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Purification and Characterization of Extracellular Invertase from the Hypocotyls of Mung Bean (*Phaseolus radiatus* L.)

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The extracellular invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) was isolated and characterized from the hypocotyls of mung bean (*Phaseolus radiatus* L.). The enzyme was purified to apparent homogeneity by ammonium sulfate fractionation and sequential chromatography over diethylaminoethyl (DEAE)-cellulose anion exchange, Concanavalin (Con) A-Sepharose 4B affinity and Sephadex G-200. The overall purification was about 77-fold with a recovery of about 11%. The finally purified enzyme exhibited a specific activity of about 113 μmol of glucose produced mg^{-1} protein min^{-1} at pH 5.0 and appeared to be a single protein by nondenaturing polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE. The enzyme had the native molecular mass of 134 kD and subunit molecular weight of 67 kD as estimated by Sephadex G-200 chromatography and SDS-PAGE, respectively, suggesting that the enzyme was composed of homodimeric proteins. On the other hand, the enzyme appeared to be a glycoprotein containing mannosyl residues on the basis of its ability to interact specifically with the immobilized Con A and the separability of invertase-Con A complex by methyl-α-D-mannopyranoside. The enzyme had a K_m for sucrose of 3.4 mM and its pH optimum of 4.0. The enzyme showed highest enzyme activity with sucrose as substrate. Raffinose and cellobiose were hydrolyzed at a low rate, maltose and lactose were not cleaved by the enzyme. These results indicate the extracellular invertase is a β-fructofuranosidase.

Keywords: extracellular invertase, purification, mung bean, hypocotyls

INTRODUCTION

Invertases (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) play an important role for hydrolysis of sucrose, processing a terminal unsubstituted β-D-fructofuranosyl residue into its constituent monosaccharides, glucose and fructose. They are present in multiple forms in given tissues (Avigad, 1982). Two major types are usually distinguished based on their optimal pH to be nomenclatured to acid invertase (pH 4.0~5.0) and alkaline invertase (pH 7.0~8.0) (Chin and Weston, 1973; Matsushita and Uritani, 1974). The former can be subdivided into intra- and extracellular forms according to its subcellular localization (Chin and Weston, 1973; Unger *et al.*, 1992).

Extracellular invertase bound ionically to cell wall gives rise to maintain a steep sucrose concentration gradient between source and sink organs in phloem unloading in the case of sucrolysis prior to sucrose uptake (Fahrendorf and Beck, 1990; Sturm and Chrispeels, 1990). The enzyme known to play a role for sucrose translocation and cleavage, occurs in rapidly growing tissues with a high demand for hexoses, such as in extension zone of root tip and extending internodes, in the sites of emerging secondary roots, in developing tap roots, and in developing and expanding leaves (Lemoine *et al.*, 1988; Sung and Huang, 1994). Also, high activity of the enzyme is found in barley elongating stem tissues (Karuppiah *et al.*, 1989), carrot cells (Lauriere *et al.*, 1988), suspension-cultured tobacco cells (Weil and Rausch, 1994) and spruce root cells (Salzer and Hager, 1993).

In order to better understand the biochemical characteristics of the extracellular invertase partially purified preparations of the enzyme have been obtained from some plants using ammonium sulfate fractionation and DEAE-cellulose chromatography

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(Krishnan *et al.*, 1985). The enzyme has been successfully purified from barley elongating stem tissues, and suspension-cultured tobacco cells (Salzer and Hager, 1993; Weil and Rausch, 1994; Weil *et al.*, 1994). Despite of the extensive physiological and biochemical works on the extracellular invertase, the biochemical and molecular properties of these enzymes purified from plant tissues are still limited. Recently, a full length of cDNA encoding maize cell wall-bound invertase has been isolated and sequenced to clarify the molecular structure (Shanker *et al.*, 1995). Also, a detailed enzymological comparison of the intracellular (soluble) and extracellular (cell wall-bound) acid invertases in the same tissue is still lacking though the only sources, intra- or extracellular invertase, having been intensively investigated (Weil and Rausch, 1994; Isla *et al.*, 1995; Tang *et al.*, 1996). Therefore, we performed the isolation and extensive purification of extracellular invertase from the hypocotyls of mung bean (*Phaseolus radiatus*) to understand the biochemical characteristics.

In the present report, we describe the purification and biochemical characterization of extracellular invertase from the hypocotyls of mung bean as well as a comparison of the biochemical properties of extracellular invertase and intracellular acid invertase extensively purified in the same plant tissues (Lee and Kim, 1995).

