

A novel bound form of plant invertase in rice suspension cells

Cheng-Chung Hsiao¹, Ru-Huei Fu¹, and Hsien-Yi Sung*

Biochemistry Laboratory, Department of Agricultural Chemistry, National Taiwan University, 1 Roosevelt Road Section 4, Taipei, Taiwan 106, Republic of China

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Abstract. A novel alkaline bound invertase (ITab) was purified 116 fold from crude extract of rice suspension cells (*Oryza sativa* L. cv. Tainong 67). The enzyme can be released by treating the cell wall and membrane fragments with 1.0 M NaCl and purified to homogeneity through the steps of ammonium sulfate precipitation, Con A-Sepharose affinity (non-retained), Sepharose CL-6B, and DEAE-Sepharose chromatographies. The FPLC and SDS-PAGE analyses revealed ITab to have a native molecular mass of about 250 kDa, and it may therefore be homotetramer composed of subunit with a mass of 60 kDa. Isoelectric focusing analysis indicated that the pI value was 4.7. The optimum pH was 7.6 and the optimum temperature was 40-50°C. ITab can hydrolyze sucrose and raffinose, but not maltose. The enzyme did not have affinity toward Con A. So it is not a glycoprotein. The Km for sucrose was 15.28 mM. Fructose and glucose were inhibitors. The enzyme was activated by additional protein such as bovine serum albumin. Tris, metal ions, and thiol reagents inhibited the enzyme activity, but activity could be restored with DTT. It is suggested that sulfhydryl-group(s) exist either in the active site or nearly active site.

Keywords: Bound form invertase; Rice suspension cells; Sucrose metabolism.

Abbreviations: Con A, concanavalin A; DTT, 1,4-dithiothreitol; ITab, alkaline bound invertase; PCMBs, p-chloromercuribenzenesulphonic acid.

Introduction

Sucrose is one of the predominant initial products of photosynthesis and serves as the major carbohydrate translocator, storage component, osmoticum, and regulator of gene expression (Koch, 1996) in higher plants. Utilization of the most sucrose depends on its cleavage into glucose and fructose, and in plants it is either invertase, also called β -D-fructofuranoside fructohydrolase (EC 3.2.1.26), or sucrose synthase (EC 2.4.1.13) that catalyzes this reaction.

Invertase isozymes can be distinguished by their subcellular localization, solubility, and optimum pH. The acid bound invertase (insoluble) with a basic pI is ionically or covalent bound to the cell wall. The acid unbound invertase (soluble) with an acidic pI is localized in the vacuole; and the alkaline unbound invertase (soluble) is thought to be cytoplasm (Tymowska-Lalanne and Kreis, 1998). Acid invertases are glycosylated forms and also hydrolyze other β -fructofuranoside-containing oligosaccharides such as raffinose and stachyose, while alkaline invertases are most likely a non-glycosylated form and appear to be sucrose specific (Sturm, 1999).

Acid invertases are synthesized as preproteins, with long leader sequences, which are cleaved off during transport and protein maturation. The amino acid sequences of the plant acid invertases share a few highly conserved motifs: β -fructosidase motif (NDPNG/A), close to the N terminus of the mature proteins, and cysteine catalytic site (MWECV/PDF), located closer to the middle of mature proteins (Sturm, 1999). It is pertinent that almost all cell wall bound invertases have a proline residue in the cysteine catalytic site while vacuolar invertases have a valine residue (Tymowska-Lalanne and Kreis, 1998). In addition, most plant invertases have a third domain, SI/VVESF, but its function is not clear.

In connection with the various roles sucrose plays in plants, invertases may have several different functions (Sturm and Tang, 1999). The vacuolar invertases not only mobilize sucrose and control sugar composition in fruits and storage organs, but also play a role in osmoregulation, cell enlargement, and cold sweetening. Cell wall bound invertases are considered to be key enzymes involved in sucrose unloading, wounding, infection response, and cell differentiation. The cytosolic invertase is most likely involved in the regulation of intracellular glucose and fructose levels in mature tissues and channeling sucrose into catabolism, in which sucrose synthase activity is low. The presence of multiple isoforms of invertase might confer a great flexibility to the control of sugar metabolism,

¹Both authors contributed equally to this work.

