Purification and characterization of soluble invertases from suspension-cultured bamboo (*Bambusa edulis*) cells

Chia-Chen Liu [a], Li-Chun Huang [b], Chen-Tien Chang [c], Hsien-Yi Sung [a,*]

[a] *Department of Biochemical Science and Technology, National Taiwan University, Taipei 106, Taiwan, ROC*
[b] *Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, ROC*
[c] *Department of Food and Nutrition, Providence University, Shalu, Taiwan, ROC*

Abstract

An alkaline invertase (IT I) and an acid invertase (IT II) were purified from the soluble fraction of suspension cultured bamboo cells. Both purified invertases were homogeneous as examined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and were identified as β-fructofuranosidases able to attack the β-fructofuranoside from the fructose end. With respect to sucrose hydrolysis, the optimal pHs were 7.0 and 4.5 for IT I and IT II, respectively. The Km's were 10.9 and 3.7 mM. The IT I and IT II molecular masses were 240 and 68 kDa, respectively, as estimated by gel filtration. The isoelectric points were 4.8 and 7.4. IT I was a homotetrameric enzyme activated by bovine serum albumin (BSA). IT II was a monomeric enzyme activated by BSA, concanavalin A (ConA) and urease. Both isoforms were significantly inhibited by heavy metal ions Ag^+ (5 mM) and Hg^{2+} (1 mM), and mercaptide forming agent ρ-chloromercuribenzoic acid (PCMB; 0.5 mM).
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Bamboo (*Bambusa edulis*) suspension cells; Invertase; Purification; Characterization

1. Introduction

Sucrose is one of the predominant initial photosynthesis products and serves as the major form of carbohydrate translocation in higher plants. Sucrose is synthesized in the photosynthetic leaf tissue (source organ), from where it is transported to the heterotrophic parts of the plant (sink organs). Before utilization, sucrose is cleaved either by invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) or by sucrose synthase (EC 2.4.1.13). Invertase catalyzes the irreversible hydrolysis of sucrose into D-glucose and D-fructose, the main forms of carbon and energy supplies in plant metabolism. Plant invertases include a variety of forms, which can be categorized in terms of solubility, optimum pH,

isoelectric point, and subcellular localization (ap Rees, 1984; Sturm & Chrispeels, 1990). Two types of invertases have been isolated from plants, the soluble forms and cell wall-bound enzymes. Soluble invertases are classified into acid and alkaline/neutral invertases according to the optimum catalysis pH (Copeland, 1990; Pollock & Lloyd, 1977). Soluble acid invertase (at an optimum pH of 3.5–5.0) is involved in sucrose metabolism and storage in the vacuole of young plant organs (Yelle, Chetelat, Dorais, DeVernaj, & Bennett, 1991; Lin & Sung, 1993; Obenland, Simmen, Boller, & Wiemken, 1993). Alkaline invertase (pH 7.0–8.0) may be present exclusively in the cytoplasm of mature tissues (Ricardo, 1974) and may regulate hexose and sucrose levels the in cytoplasm (Hatch, Sacher, & Glasziou, 1963; Masuda, Takahashi, & Sugawara, 1988). In the apoplast, a cell wall invertase with an acidic pH optimum may play an important role in photosynthetic

assimilates uptake by maintaining a sucrose concentration gradient between the source and sink tissues (Dickinson, Altabella, & Chrispeel, 1991). The presence of multiple invertase isoforms is probably physiologically advantageous to the plant in that it provides a means for optimizing sucrose metabolism, partitioning and storage control within different cells, at different developmental stages and under different physiological conditions (Stommel & Simon, 1990; Unger, Hofsteenge, & Sturm, 1992). Invertase activity has been detected in many plant tissues, such as wheat (Krishnan, Blanchette, & Okita, 1985), maize (Doehlert & Felker, 1987), barley (Obenland et al., 1993), mung bean (Arai, Mori, & Imaseki, 1991), sugar beets (Masuda & Sugawara, 1980), carrot (Stommel & Simon, 1990), lily (Tymowska-Lalanne & Kries, 1998; Singh & Knox, 1984), bamboo shoots (Cheng, Mitsuya, Juang, & Sung, 1990), rice (Charng, Juang, Su, & Sung, 1994; Chen & Sung, 1996; Hsiao, Fu, & Sung, 2002; Lin & Sung, 1993) and sweet potato (Matsushita & Uritani, 1974; Wu, Huang, & Sung, 2002). They accumulate as either soluble proteins in the cell or are ionically bound to the cell wall.

Cell-suspension cultures have the advantage that growth conditions may be strictly controlled, thereby preventing experiments from being complicated by differentiation processes. Suspension plant cell cultures are suitable systems for a variety of physiological studies and metabolic investigations (Masuda et al., 1988; Stommel & Simon, 1990). Multiple invertase forms have been purified from sugar beet (Masuda et al., 1988), carrot (Stommel & Simon, 1990), rice (Chen & Sung, 1996) and sweet potato (Wu et al., 2002) cell cultures. Recently, we found that both acid and alkaline invertases were induced in bamboo suspension cells during cultivation. This is an ideal system for study of roles of invertase isoforms in sucrose metabolism in bamboo cells. In the present study, we report on the purification and characterization of alkaline invertase (IT I) and acid invertase (IT II) isoforms from cultured bamboo cells.

2. Materials and methods

2.1. Bamboo cell suspension cultures

Two grams of bamboo (*B. edulis*) suspension cells were incubated in Murashige-Skoog (MS) liquid medium (Murashige & Skoog, 1962) supplemented with 3% (w/v) sucrose and 3 ppm 2, 4-dichlorophenoxyacetic acid (2, 4-D) in a 125 ml flask. The mixture was agitated in a shaking incubator (110 rpm) at 25–27 °C under illumination (16 h light/day, light intensity: 1000 lux) for 2–14 days. The suspension cell cultures were subcultured every 7 days.

2.2. Enzyme isolation

Bamboo suspension cells, subcultured for 5 days, were separated from the medium by vacuum filtration and washed with deionized water several times. Two hundred grams (fresh weight) of cells were frozen by adding liquid nitrogen, pulverized with a blender and extracted with two volumes of 50 mM sodium phosphate buffer, pH 7.0 (PB-7.0) containing 1 mM EDTA, 1 mM β-mercaptoethanol and 1 mM benzamidine. After centrifugation (Beckman JA 16.25, 10,000 rpm, 30 min), the resulting supernatant was collected and designated as crude soluble invertase.