MATERIALS AND METHODS

Plant Material and Reagents

Seeds of mung bean (*Phaseolus radiatus* L.) were germinated and grown in the dark as described previously (Lee and Kim, 1995). The elongating hypocotyls of 10 day-old seedlings were used as experimental materials.

Chemicals and assay enzymes were obtained from Sigma and Boehringer Chemical Co. Reagents for electrophoresis and chromatography such as DEAE-cellulose, Con A-Sepharose 4B and Sephadex G-200 chromatography were purchased from Sigma.

The buffer solutions used were as follows: buffer A, 20 mM sodium phosphate (pH 7.0), 1 mM Mg-acetate, 1 mM Na-ethylenediaminetetraacetic acid (EDTA), 0.3 M NaCl, 1% Triton X-100, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonylfluoride (PMSF); buffer B, 10 mM sodium phosphate (pH 7.0), 1 mM Na-EDTA and 1 mM PMSF; buffer C, 100 mM sodium phosphate (pH 7.0), 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM PMSF; buffer D, 50 mM sodium phosphate (pH 7.0) and 1 mM PMSF.

Assays for Invertase Activities and Protein

Invertase activity was determined by measuring glucose content formed from sucrose hydrolysis as previously described (Lee and Kim, 1995). For the assay of extracellular invertase, reaction mixtures containing 50 mM phosphate-citrate buffer, pH 5.0, 1 mM Mg-acetate, 100 mM sucrose and a suitable amount of enzyme solutions were incubated at 25°C for 30 min, and then boiled for 3-5 min to stop reaction. Glucose formed was detected by the modified glucose oxidase-peroxidase method (Bergmeyer and Bernt, 1974), and quantitated by measurement of absorbance at 540 nm. A unit is defined as the formation of 1 mol glucose from sucrose per min per 1 mL of enzyme solution at 25°C at pH 5.0 for extracellular invertase.

The amount of protein was determined according to the modified Bradford method (1976) using BSA (bovine serum albumin) as the standard protein.

Crude Invertase Extraction

Approximately 5 kg of elongating hypocotyls rinsed three times with distilled water were homogenized in buffer A with a ratio of elongating hypocotyls to buffer A of 1:1 (w/v). The homogenate was filtered through four layers of cheesecloth, then the slurry residues, which was used for the preparation of extracellular invertase, was extracted two times with the same buffer and subsequently rinsed with distilled water until the effluent was free of soluble protein. From the suspension, extracellular invertase was extracted by 12~18 h incubation in 25 mM Tris-HCl buffer (pH 8.0) which contained a high concentration (1.5 M) of NaCl, in addition to 1 mM Mg-acetate, 1 mM Na-EDTA, 1 mM DTT and 1 mM PMSF. The suspension was centrifuged at 12,000 rpm for 20 min, and then the supernatant was designated as the crude extract of extracellular invertase (Lauriere *et al.*, 1988). All purification steps were carried out at 4°C.

Separation of Extracellular Invertase by Ammonium Sulfate Precipitation

Crude extract, which was prepared from about 5 kg of mung bean hypocotyls, was precipitated from 30 to 80% saturation with enzyme grade $(NH_4)_2SO_4$

powder. The precipitates were collected after centrifugation, and dissolved in buffer B, then dialyzed overnight against the same buffer.

DEAE-Cellulose Chromatography

The protein solutions with invertase activity were applied to DEAE-cellulose column (3×15 cm) preequilibrated with buffer B. The column was washed with the same buffer to remove unaimed proteins, then eluted to a 1 L linear gradient, 0 to 1.0 M NaCl in buffer B at a flow rate of 0.8 mL/min. 8 mL fractions, which occurred activity peak at pH 5.0, were precipitated to 80% saturation with ammonium sulfate, and then dissolved in buffer C. The precipitates were dialyzed against the same buffer or desalted with a Sepadex G-25 column.

Con A-Sepharose 4B Chromatography

About 10 mL of concentrated enzyme solutions obtained from DEAE-cellulose chromatography were applied to the Con A-Sepharose 4B column (1.5×10 cm) previously equilibrated with buffer C. The column was washed with the same buffer until the A_{280} was decreased to minimum level, and then eluted with 0.2 M methyl-α-D-mannopyranoside in buffer C at a flow rate of 0.5 mL/min. 5 mL fractions were concentrated by ammonium sulfate precipitation as above. The precipitates dissolved in buffer D were dialyzed against the same buffer.