*Corresponding author. Tel: 886-2-23634729; Fax: 886-2-23634729; E-mail: SUNGHY@ccms.ntu.edu.tw

translocation, and storage in different tissues, organs, stages of plant development, and under various environmental conditions.

Plant invertases gene expression and enzyme activity are both known to be influenced by a variety of intracellular and extracellular factors such as end-products, substrate (Isla et al., 1991; Burch et al., 1992; Roitsch et al., 1995), hormones (Ehness and Roitsch, 1997), wounding, pathogens (Sturm and Chrispeels, 1990), temperature (Bournay et al., 1996), gravity (Wu et al., 1993), light (Krishnan et al., 1985), water stress (Hatch et al., 1963), low oxygen (Zeng et al., 1999), and proteinaceous inhibitors (Weil et al., 1994; Greiner et al., 1998). Analysis of some of the purified proteins on denaturing SDS gels under reducing conditions revealed the presence of proteolytic fragments (Unger et al., 1994). Whether fragmentation has a physiological function is not clear.

The purification of cell-wall invertases from several plant tissues and organs has been described (Jaynes and Nelson, 1971; Ricardo and ap Rees, 1970; Doehlert and Felker, 1987; Weil and Rausch, 1990). Several types of invertase have been purified and characterized from rice (*Oryza sativa*) (Lin and Sung, 1993; Sung and Huang, 1994; Chen and Sung, 1996; Lin et al., 1999). Suspension cultures of plant cells are suitable for a variety of biochemical investigations. Such a system has been used to investigate the various types of invertase in suspension cell cultures derived from rice grain. This paper reports the purification to apparent homogeneity and characterization of a novel alkaline bound invertase (ITab) from rice suspension cells. Molecular, kinetic properties and the mechanism of protein activation are investigated, and the comparison of ITab with other cell wall invertases is discussed.

Materials and Methods

Plant Materials and Reagents

Rice (*Oryza sativa* L. cv. Tainong 67) was grown in the field at the Experimental Farm of the National Taiwan University. The mature grains were harvested and dehydrated under sunlight. The dry grains were frozen for storage at -20°C. Chemicals and enzymes for the coupling assay of invertase activity were purchased from Sigma and Boehringer. Reagents for PAGE were purchased from Bio-Rad. Con A-Sepharose and DEAE-Sephacel were obtained from Pharmacia. All chemicals were of reagent grade.

Induction of Callus and Suspension Cells from Grains

Calli from rice grains were initiated by the following method. The rice grains were surface-sterilized in 70% ethanol for 10 min, then in a solution of 1% sodium hypochlorite for hypersonication at 4°C for 45 min, and washed three times with sterile water. The basal medium was solid MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 2 ppm 2, 4-D, and 0.8%

agar. The pH was adjusted to 5.8 with 1M KOH before autoclaving (20 min at 120°C). Rice grains were placed on solid medium in a Petri dish (145×20 mm) with 80-90 mL of medium and cultured in dim light at 25°C. Callus cultures were initiated for 3-4 weeks after inoculation of rice grains. Buds were removed and then subcultured every 4 weeks on MS medium. Seven g rice calli were cut into small pieces, inoculated into 200 mL of N6 liquid medium including 3% sucrose in a 1,000 mL Erlenmeyer flask, and agitated on an orbital shaking incubator at 120 rpm under dim light at 25°C. The suspension cultures thus obtained were subcultured every 7 days.