2.3. Enzyme purification

2.3.1. Ammonium sulfate fractionation
Crude enzyme extract was fractionated by adding $(NH_4)_2SO_4$. The precipitate formed between 20% and 60% saturation of $(NH_4)_2SO_4$ was collected by centrifugation (Beckman JA 25.5, 15,000 rpm, 30 min) and dissolved in a small amount of PB-7.0.

2.3.2. Anionic chromatography
After dialysis against PB-7.0 overnight and centrifugation, the supernatant was applied to a DEAE–Sephacel column (2.6 × 15 cm) pre-equilibrated with PB-7.0. After sample absorption, the column was washed with the equilibrium buffer, until most of the non-bound protein was eluted. The column was then stepwise eluted with 0.1 M, 0.2 M, 0.3 M and 0.5 M NaCl in PB-7.0 at a flow rate of 30 ml/h. Five ml fractions were collected. Non-bound protein fractions containing acid invertase activity (60 ml) and bound protein fractions containing alkaline invertase activity (65 ml) were pooled for the subsequent purification of the two invertase isoforms.

2.4. Alkaline invertase purification

2.4.1. Gel filtration
Bound protein fractions containing alkaline invertase activity obtained from the DEAE–Sephacel column were concentrated in an Amicon cell (YM-10 membrane >10,000 MW) and loaded onto a Sephacryl S-200 column (1.6 × 95 cm) pre-equilibrated with PB-7.0 containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 25 ml/h and fractions of 2 ml were collected. Those containing invertase activity were pooled (20 ml), concentrated in an Amicon cell (YM-10 membrane) to a volume of 1.2 ml and dialyzed against PB-7.0.

2.4.2. Preparative polyacrylamide gel electrophoresis
The above dialyzed solution was loaded on a preparative polyacrylamide gel (7.5% separation gel, BRL V-16 Vertical gel electrophoresis system). After electrophoresis

the protein band with invertase activity was sliced out and put in an electrophoretic concentrator (ISCO Model 1750) to elute the enzyme (0.6 ml). The eluted enzyme was stored frozen for further characterization.

2.5. Acid invertase purification

2.5.1. Cationic chromatography

The acid invertase obtained from the DEAE–Sephacel column (non-bound fraction) was concentrated and applied to a CM–Sepharose column (2.6×13 cm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.0 (PB-6.0). Non-bound protein was removed with PB-6.0 buffer. Bound protein was stepwise eluted with 0.1, 0.2 and 0.4 M NaCl in PB-6.0 at a flow rate of 18 ml/h. Three ml fractions were collected. The main fractions containing invertase activity (fractions 98–104; 21 ml) were pooled.

2.5.2. ConA–Sepharose affinity chromatography

The enzyme obtained from CM–Sepharose column was loaded on a ConA–Sepharose column (1.6×12 cm) pre-equilibrated with PB-7.0 containing 0.5 M NaCl. The column was then washed with PB-7.0; invertase was subsequently eluted with 250 ml of α-methyl-D-mannoside (0–0.3 M) linear gradient in PB-7.0 containing 0.5 M NaCl at a flow rate of 18 ml/h. Two ml fractions were collected. Fractions containing invertase activity were pooled (40 ml).

2.5.3. Mono Q column chromatography

The enzyme obtained from ConA–Sepharose column was concentrated in an Amicon cell (YM-10 membrane), dialyzed overnight against 50 mM sodium phosphate buffer, pH 7.5 (PB-7.5) and applied to a Mono Q column (HR 5/5, Pharmacia), that had been pre-equilibrated with PB-7.5. The column was washed with 8 ml of PB-7.5 and then eluted with 12 ml of a linear gradient of 0–0.5 M NaCl and 8 ml of 1 M NaCl in PB-7.5 at a flow rate of 30 ml/h. One half ml fractions were collected. Fractions containing invertase activity were pooled (2 ml). The purified enzyme was stored frozen for further characterization.

2.6. Invertase activity measurement

Invertases were assayed in a 0.36 ml mixture of 0.1 M sucrose in either 50 mM sodium phosphate (pH 7.0) for alkaline invertase or 100 mM sodium acetate (pH 5.0) for acid invertase. The reaction mixture was incubated at 37 °C for 10 min. The reaction was stopped by adding 0.3 ml of arsenomolybdate color reagent. The amount of reducing sugar produced was measured using the Somogyi–Nelson' s method (Nelson, 1944). A standard curve was established for an equimolar mixture of glucose and fructose.

2.7. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on slab gels (Hoefer mighty small SE 250) using the Laemmli method (Laemmli, 1970) with a 4% stacking gel and 12.5% separating gel. The same system was used for native basic gels, except SDS was omitted and a 7.5% polyacrylamide gel was used. After electrophoresis, the separated proteins were stained with either Coomassie Brilliant Blue R-250 or silver staining kit (PlusOne Amersham Pharmacia Biotech).

2.8. Isoelectric point (pI) measurement

The pI of the purified invertase was measured using isoelectric focusing (IEF) on a Pharmacia Ampholine PAG plate (Multiphor II electrophoresis unit, Amersham Pharmacia Biotech) (pH 3.5–9.5) and compared with standards from an IEF calibration kit according to the manufacturer's instructions.

2.9. Molecular mass estimation

The IT I and IT II molecular masses were estimated using gel filtration on a Sephacryl S-200 column according to Whitaker (1963). Thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa) were used as standards (Sigma molecular weight markers for gel filtration). The subunit molecular masses of IT I and IT II were estimated using SDS–PAGE. Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) (MW SDS-200 kit, Sigma) were used as standards.

2.10. N-terminal amino acid sequence analysis

The IT II isoform was electroblotted onto an Immobilon PVDF membrane (Hoefer transphor electrophoresis unit TE 22; Immobilon-P transfer membrane, Millipore) and sequenced by automated Edman degradation using an Applied Biosystems 477 A protein sequencer.