Sephadex G-200 Chromatography

About 5 mL of the enzyme solutions obtained from Con A step were carefully loaded to the top of the Sephadex G-200 column (1.5×90 cm) preequilibrated with buffer D. The column was run at a flow rate of 0.1 mL/min. 2 mL fractions, which showed activity peak at pH 5.0, were pooled and concentrated by Amicon ultracentrifugation (Amicon Diaflo ultrafiltration membranes, 10 XM50, 43 mm).

Native Molecular Weight Estimation

The native molecular weight of the enzyme was determined by using Sephadex G-200 chromatography as described in Lee and Kim (1995) except that 2 mL fractions were used to measure the A_{280} and extracellular invertase activity after collection. The column was calibrated with ferritin (450 kD), catalase (240 kD), aldolase (158 kD), BSA (68 kD), albumin from hen egg (45 kD), chymotrypsinogen A (25 kD), and cytochrome C (12.5 kD) as standard markers.

Electrophoresis

SDS-PAGE was performed by the modified method of Ausubel *et al.* (1987). Proteins were separated on a 12% resolving gel at 120V. After electrophoresis, the gels were stained with silver or Coomassie brilliant blue. The standard proteins used for SDS-PAGE were phosphorylase b (97.4 kD), albumin (66.2 kD), ovalbumin (42.7 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD) and α-lacalbumin (14.4 kD).

Nondenaturing PAGE were performed in a similar way to SDS-PAGE except that SDS and β-mercaptoethanol were not added to the gels.

RESULTS

Purification of Extracellular Invertase

The extracellular invertase could be isolated from the hypocotyls of mung bean with a high-ionic concentration buffer containing 1.5 M NaCl, and then fractionated by ammonium sulfate. Extracellular invertase activity measured at pH 5.0 mainly showed from 30 to 80% ammonium sulfate saturation, while intracellular acid invertase activity measured at pH 5.0 mainly showed from 50 to 70% ammonium sulfate saturation as described in Lee and Kim (1995). The similar separations using ammonium sulfate frac-

Table 1. Purification protocol of extracellular invertase in the hypocotyls of *Phaseolus radiatus*[a]

Steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Purfication (fold)	Yield (%)
Crude extract	188.67	280	1.48	1	100
30-80% ammonium sulfate precipitate	56.01	105	1.88	1.27	37.5
DEAE-cellulose chromatography	7.36	58	7.88	5.32	20.7
Con A chromatography	0.72	49	68.06	45.00	17.5
Sephadex G-200 chromatography	0.27	30.6	113.33	76.57	10.9

[a]From about 5 kg of elongationg hypocotyls

tionation have been obtained in some plants such as stinging nettles (Fahrendorf and Beck, 1990). Table 1 shows the protocol of extracellular invertase from mung bean hypocotyls, the enzyme was purified by consecutive step of DEAE-cellulose anion exchange chromatography, Con A affinity chromatography and gel filtration through Sephadex G-200 column. The purification of extracellular invertase was about 77-fold with a yield of about 11% from starting materials, while that of intracellular acid invertase was about 148-fold with a yield of about 15% (Lee and Kim, 1995). The low recovery may be a consequence of the small quantities of protein with which to work. The finally purified enzyme exhibited a specific activity of approximately 113 μmol of glucose produced mg^{-1} protein min^{-1} at pH 5.0, and showed a single band in nondenaturing PAGE (data not shown). The purified enzyme was composed of single type of subunits by SDS-PAGE (Fig. 1).

The extracellular invertase bound to DEAE-cellulose matrix was eluted with NaCl gradient ranging from 0.2 to 0.45 M because the enzyme was strongly bound to DEAE matrix compared to intracellular acid invertase which eluted with NaCl gradient ranging from 0.05 to 0.1 M (Fig. 2).

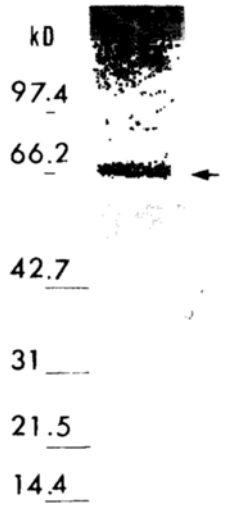

Fig. 1. SDS-PAGE of the purified extracellular invertase (indicated by the arrow) obtained from Sephadex G-200 chromatography. The gel was stained by silver. The standard proteins used for SDS-PAGE were phosphorylase b (97.4 kD), albumin (66.2 kD), ovalbumin (42.7 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD), α-lactalbumin (14.4 kD).