Extraction and Purification of Bound form of Invertase

Unless otherwise stated, all procedures were performed at 4°C. Cells, cultured for 7 days, were separated from medium by vacuum filtration and washed with deionized water several times. They (100 g fresh weight) were then homogenized with 2 volumes of buffer A (50 mM sodium phosphate, pH 7.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol) containing sea sand in a mortar. The crude homogenate was centrifuged at 22,000 g for 30 min. The precipitate, containing cell-wall and membrane fragments, was washed several times with buffer A and collected by centrifugation at 22,000 g for 20 min until no invertase activity was detectable in the supernatant. Solids were resuspended in buffer A containing 1 M NaCl with gentle stirring for 5 h. After centrifugation (22,000 g, 20 min), proteins in the supernatant were fractionated by the addition of solid ammonium sulfate. 0-40% saturation fractionation was dissolved in a small volume of buffer A and dialyzed overnight against the same buffer. After centrifugation of dialyzed solution, the supernatant was applied to a Con A-Sepharose column (Pharmacia, 1.6×10.0 cm) equilibrated with buffer A containing 0.5 M NaCl. This step separated the alkaline invertase (non-retained) from the acid invertases (retained). The column was washed with buffer A (containing 0.5 M NaCl) at a flow rate of 25 mL/hr. The acid invertase was eluted with a linear gradient of 0-0.3 M α -methyl-D-mannoside in buffer A (containing 0.5 M NaCl). Fractions containing alkaline invertase activity (non-retained) were pooled, dialyzed, and concentrated by ultrafiltration with an Amicon YM-10 membrane. The solution was applied to a Sepharose CL-6B column (2.6 cm × 90 cm) pre-equilibrated with buffer A. The column was eluted with the same buffer at a flow rate of 25 mL/hr, and fractions (3 mL each) in the active peak were collected and pooled. The alkaline invertase fraction was loaded on a DEAE-Sepharose column (2.6 × 18 cm) pre-equilibrated with buffer A. The column was washed with buffer A at a flow rate of 25 mL/h until A_{280} value decreased to a steady level. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl in buffer A, and 3 mL fractions were collected. The fractions containing alkaline invertase activity were pooled and concentrated by ultrafiltration (Amicon YM-10).

Invertase Assay

In a total volume of 0.36 mL, the assay mixture contained 60 μ L of enzyme solution, 0.05 M sodium phosphate, pH 8.0 (for alkaline invertase) or 0.1 M sodium acetate, pH 5.0 (for acid invertase), and 0.2 M sucrose. The reaction was allowed to proceed for 10 min at 37°C. The amount of reducing sugar produced was measured using the Somogyi-Nelson method (Nelson, 1944). One enzyme unit was defined as the amount of enzyme that catalyzed the production of 1 μ mole of reducing sugar per min at 37°C and at the optimum pH. A standard curve was established for an equimolar mixture of glucose and fructose.

Protein Determination

Protein concentration was determined by the method of Bradford (Bradford, 1976) using BSA as standard and by absorption at 280 nm in the column fractions.

Inhibition Kinetics Study

In a total volume of 0.36 mL, the reaction mixture contained 60 μ L of enzyme solution, 50 mM sodium phosphate, pH 8.0, 0.025–1.0 M sucrose, and 60 μ L of 0–10.0 mM fructose or 0–10.0 mM glucose. Incubations were done at 37°C for selected time lengths. The amount of glucose released was determined by the glucose oxidase method (Ebell, 1969).

Molecular Mass Determination

The molecular mass of the enzyme was measured on a calibrated Superose 6 column. 200 μ L of purified enzyme, together with the molecular weight standard proteins, thyroglobulin (669 kDa), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), and carbonic anhydrase (29 kDa) were loaded. The enzyme activity and A_{280} in each fraction were measured and elution volumes for marker proteins and the enzyme were calculated.

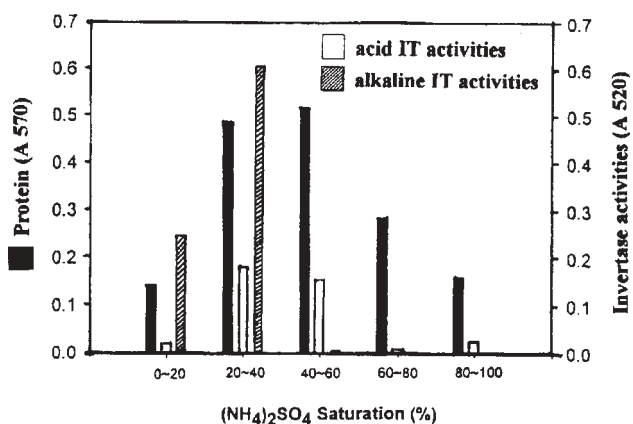


Figure 1. Distribution of ammonium sulfate fraction profile of invertase activity from rice suspension cells. Acid ($\square$) and alkaline (hatched) bound invertases activities and protein content ($\blacksquare$) are shown.