3. Results and discussion

3.1. Changes in fresh weight and soluble invertase activities in suspension cells during cultivation

Bamboo suspension cells were incubated in MS medium containing 3% sucrose and 3 ppm 2,4-D, and agitated at 25–27 °C. Fig. 1 shows the time course for intracellular soluble invertase activity and fresh
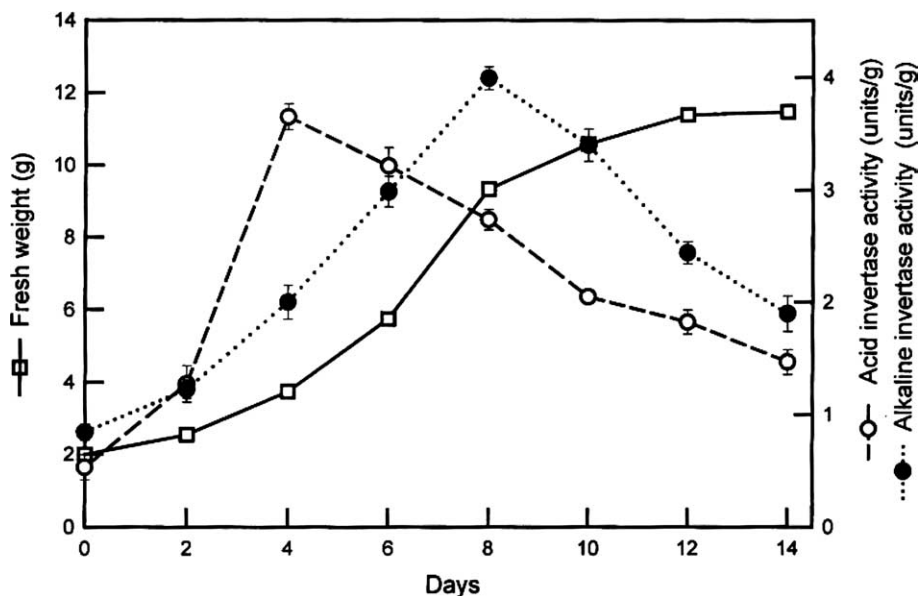
Fig. 1. Changes in fresh weight and soluble invertase activities from bamboo suspension cells which were collected and weighted at various time intervals after subculture. The supernatants from the crude suspension cell extracts were used for invertase activity assay.

suspension cell weight. A logarithmic cell growth rate was observed between approximate 3 and 9 days and reached a maximal level on day 12 after subculture. During cultivation, soluble acid and alkaline invertases could be detected in the initial suspension cells and reached maximal levels at the logarithmic growth phase. Acid invertase increased rapidly during the first several days to a maximal level on day 4 and then decreased. However, alkaline invertase increased gradually to a maximal level on day 8 and then decreased. There was no extracellular invertase activity in the medium. These results implied that bamboo suspension cells division during cultivation was apparently accompanied by acid and alkaline invertases induction and that the sucrose added to the medium was hydrolyzed by intracellular acid and alkaline invertases during cell growth. Two classes of invertases are believed to play different roles in plants. Acid invertase isoforms are present in the vacuole and cell wall, and have been shown to constitute the majority of invertase activity within plant cells. High acid invertase activity was observed in tissue containing low sucrose concentrations (ap Rees, 1984). Alkaline invertase isoforms are only present in the cytoplasm, and have been proposed as "maintenance" enzymes involved in providing a substrate for the tricarboxylic acid cycle in tissues in which acid invertase or sucrose synthase activities are low (ap Rees, 1984). High alkaline invertase activity has been reported to be closely related to sucrose accumulation during growth of sugar beet roots (Masuda, Takahashi, & Sugawara, 1987) and cultivation of cells from leaf explants of sugar beets (Masuda et al., 1988).

3.2. Distribution of different invertase types

Bamboo suspension cells subcultured for 5 days were used for soluble and cell wall-bound invertase analysis. The soluble and cell wall-bound acid invertases in the suspension cells were 62.4% and 37.6% of the total acid invertase activity. About 40% of the cell wall-bound invertases were released by 1 M NaCl and 60% of the enzyme activity still remained in the residual cell-wall fragments. These results indicated that at least two types of cell wall bound invertases existed in bamboo suspension cells. The first type bound weakly and the second bound strongly or covalently on cell walls. Different soluble and cell wall-bound invertases isoforms have also been found in rice and sweet potato suspension cells (Chen & Sung, 1996; Wu et al., 2002).

3.3. Soluble alkaline and acid invertase isoform purification

Bamboo suspension cells cultured for 5 days were used for soluble alkaline and acid invertases isolation and purification. The soluble invertases were recovered by fractionating the crude bamboo suspension cell extract, at 20–60% saturation with $(NH_4)_2SO_4$, and separating them into two isoforms using ion-exchange chromatography on a DEAE–Sephacel column. As shown in Fig. 2, a protein peak with acid invertase activity was not adsorbed by DEAE–Sephacel at pH 7.0 (non-bound acid invertase containing IT II and IT III isoforms, as further purified) and therefore eluted immediately from the column. Hence, the non-bound acid
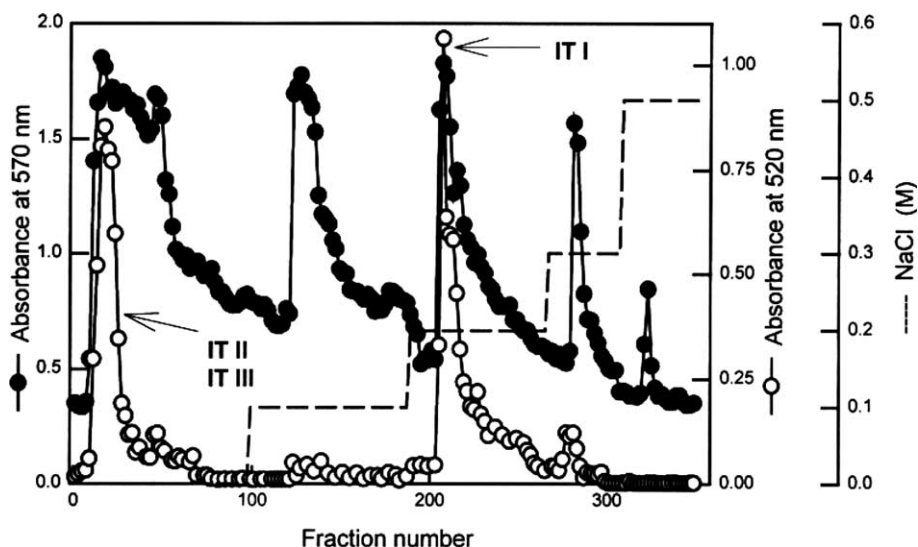
Fig. 2. Ion-exchange chromatography of bamboo invertases on DEAE–Sephacel. The column (2.6 × 15 cm) was equilibrated with 50 mM sodium phosphate buffer, pH 7.0. Proteins bound to the column was stepwise eluted with 0.1 M, 0.2 M 0.3 M and 0.5 M NaCl in equilibrium buffer at a flow rate of 30 ml/h.; 5 ml fractions were collected. Protein profile (●) and invertase activity (○) were separately monitored.

invertase was positively charged at pH 7.0 (isoelectric point >7.0). However, a bound protein peak with alkaline invertase activity (IT I isoform) was eluted from the DEAE–Sephacel column using a stepwise NaCl gradient (0.1–0.5 M). This implied that the bound IT I isoform alkaline invertase was negatively charged at pH 7.0 (isoelectric point <7.0). The IT I isoform obtained was further purified using gel filtration on a Sephacryl S-200 column (Fig. 3), and preparative gel electrophoresis. The purification results are summarized in Table 1.