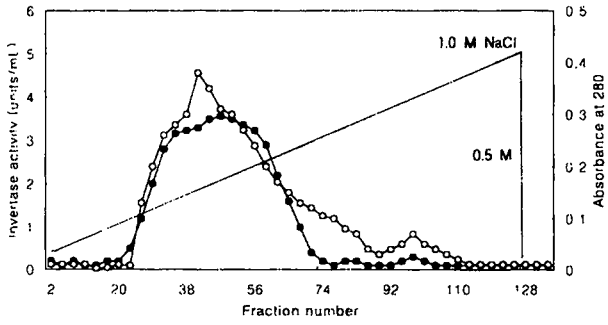

Fig. 2. DEAE-cellulose anion exchange chromatography of extracellular invertase in the hypocotyls of *Phaseolus radiatus*, with elution with a linear gradient from 0 to 1.0 M NaCl. Fractions (8 mL) were assayed for extracellular invertase activity (●) and protein content (○) by A_{280}.

The fractions with extracellular invertase activity peak were precipitated to 80% with ammonium sulfate, and the precipitates were loaded onto Con A-Sepharose 4B column (Fig. 3). Extracellular invertase bound to Con A-Sepharose 4B was eluted with 0.2 M methyl α-D-mannopyranoside. The strong binding of both enzyme onto Con A and the separability of invertase-Con A complex by methyl α-D-mannopyranoside suggest extracellular invertase is glycoprotein containing mannosyl residues.

There were some impurities in the pooled Con A-Sepharose 4B fractions. To remove contaminating proteins the fractions were subjected to gel filtration on Sephadex G-200 column (Fig. 4). The extracellular invertase, which was finally separated from other proteins, represented a single band as shown by nondenaturing PAGE (data not shown).

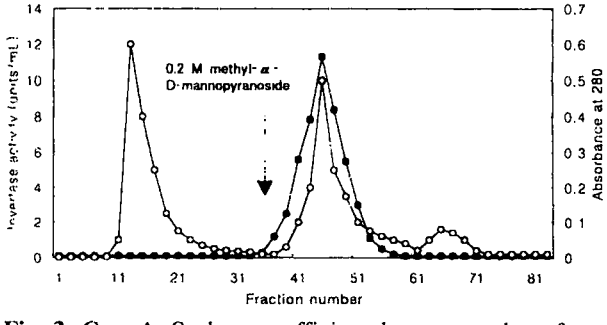

Fig. 3. Con A Sepharose affinity chromatography of extracellular invertase in the hypocotyls of *Phaseolus radiatus*, with elution with 0.2 M methyl-α-D-mannopyranoside. Fractions (5 mL) were assayed for extracellular invertase activity (●) and protein content (○) by A_{280}.

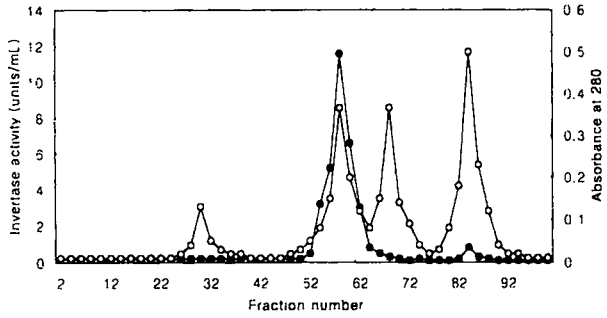

Fig. 4. Sephadex G-200 chromatography of extracellular invertase in the hypocotyls of *Phaseolus radiatus*. Fractions (2 mL) were assayed for extracellular invertase activity (●) and protein content (○) by A_{280}.

Molecular Weight Determination

The purified extracellular invertase, which shown a single band using native gel system, had a molecular mass of 134 kD in its native form, as determined by gel filtration through Sephadex G-200 column with protein standards (Fig. 5). A further separation of the enzyme using SDS-PAGE revealed that it contained single polypeptide chains of 67 kD on the basis of their mobility relatively to those of standard proteins (Fig. 1). There results indicate that the extracellular invertase is composed of single type of subunits, thus seems to be homodimeric protein of 67 kD polypeptides

pH and Substrate Specificity

The extracellular invertase exhibited highest levels

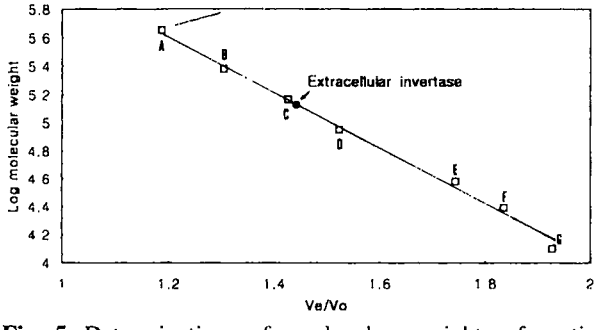

Fig. 5. Determination of molecular weight of native extracellular invertase by gel filtration in the hypocotyls of *Phaseolus radiatus*. Standard proteins (□) and extracellular invertase (●) eluted from Sephadex G-200 column. Fractions (2 mL) were assayed for extracellular invertase. A, ferritin (450 kD); B, catalase (240 kD); C, aldolase (158 kD); D, albumin from bovine serum (68 kD); E, albumin from hen egg (45 kD); F, chymotrypsinogen A (25 kD); G, cytochrome C (12.5 kD).