Gel Electrophoresis and Isoelectric Focusing

SDS-PAGE and Native-PAGE were performed according to Laemmli (Laemmli et al., 1970). For isoelectric focusing (IEF), the procedure of the instruction manual of Pharmacia Ampholine PAGplate was followed. The Pharmacia gel plate containing Ampholine in the range of pH 3.5–9.5 was used. Proteins were stained with either Coomassie Blue R-250 or a silver staining kit (for protein).

Results

Purification of Alkaline Bound Invertase

Bound invertases are assumed to be immobilized on the cell wall or cell membrane by ionic bonding. Some of them can be solubilized by treatment with a buffer solution of high ionic strength (1 M NaCl) or one containing a chelating agent such as EDTA, but considerable activity often remains in the residual cell fragments and is not extractable and is, therefore, regarded as being covalently bound to the cell fragments. The cell-wall and membrane fragments of rice suspension cells obtained after extracting soluble invertases were suspended in buffer A containing 1 M NaCl and gently shaken at 4°C for 5 h. One of the bound forms of the invertases could be released with 1 M NaCl solution. The results of purification steps, ammonium sulfate fractionation, Con A-Sepharose affinity chromatography, and Sepharose CL-6B chromatography are presented in Figures 1, 2 and 3, respectively. The alkaline bound invertase (ITab) did not have affinity toward Con A and thus could be separated from the Con A binding acid forms. The final step of DEAE-Sepharose chromatography was an effective step; a large amount protein could not be retained on the DEAE matrix, while the enzyme was eluted at 0.42 M NaCl (Figure 4). Its native-PAGE and SDS-PAGE patterns are shown in Figure 5. The results of the stepwise purification of the NaCl-released invertase are summarized in Table 1.

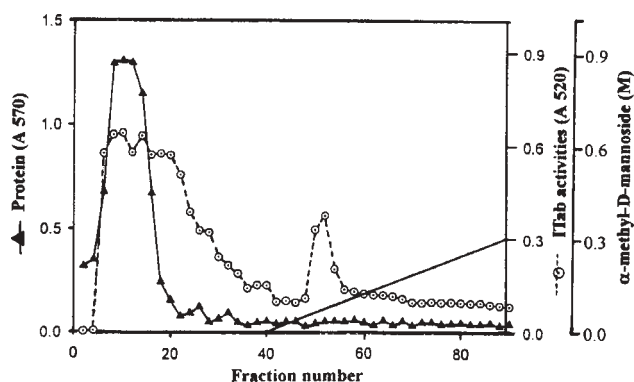


Figure 2. Affinity chromatography of alkaline bound invertase (ITab) from rice suspension cells on a Con A-Sepharose column. ITab ($\odot$) activity and protein content ($\blacktriangle$) are shown. The full line represents the α -methyl-D-mannoside gradient from 0 to 0.3 M.

Table 1. Purification of ITab from rice suspension cells.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	56				
1 M NaCl elution	20.8	278.8	0.075	1	100
Ammonium Sulfate precipitation	15.1	118.5	0.13	1.7	79.5
Con A-Sepharose unbound	11.7	64.6	0.181	2.4	56.1
Sepharose CL-6B	7.9	4.7	1.6	21.4	36.4
DEAE-Sepharose	6.6	0.76	8.7	116.4	31.6

^a Data are obtained from 100 g rice suspension cells.

^b One unit of enzyme was defined as the amount of enzyme required for formation 1 μ mole of reducing sugars from sucrose at 37°C and pH 8.0 per min.

Molecular Mass and pI Value

The results showed that the molecular mass of native enzyme was 250 kDa after fast protein liquid chromatograph (Superose 6). In denaturing SDS-PAGE, it produced a single band with the same subunit apparent molecular mass (60 kDa) (Figure 5B). The pI value of the enzyme as analyzed by IEF was 4.7 (Figure 6).