Using these steps, the purity of the IT I isoform was increased 144 fold, with a yield of 2.4%. The purified IT I isoform was homogeneous, as determined by native–PAGE (Fig. 4(a)) and SDS–PAGE (Fig. 4(b)).

Acid invertase obtained from DEAE–Sephacel column (non-bound acid invertase) was dialyzed against 50 mM sodium phosphate buffer (pH 6.0) and applied to a CM–Sepharose column. As shown in Fig. 5, the acid invertase was adsorbed onto the gel and separated into a major isoform (IT II) and a minor isoform (IT
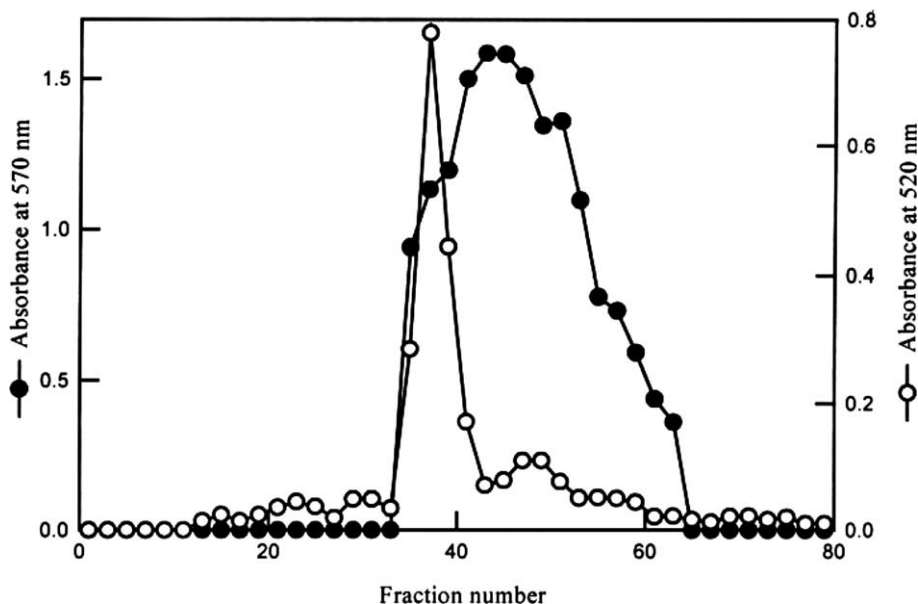

Fig. 3. Gel filtration chromatography of bamboo alkaline invertase (IT I) on Sephacryl S-200. The column (1.6 × 95 cm) was equilibrated with 50 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.0. Proteins bound to the column was eluted with the same buffer at a flow rate of 35 ml/h; 2 ml fractions were collected. Protein profile (●) and invertase activity (○) were separately monitored.

Table 1
Purification of IT I from bamboo suspension cells[a]

Procedure	Total activity (units)[b]	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	488	3203	0.15	1	100
20 ∼ 60% saturation ammonium sulfate fractionation	323	543	0.60	3	66
DEAE–Sephacel ion-exchange chromatography	247	42	5.8	30	51
Sephacryl S-200 gel filtration	128	10	12.8	64	26
Preparative gel electrophoresis	11.5	0.4	28.8	144	2.4

[a] Data were obtained from 200 g of bamboo suspension cells.

[b] One unit is defined as the amount of enzyme producing 1 μmole of reducing sugar from sucrose per minute at 37 °C at pH 7.0.
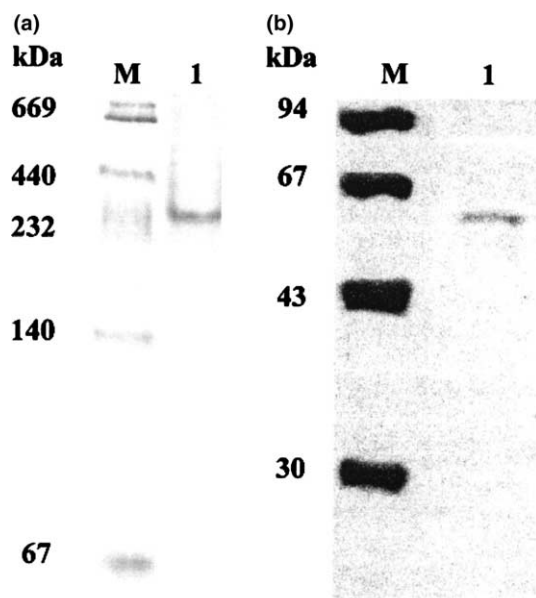

Fig. 4. Native–PAGE and SDS–PAGE of bamboo alkaline invertase (IT I). Panel a (native–PAGE): lane 1, preparative gel electrophoresis purified IT I; lane M, high molecular weight standard proteins. Panel b (SDS–PAGE): lane 1, preparative gel electrophoresis purified IT I; lane M, low molecular weight standard proteins.

