enzyme. Similar inactivation of wheat invertases with $HgCl_2$ has also been reported (8).

In the present study we have been able to show that the soluble and cell wall isoforms of invertase in barley can be differentiated on the basis of their pH optima and kinetic constants (K_m and V_{max}). The purification scheme employed in the present investigation is a modification of Krishnan *et al.* (8), and does not seem to have the same order of magnitude in the purification factor observed for wheat invertases. However, when the purified enzymes from barley were analyzed by SDS-PAGE, a single polypeptide band at about 60,000 D was observed, unlike that reported by Krishnan *et al.* (8). An activity of a third invertase isoform found tightly associated with the cell wall can be detected but cannot be isolated yet.

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