Kinetic Properties and Substrate Specificity

The optimum pH of alkaline bound invertase was 7.6. It also had the highest stability at pH 6.0. Thermal stability of the enzyme decreased as the incubation time and temperature increased. When the incubation temperature reached 70°C, the enzyme lost over 95% of its activity. The purified enzyme responded to sucrose concentration according to Michaelis-Menten kinetics up to 0.1 M, but showed substrate inhibition when the sucrose concentration was higher than 0.5 M (Figure 7). The apparent Km value for sucrose determined from the Lineweaver-Burk double reciprocal plot was 15.28 mM (Figure 7). In that case, sucrose was the preferred substrate, but the affinity for raffinose and stachyose was lower. None of the preparations hydrolyzed α -glucosides such as maltose.

Table 2. Effect of some compounds on alkaline bound invertase.

Compounds	Concentration (mM)	Relative activity (%)
Control	-	100
Aniline	5	92
	10	85
Pyridoxine.HCl	1	87
	2	84
	4	83
AgNO ₃	0.01	94
	0.05	56
	0.1	15
CuSO ₄	1	95
	2.5	85
	5	83
HgCl ₂	0.05	76
	0.1	66
	0.2	26
PCMBS	0.5	80
	1	60
DTT	1	107
	2	110
	4	117

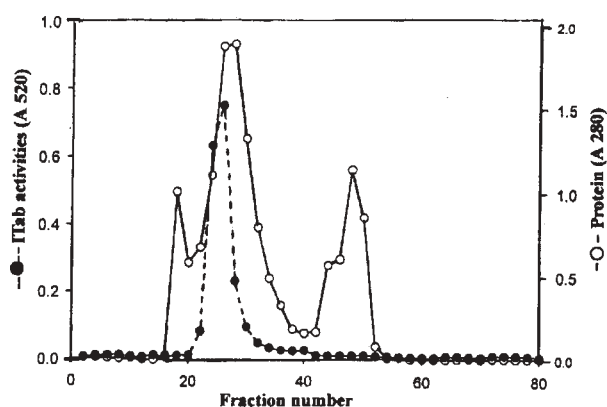


Figure 3. Gel filtration of alkaline bound invertase (ITab) from rice suspension cells on a Sepharose CL-6B column. ITab activity (●) and protein content (○) are shown.

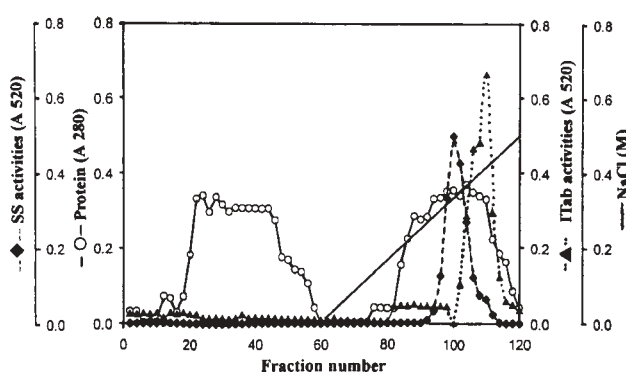


Figure 4. Anion exchange chromatography of alkaline bound invertase from rice suspension cells on a DEAE-Sepharose column. ITab (▲) and sucrose synthase: SS (◆) activities and protein content (○) are shown. The full line represents the NaCl gradient from 0 to 0.5 M.