III) after being eluted with a stepwise gradient of NaCl (0.1–0.4 M). From the elution profile, a protein peak (–●–A_{570}) of IT II seems to be not completely eluted with 0.1% NaCl before adding 0.2% NaCl for elution of IT III. It is probable that IT III is a portion of uneluted IT II. The major acid invertase (IT II) isoform was further purified using affinity chromatography on a ConA–Sepharose column. As shown in Fig. 6, a protein peak without invertase activity emerged immediately from the column. The invertase was adsorbed onto the gel and emerged after being eluted with a gradient of α-methyl-D-mannoside (0–0.3 M). After ConA–Sepharose affinity chromatography, the IT II was purified on a Mono Q column using an FPLC System. As shown in Fig. 7, a single protein peak with invertase activity and several peaks without enzyme activity were obtained. The purification results are summarized in Table 2. Using these steps the IT II isoform purity was increased 130-fold with a yield of 2.3%. The purified IT II isoform was homogeneous, as determined by SDS–PAGE (Fig. 8). ConA could bind molecules that contained α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related residues, suggesting that IT II is
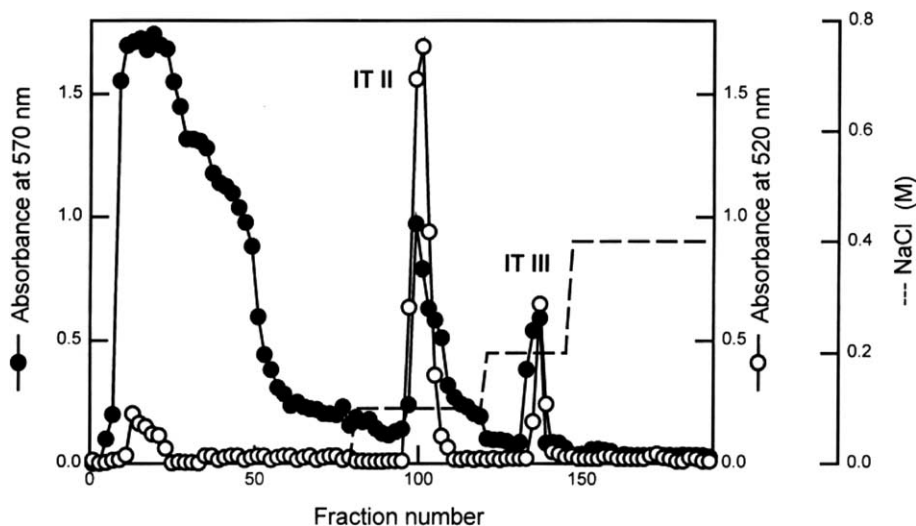

Fig. 5. Ion-exchange chromatography of bamboo acid invertase (IT II) on CM–Sepharose. The column (2.6 × 13 cm) was equilibrated with 50 mM sodium phosphate buffer, pH 6.0. Proteins bound to the column was stepwise eluted with 0.1 M, 0.2 M and 0.4 M NaCl in equilibrium buffer at a flow rate of 18 ml/h; 3 ml fractions were collected. Protein profile (●) and invertase activity (○) were separately monitored.
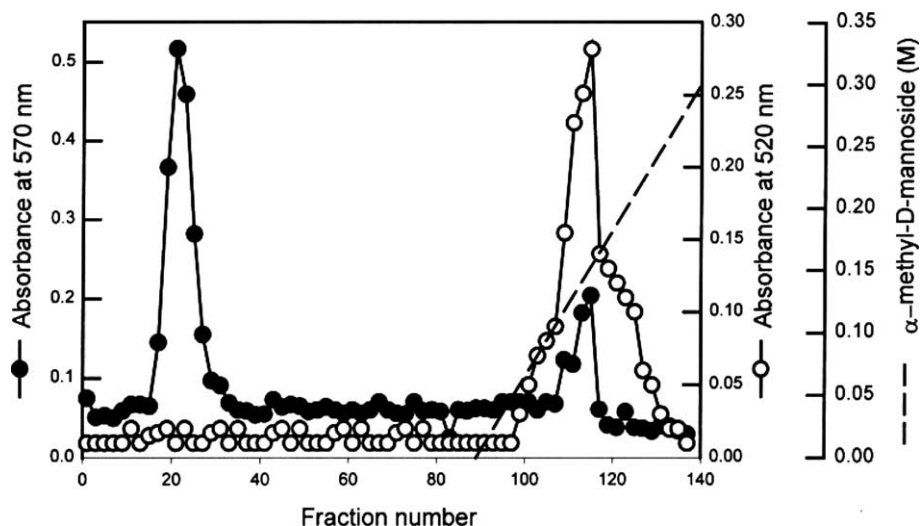
Fig. 6. Affinity chromatography of bamboo acid invertase (IT II) on ConA–Sepharose. The column (1.6 × 12 cm) was equilibrated with 50 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.0. Protein bound to the column was eluted with a linear gradient of α-methyl-D-mannoside (0–0.3 M) in 50 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.0 at a flow rate of 18 ml/h; 2 ml fractions were collected. Protein profile (●) and invertase activity (○) were separately monitored.
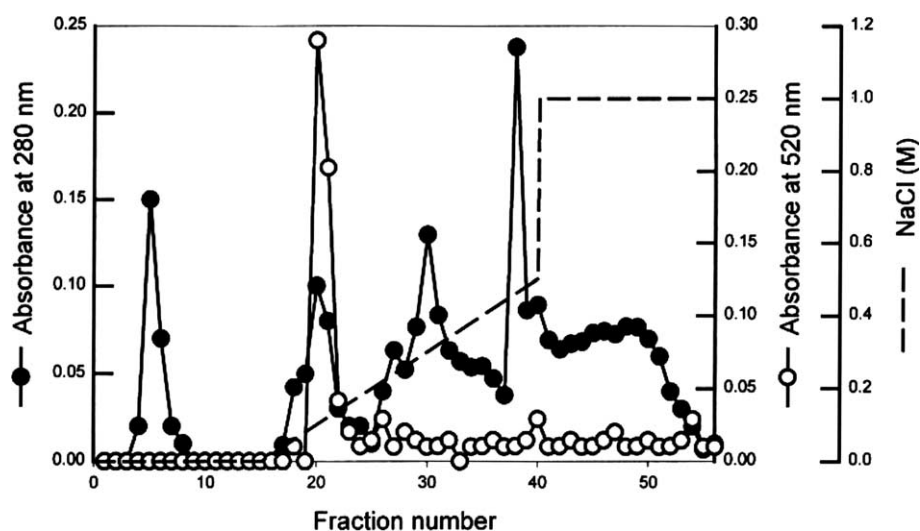

Fig. 7. Ion-exchange chromatography of bamboo acid invertase (IT II) on a Mono Q HR 5/5 column. The column (0.5 × 5 cm) was equilibrated with 50 mM sodium phosphate buffer, pH 7.5. Proteins bound to the column was eluted with a linear NaCl gradient (0–0.5 M) and 0.1 M NaCl in equilibrium buffer at a flow rate of 30 ml/h using an FPLC System (Pharmacia); 0.5 ml fractions were collected. Protein profile (●) and invertase activity (○) were separately monitored.