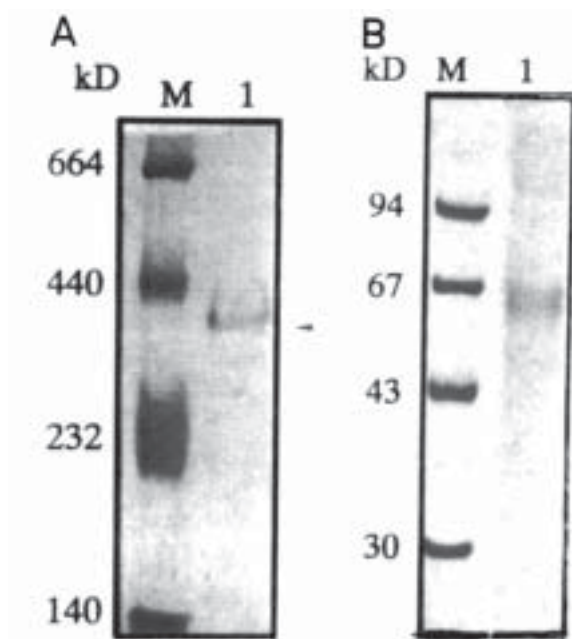


Figure 5. Native-PAGE and SDS-PAGE of alkaline bound invertase (ITab) fraction separated after DEAE-Sepharose chromatography. Approximately 10 μ g of total protein were loaded in each lane. Proteins were stained with Coomassie blue. Panel A: Native-PAGE. Mr, molecular mass markers in kDa; Lane 1, fraction no. 110. Panel B: SDS-PAGE. Mr, molecular mass markers in kDa; Lane 1, fraction no. 110.

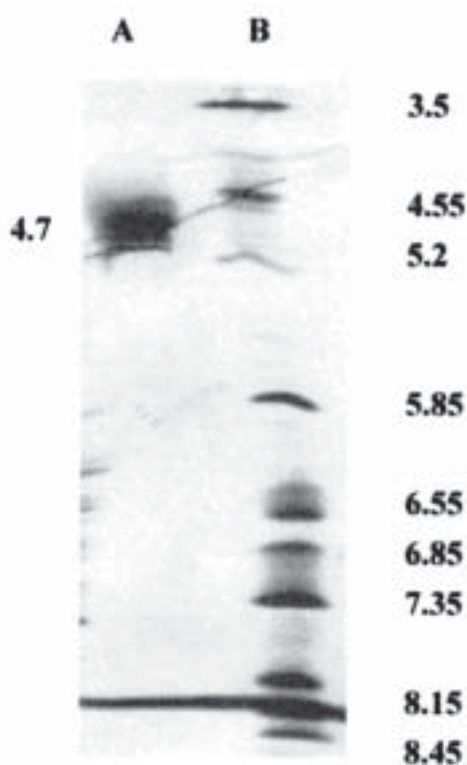


Figure 6. Isoelectric focusing of alkaline bound invertase (ITab) from rice suspension cells in the Ampholine PAGplate, pH 3.5–9.5. Lane A: ITab. Lane B: IEF marker.

Effect of Reaction Products on Invertase Activities

Fructose and glucose were inhibitors (Figure 8). Five mM of fructose or glucose was able to inhibit 50% of ITab activity. These results indicated that the enzyme activity could be modulated by end-products.

Absence of Glycoconjugates

Most purified plant acid invertases were reported to be glycoproteins, but the glycosylation status of alkaline invertase was uncertain. Acid and alkaline invertases of rice with and without glycoconjugates could be distinguished by their behaviors in the Con A-Sepharose chromatography (Figure 2). We thus conclude that the rice alkaline bound invertase is not a glycoprotein.

Effect of Metal Ions and Thiol Reagents on Invertase Activities

The effects of metal ions and various chemicals on the activity of the alkaline invertase are shown in Table 2. Cu^{2+} had no effect on the enzyme activity, while heavy metal ions, especially Ag^+ and Hg^{2+} , reduced the activity. Several reagents known to inhibit plant invertases (Karupiah et al., 1989; Lin and Sung, 1993; Sung and Huang, 1994; Chang et al., 1994) were tested (Table 2). At a high concentration level of 5 mM, pyridoxine and aniline barely reduced the enzyme activity, but at a low concentration level (1 mM), the PCMBs reduced the activity to 60%. The thiol reagents inhibited enzyme activity, but DTT restored it. Treatment with BSA enhanced enzyme activity.

Discussion

This is the first report to study alkaline bound invertase (ITab). The unbound invertase (38.5% of total inver-

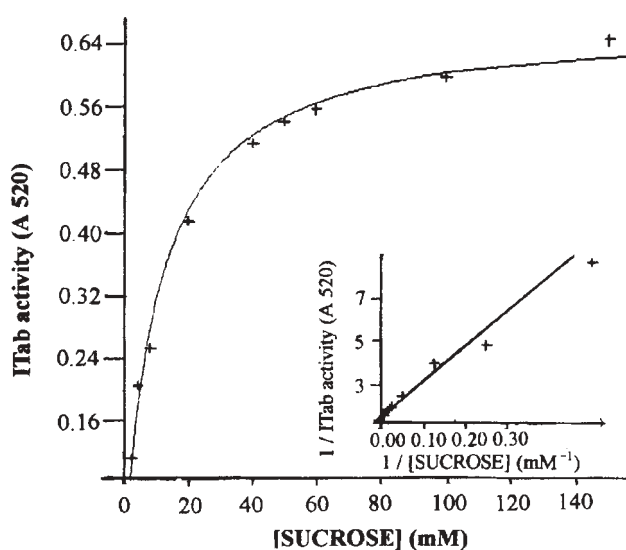


Figure 7. Effect of sucrose concentrations on activity of alkaline bound invertase (ITab) and double reciprocal plot of the sucrose concentration dependence of alkaline bound invertase.

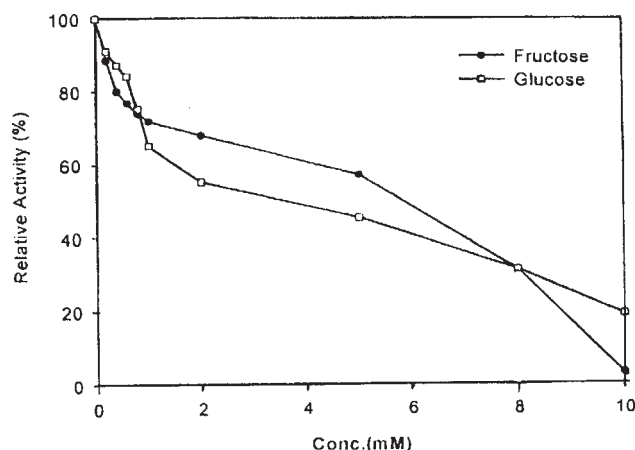


Figure 8. Effect of glucose and fructose concentration on alkaline bound invertase (ITab) activity.

tase activity) and bound invertase (61.5% of total invertase activity) were analyzed in rice suspension cells. During cultivation, both alkaline bound and acid bound invertase activities could be detected on the second day and reached maximal levels on the fourth day. ITab activity could be solubilized stepwise by treatment with 1 M NaCl and purified to apparent homogeneity through the steps of ammonium sulfate fractionation, Con A-Sepharose affinity chromatography (non-retained), Sepharose CL-6B chromatography, and DEAE-Sepharose chromatography (Figures 1, 2, 3 and 4). It was purified 116 fold from crude extract of rice suspension cells (Table 1).

The molecular mass of the enzyme is 250 kDa, and that of the subunit is 60 kDa (Figure 5B), indicating the enzyme is homotetrameric. The ITab could not bind to the Con A-Sepharose column (Figure 2), so it is not a glycoprotein. The pI value of the enzyme is 4.7 (Figure 6). The optimal reaction temperature was 40°C.

The phenomenon of product inhibition for invertases has long been recognized. We found that fructose and glucose inhibited ITab (Figure 8). Kinetic studies (Lin et al., 1999) have shown fructose was a competitive inhibitor while glucose was a non-competitive inhibitor. Our data showed substrate active ITab when the sucrose concentration was lower 0.5 M (Figure 7). This supports the view that the enzyme is a regulatory agent of hexose and sucrose levels in plant cells.

The inhibitory effects (Table 2) exerted by heavy metal ions, Tris and basic and acidic amino acid-affecting reagents are all in agreement with enzymes from other plants (Chen and Black, 1992). Thiol reagents inhibited enzyme activity, but DTT restored it, suggesting the presence of sulfhydryl group(s) either in an active or nearly active site. The sulfhydryl group of a cysteine residue was found in the highly conserved catalytic domain (WECV/PD) of putative invertase proteins (Sturm, 1999), which has been proposed to be important for the catalytic function of invertase. Details on the mechanism of inhibition are unknown and need further study.