Table 2
Purification of IT II from bamboo suspension cells[a]

Procedure	total activity (units)[b]	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	560	3203	0.2	1	100
20–60% saturation ammonium sulfate fractionation	423	543	0.8	4	76
DEAE–Sephacel ion-exchange chromatography	258	68	3.8	19	46
CM–Sepharose ion-exchange chromatography	80	9.2	8.7	44	14
ConA–Sepharose affinity chromatography	45	3.0	15	75	8.0
Mono Q FPLC	13	0.5	26	130	2.3

[a] Data were obtained from 200 g of bamboo suspension cells.
[b] One unit is defined as the amount of enzyme producing 1 μmole of reducing sugar from sucrose per minute at 37 °C at pH 5.0.
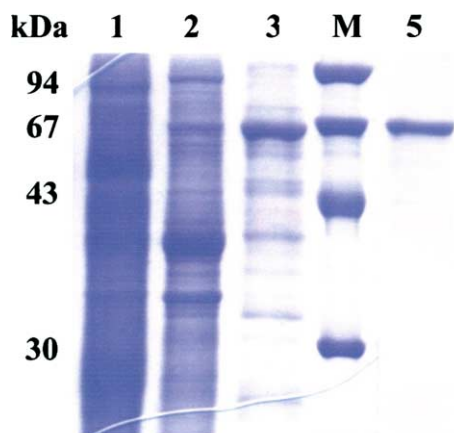
Fig. 8. SDS–PAGE of bamboo acid invertase (IT II). Lane 1, DEAE–Sephacel non-bound acid invertase; lane 2, CM–Sepharose column purified acid invertase; lane 3, ConA–Sepharose column purified acid invertase; lane 5, Mono Q column purified acid invertase; lane M, low molecular weight standard proteins.

a glycoprotein with mannose, and/or glucose residues in its carbohydrate moiety. Most of the purified invertases, especially the acidic forms, are reported to be glycoproteins (Copeland, 1990; Fahrendorf & Beck, 1990). The glycosylation of acid invertase synthesized in the cytoplasm would be necessary for its transport across either the tonoplast or the plasma membrane. In plants, vacuolar proteins are synthesized at the rough endoplasmic reticulum and transported via the Golgi apparatus to the acidic compartment. Thus, soluble acid invertase IT II most likely accumulates in vacuoles after transport through the endomembrane system.

At the final stage of purification, the recovery of enzyme activity of both IT I and IT II was rather low. This may be due to the removal of some fractions with lower enzyme activity during column chromatography. It is also possible that some of the activity loss occurred during purification since, especially at the final stage of purification both isoforms are rather unstable.

3.4. Properties of IT I and IT II

3.4.1. Effect of pH

The optimal pHs for IT I and IT II were 7.0 to 7.5, and 4.5, respectively. Hence, IT I is an alkaline invertase while IT II is an acid invertase.

3.4.2. Effect of temperature and thermostability

Both IT I and IT II had an optimal temperature at approximate 30 to 40 °C and retained over 70% of their activity after being heated to 50 °C for 10 min.

3.4.3. Molecular masses, isoelectric point (pI) and N-terminal amino acid sequence

The molecular masses of IT I and IT II were 240 and 68 kDa, respectively, as estimated by Sephacryl S-200 gel filtration and 60 and 67 kDa, respectively, as estimated by SDS–PAGE (Figs. 4(b) and 8). These results indicate that IT I is a homotetrameric enzyme while IT II is a monomeric enzyme. The pIs of IT I and IT II were 4.8 and 7.4, as estimated by isoelectrofocusing electrophoresis (data not shown). Soluble acid invertases have been characterized from several plant species (Doehlert & Felker, 1987; Fahrendorf & Beck, 1990; Faye & Ghorbel, 1983; Krishnan et al., 1985; Masuda & Sugawara, 1980; Roberts, 1973; Singh & Knox, 1984). Most of characterized soluble acid invertases have a native molecular mass in the range of $50 \sim 450$ kDa and an pI value in the range of $3.2 \sim 8.7$. The high molecular masses forms of soluble acid invertase may exist in oligomeric forms, or they are generated complexation with phenolic compounds (Spencer et al., 1988). The native molecular mass and pI value of IT II acid invertase are in the reported value range. However, its molecular mass is much less than that of soluble acid invertase characterized from green bamboo shoots (Cheng et al., 1990). The molecular masses of characterized plant alkaline invertases are in the range of $60 \sim 280$ kDa. The IT I alkaline invertase molecular mass is also in the reported value range, and near that of alkaline invertase characterized from rice (Lin, Lin, Wang, & Sung, 1999) and soybean hypocotyls (Chen & Black, 1992). The N-terminal amino acid sequence of IT II is ARPQPWEDIVLSWVQASLQRIIA. It shows 38% of identity with that of residues 1–23 of the carrot invertase (Sturm, 1996).

3.4.4. Substrate specificity and Michaelis constant (Km)

Both IT I and IT II showed activities toward sucrose and raffinose but no activity toward maltose. Therefore, both isoforms are a β-frutofuranosidase, able to attack the β-fructofuranoside from the fructose end. For sucrose hydrolysis, the IT I and IT II Km values were 10.9 and 3.7 mM, respectively. The IT II Km value is higher than that of type I acid invertase (7.9 mM) isolated from green bamboo shoots. The IT I Km value is higher than that of rice alkaline invertase (Lin et al., 1999) but close to that of alkaline invertases from sugar beet suspension cells (Masuda et al., 1988) and soybean hypocotyls (Chen & Black, 1992).

3.4.5. Effectors

The effects of substrate, end-products, metal ions and various chemicals on the IT I and IT II activities are shown in Tables 3–5. As show in Table 3, substrate sucrose at concentrations above 0.4 M significantly inhibited IT II activity but only slightly decreased IT I activity. The glucose and fructose end products significantly decreased activities of both isoforms at concentrations above 2.5 mM. As shown in Table 4, Mg^{2+} had no effect on both isoform activities, whereas heavy

Table 3
Effects of substrate and end-products on the activities of IT I and IT II[a]

Substrate (mM)	End-products (mM)		Relative activity (%)	
Sucrose	Glucose	Fructose	IT I	IT II
100	–	–	100	100
400	–	–	90	80
1000	–	–	90	38
100	2.5	–	42	28
100	5.0	–	20	10
100	10.0	–	10	2
100	–	2.5	38	30
100	–	5.0	30	15
100	–	10.0	20	10

[a] The amount of glucose released was determined by the glucose oxidase method (Ebell, 1969).