We have isolated several invertase isoforms in rice. They include one soluble alkaline form (IT7), two soluble acid forms (IT4 and IT5) and one cell wall-bound form (ITb) in the milky stage grains (Charng et al., 1994; Sung and Huang, 1994). In leaves, there are three soluble acid invertase forms (IT I, IT II and IT III) and one cell wall-bound form (IT IV) (Lin and Sung, 1993). Suspension cells possess two soluble acid invertases (Type I and Type II) (Chen and Sung, 1996). Etiolated seedlings have one soluble alkaline form (AIT) (Lin et al., 1999). ITab, IT7 and AIT are similar in optimum pH, molecular mass, pI value, and in not being glycoproteins (not bound to Con A-Sepharose), but their distributions in the cell compartment are different. ITab is different from the other cell wall invertases of rice like ITb and IT IV in optimum pH, molecular mass, pI value, and glycosylation although they could be released from cell-walls and membranes by treatment with 1 M NaCl or 5% EDTA.

Four putative genes of cell-wall invertase (*Incw1,2,3* and 4) in maize have been isolated (Kim et al., 2000). It is interesting that an acid isoelectric point deduced from the INCW4 protein sequence is neutral or negatively charged in a cell-wall environment where the pH is acidic. Most cell wall invertases have positive charges leading to ionic binding with cell-wall components. This suggests that the *Incw4* gene may encode a new type of cell wall invertase. This observation is consistent with the report of contaminating cell-wall invertase in the soluble fraction in suspension cultures and developing maize kernels (Carlson and Chourey, 1999). Like INCW4, ITab has an acid pI, in contrast to most cell wall invertase pI values (pI value > 7). Because membrane fragments besides cell wall fragments are pelleted by centrifugation at 22,200 g for 30 min, we think that ITab may be a novel membrane-bound, as opposed to cell-wall bound, invertase and that it combines with four INCW4-like subunit (60 kDa) proteins. Reasons for the difference in pI values are unknown and need further study.

Our results indicate that ITab only exists in cell culture. It is possible that suspension cells are not grown in normal physiological conditions and ITab in this case may be also affected by culture conditions such as hormones and nutrients. What is the function of alkaline bound form invertase and why does it exist in cell membrane? We still don't know the exact reason. Answers to these questions may only determine the N-terminal amino acid residue of the enzyme. The partial amino acid sequence could be used to prepare specific probe and screen full-length cDNA of ITab from rice suspension cells. Finally, the antibody could be obtained from recombinant protein of ITab. Using immunochemistry and in-situ hybridization, the subcellular localization and function of ITab could become known. Alternatively, protoplasts studies derived from rice suspension cells could indirectly confirm the localization of ITab.

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水稻懸浮細胞內一種新型態的結合性蔗糖轉化酶

蕭正忠 傅如輝 宋賢一

國立台灣大學農業化學系

以台農 67 號水稻懸浮細胞為材料，酵素粗抽後所得的細胞碎片，以 1.0 M NaCl 溶離，溶離液經硫酸銨分劃、Con A-Sepharose 親和層析（未吸附部分）、Sepharose CL-6B 膠體過濾及 DEAE-Sepharose 陰離子交換等管柱層析進行酵素純化，可得一純化倍數約 116 倍的新型態鹼性結合性轉化酶（alkaline bound invertase，簡稱 ITab）。以 FPLC 管柱（Superose 6）測定蛋白質的原態分子量為 250 kDa，以 SDS-PAGE 分析推測其單元體分子量約為 60 kDa，得知 ITab 可能由同質四元體組成。其 pI 值為 4.7，最適作用 pH 值為 7.6，最適溫度在 40-50°C 之間。能夠水解蔗糖與棉仔糖，但對麥芽糖沒有作用，故為 β -fructosidase。此酵素不被 Con A 所吸附，可能不為醣蛋白。對蔗糖的 K_m 值為 15.28 mM。產物果糖及葡萄糖對其活性有抑制作用。外加 BSA 對酶素有活化的效果，而 Tris、重金屬離子及影響硫氫基的試劑對其活性有抑制作用，而 DTT 對其有活化作用，表示 ITab 的活性部位可能有硫氫基直接參與蔗糖的水解，或是活性部位附近的硫氫基和試劑鍵結，間接影響水解蔗糖的作用。

關鍵詞：水稻；結合性蔗糖轉化酶；蔗糖代謝；酵素純化。