Table 4
Effects of metal ions on the activities of IT I and IT II

Metal ions	Concentration (mM)	Relative activity (%)	
		IT I	IT II
None		100	100
Mg^{2+} $(MgCl_2)$	1.0	89	86
	5.0	88	109
Ca^{2+} $(CaCl_2)$	1.0	80	65
	5.0	65	16
Zn^{2+} $(ZnCl_2)$	1.0	98	14
	5.0	94	12
Cu^{2+} $(CuSO_4)$	1.0	47	15
	5.0	30	12
Hg^{2+} $(HgCl_2)$	1.0	7	5
	5.0	3	1
Ag^{+} $(AgNO_3)$	1.0	44	19
	5.0	15	19

metal ions, especially Hg^{2+}, significantly or almost completely inhibited both isoform activities.

As shown in Table 5, pyridoxine (5 mM), p-chloromercuribenzoic acid (PCMB, 0.5 mM) and aniline (15.0 mM) decreased IT I activity to 52%, 38% and 34%, respectively. Other reagents had little effect on IT I activity. These results indicated that cysteine, acidic and basic amino acids are important for the IT I catalytic activity. Iodoacetamide (5 mM), aniline (15 mM), PMSF (0.5 mM), pyridoxine (5 mM) and PCMB (0.5 mM) decreased IT II activity to 56%, 52%, 40%, 25% and 14%, respectively. These results indicated that cysteine, acidic and basic amino acids, serine and tyrosine are important for the IT II catalytic activity. As shown in Table 6, protein BSA (100 mg/ml) enhanced IT I activity by 18%, but other proteins (ConA and ure-

Table 5
Effects of chemical modification agents on the activities of IT I and IT II

Chemicals	Concentration (mM)	Relative activity (%)	
		IT I	IT II
None		100	100
PMSF	0.1	91	76
	0.5	80	40
Iodoacetamide	1.0	77	80
	5.0	83	56
Pyridoxine	1.0	82	54
	5.0	52	25
PCMB	0.1	71	45
	0.5	38	14
DTNB	0.1	85	109
	0.5	76	111
Aniline	5.0	64	83
	15.0	34	52

PMSF: phenylmethylsulfonyl fluoride.
PCMB: ρ-chloromercuribenzoic acid.
DTNB: 5,5′-dithiol-bis-(2-nitrobenzoic acid).

ase) did not. However, proteins BSA (0.2 mg/ml), ConA (0.9 mg/ml) and urease (0.1 mg/ml) enhanced IT II activity by 90, 40 and 30%, respectively. Therefore, BSA, ConA and urease are acid invertase (IT II) activators, whereas BSA is an alkaline invertase (IT I) activator. This activation is a consequence of the protein–protein interaction between the protein and the invertase (Masuda & Sugawara, 1980). Isla, Salerno, Pontis, Vattuone, and Sampietro (1995) reported that treatment with proteins BSA, ConA, urease and alkaline phosphatase enhanced rice acid invertase activity. Lin et al. (1999) reported that treatment with alkaline phosphatase enhanced rice alkaline invertase activity, but other proteins (BSA, ConA and urease) did not. Some plant acid invertases have been reported as located in the vacuole together with sucrose, fructose, glucose and protein.(Leight, ap Reess, Fuller, & Banfield, 1979; Vinals & Sampietro, 1983) The functional state of the plant soluble acid invertase probably occurs as a complex with other proteins or enzyme. Alkaline invertase is considered to be a cytoplasmic enzyme, more important for

Table 6
Effect of some proteins on the activities of IT I and IT II

Protein	POA (mg/ml)[*]		Maximal activation (%)	
	IT I	IT II	IT I	IT II
Control	–	–	–	–
BSA	100	0.2	18	90
ConA	–	0.9	–	40
Urease	–	0.1	–	30

[*] The point of optimal activation (POA) is the minimal concentration of effector that produces the maximal enzyme activation. –: no effect.

sucrose hydrolysis in mature tissues where acid invertase levels are typically low (Ricardo, 1974). We found that fructose and glucose inhibited bamboo alkaline invertase IT I. This finding supports the view that the enzyme is a regulatory agent of hexose and sucrose levels in plant cells. The inhibitory effects exerted by heavy metal ions and mercaptide forming agents are in agreement with enzymes from other plants (Chen & Black, 1992; Pollock & Lloyd, 1977).

4. Conclusion

Both acid and alkaline invertases were induced in bamboo suspension cells during cultivation. Alkaline invertase was only present in the cytoplasm of the cultured cell, whereas acid invertase was present in the cytoplasm and cell wall. About 62% of acid invertase was soluble, and 38% was cell-wall bound. There was no extracellular invertase activity in the medium. An alkaline invertase (IT I) and an acid invertase (IT II) were identified and characterized from the soluble fraction of suspension cultures of bamboo cells. From the enzymatic properties examined, both isoforms are β-fructofuranosidases and regulatory enzymes of hexose and sucrose levels in bamboo cells. The alkaline invertase isoform (IT I) is considered to be a cytoplasmic enzyme, whereas the acid invertase isoform (IT II) probably exists in the vacuole for its acidic pH optimum and glycoprotein nature.

Acknowledgement

References

ap Rees, T. (1984). Sucrose metabolism. In *Storage carbohydrates in vascular plants* (pp. 53–57). Cambridge University Press.

Arai, M., Mori, H., & Imaseki, H. (1991). Roles of sucrose-metabolizing enzymes in growth of seedlings. Purification of acid invertase from growing hypocotyls of mung bean seedlings. *Plant and Cell Physiology, 32*, 1291–1298.

Charng, Y. Y., Juang, R. H., Su, J. C., & Sung, H. Y. (1994). Partial purification and characterization of invertase isozymes from rice grains (*Oryza sativa*). *Biochemistry and Molecular Biology International, 33*, 607–615.

Cheng, J. F., Mitsuya, N., Juang, R. H., & Sung, H. Y. (1990). Purification and characterization of invertase isozymes from bamboo shoots. *Biochemistry International, 21*, 497–506.

Chen, J. Q., & Black, C. C. (1992). Biochemical and immunological properties of alkaline invertase isolated from sprouting soybean hypocotyls. *Archieves of Biochemistry and Biophysics, 295*, 61–69.

Chen, S. Y., & Sung, H. Y. (1996). Purification and characterization of invertase isozymes from rice suspension cells. *Journal of the Chinese Agricultural Chemical Society, 34*, 673–682.

Copeland, L. (1990). Enzymes of sucrose metabolism. In P. J. Lea (Ed.). *Methods in plant biochemistry* (Vol. 3, pp. 73–85). London, UK: Academic Press.

Dickinson, C. D., Altabella, T., & Chrispeel, M. (1991). Slow-growth phenotype of transgenic tomato expressing apoplastic invertase. *Plant Physiology, 95*, 420–425.

Doehlert, D. C., & Felker, F. C. (1987). Characterization and distribution of invertase activity in developing maize (*Zea mays*) kernels. *Physiology Plant, 70*, 51–57.

Ebell, L. F. (1969). Specific total starch determination in conifer tissue with glucose oxidase. *Phytochemistry, 8*, 25–36.

Fahrendorf, T., & Beck, E. (1990). Cytosolic and cell-wall-bound acid invertases from leaves of *Urtica dioica* L. : a comparison. *Planta, 180*, 237–244.

Faye, L., & Ghorbel, A. (1983). Studies on β-fructosidase from radish seedlings. II. Biochemical and immunocytochemical evidence for cell-wall-bound forms in vivo. *Plant Science Letters, 29*, 33–48.

Hatch, M. D., Sacher, J. A., & Glasziou, K. T. S. (1963). Accumulation cycle in sugar cane. I. Studies on enzymes of the cycle. *Plant Physiology, 38*, 338–343.

Hsiao, C. C., Fu, R. H., & Sung, H. Y. (2002). A novel bound form of plant invertase in rice suspension cell. *Botanical Bulletin of Academia Sinica, 43*, 115–122.

Isla, M. I., Salerno, G., Pontis, H., Vattuone, M. A., & Sampietro, A. R. (1995). Purification and properties of the soluble acid invertase from *Oryza sativa*. *Phytochemistry, 38*, 321–325.

Krishnan, H. B., Blanchette, J. T., & Okita, T. W. (1985). Wheat invertase. *Plant Physiology, 78*, 241–245.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature., 227*, 680–685.

Leight, R. A., ap Reess, T., Fuller, W. A., & Banfield, J. (1979). The location of acid invertase activity and sucrose in the vacuoles of storage roots of beet root (*Beta vulgaris*). *Biochemical Journal, 178*, 539–547.

Lin, C. L., Lin, H. C., Wang, A. Y., & Sung, H. Y. (1999). Purification and characterization of an alkaline invertase from shoots of etiolated rice seedlings. *New Phytologist, 142*, 427–434.

Lin, S. S., & Sung, H. Y. (1993). Partial purification and characterization of soluble acid invertases from rice (*Oryza sativa*) leaves. *Biochemistry and Molecular Biology International, 31*, 945–953.

Matsushita, K., & Uritani, I. (1974). Change in invertase activity of sweet potato in response to wounding and purification and properties of its invertases. *Plant Physiology, 54*, 60–66.

Masuda, H., & Sugawara, S. (1980). Purification and some properties of cell-wall-bound invertases from sugar beet seedlings and aged slices of mature roots. *Plant Physiology, 66*, 93–96.

Masuda, H., Takahashi, T., & Sugawara, S. (1988). Acid and alkaline invertase in suspension cultures of sugar beet cells. *Plant Physiology, 86*, 312–317.

Masuda, H., Takahashi, T., & Sugawara, S. (1987). The occurrence and properties of alkaline invertase in mature roots of sugar beets. *Agricultural and Biological Chemistry, 51*, 2309–2314.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays tobacco tissue cultures. *Physiology Plant, 15*, 473–497.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for determination of glucose. *The Journal of Biological Chemistry, 178*, 539–547.

Obenland, D. M., Simmen, U., Boller, T., & Wiemken, A. (1993). Purification and characterization of three soluble invertases from barley (*Hardeum vulgare* L.) leaves. *Plant Physiology, 101*, 1331–1339.

Pollock, C. J., & Lloyd, E. J. (1977). The distribution of acid invertase in developing leaves of *Lolium temulentum* L. *Planta, 133*, 197–200.

Ricardo, C. P. P. (1974). Alkaline β-fructofuranosidases of tuberous roots: Possible physiological function. *Planta, 118*, 333–343.

Roberts, DW. (1973). A survey of the multiple forms of invertase in the leaves of winter wheat, *Triticum aestivum* L. emend Thell. ssp. *vulgare. Biochimistry and Biophysics Acta, 321*, 220–227.

Singh, M. B., & Knox, R. B. (1984). Invertases of *Lilium* pollen. Characterization and activity during *in vitro* germination. *Plant Physiology, 74*, 510–515.

Spencer, C. M., Cai, K., Martin, R., Gaffney, S. H., Goulding, P. N., Magnolato, D., Lilley, T. H., & Haslam, E. (1988). Polyphenol complexation-some thoughts and observations. *Phytochemistry, 27*, 2397–2409.

Stommel, J. K., & Simon, P. W. (1990). Multiple forms of invertase from *Daucus cartoa* cell cultures. *Phytochemistry, 29*, 2087–2089.

Sturm, A., & Chrispeels, J. (1990). cDNA cloning of carrot extracellular β-fructosidase and its expression in response to wounding and bacterial infection. *Plant Cell, 121*, 1–7.

Sturm, A. (1996). Molecular characterization and functional analysis of sucrose-cleaving enzymes in carrot (*Daucus carota* L). *Journal of Experimental Botany, 47*, 1187–1192.

Tymowska-Lalanne, Z., & Kries, M. (1998). The plant invertases: Physiology, biochemistry and molecular biology. *Advances in Botanical Research, 28*, 71–117.

Unger, C., Hofsteenge, J., & Sturm, A. (1992). Purification and characterization of a soluble β-fructofuranoside from *Daucaus carota. European Journal of Biochemistry, 204*, 915–921.

Vinals, A. L., & Sampietro, A. R. (1983). Localization of invertase activities in *Ricimus communis* leaves. *Phytochemistry, 22*, 1361–1365.

Whitaker, J. R. (1963). Determination of molecular weight of proteins by gel filtration on Sephadex. *Analytical Chemistry, 35*, 1950–1953.

Wu, L. T., Huang, W. C., & Sung, H. Y. (2002). Partial purification and characterization of soluble acid invertase from sweet potato suspension cell. *Food Science and Agricultural Chemistry, 2*, 49–54.

Yelle, S., Chetelat, R. T., Dorais, M., DeVernaj, W., & Bennett, A. B. (1991). Sink metabolism in tomato fruit. IV. Genetic and biochemical analysis of sucrose accumulation. *Plant Physiology, 95*, 1026–1035.