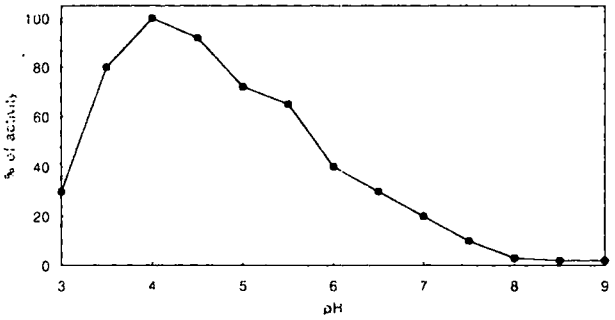

Fig. 6. The pH profile of extracellular invertase activities of *Phaseolus radiatus*. The buffers used included acetate (pH 3.0-5.0), sodium citrate (pH 5.5-6.5), HEPES (pH 7.0-8.0) and glycine (pH 8.5-9.0). The reactions were carried out at 25°C for 30 min.

of activity at pH 4.0 (Fig. 6), while intracellular invertase in the same tissues exhibited highest levels of activity around pH 5.0 (Lee and Kim, 1995). The kinetic properties of the extracellular invertase were evaluated using the Michaelis-Menten model within a sucrose concentration range of 0 to 30 mM. The enzyme activity fit Michaelis-Menten kinetics for sucrose and the K_m values for sucrose obtained from Lineweaver-Burk double reciprocal plots was about 3.4 mM (Fig. 7). Table 2 shows substrate specificity of the purified extracellular invertase from mung

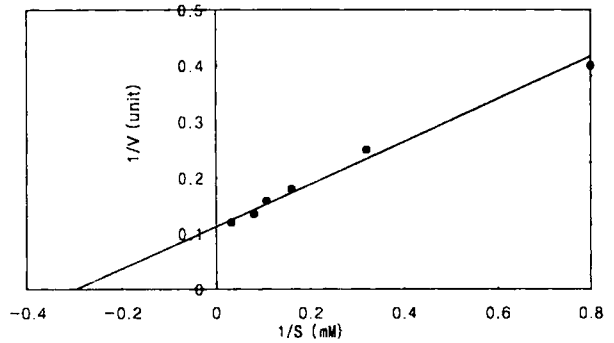

Fig. 7. Kinetics of purified extracellular invertase activities of *Phaseolus radiatus* versus sucrose concentration (0 to 30 mM).

Table 2. Substrate specificity of the purified extracellular invertase from the hypocotyls of *Phaseolus radiatus*

Substrate	Invertase activity (% with sucrose)
Sucrose	100
Raffinose	22
Cellobiose	3
Maltose	0
Lactose	0

bean hypocotyls. Its preferred substrate was sucrose where raffinose and cellobiose as substrate caused the hexose release of 22% and 3%, respectively, comparing to sucrose. Maltose and lactose were not cleaved by the enzyme. These results indicate, therefore, that the extracellular invertase is a β-fructofuranosidase.

DISCUSSION

Acid invertase activity in the hypocotyls of mung bean was shown to be associated with at least two enzyme forms. The extracellular invertase, solubilized from the cell wall in the presence of 1.5 M NaCl, exhibited a lower levels in the enzyme activity whereas intracellular invertase exhibited a higher levels in the enzyme activity at the same tissues (Lee and Kim, 1995). It appears likely that the intracellular invertase represents the major form because the evolution of its activity is correlated with changes in carbohydrate pools of some plants (Nielson *et al.*, 1991; Hubbard and Pharr, 1992). However, extracellular invertase also plays an important role for maintaining a steep sucrose concentration gradient between source and sink organs in phloem unloading in the case of sucrolysis prior to sucrose uptake (Fahrendorf and Beck, 1990; Strum and Chrispeels, 1990). To examine the biochemical characterization of extracellular invertase, the enzyme was subsequently purified by consecutive steps using ammonium sulfate fractionation, DEAE-cellulose anion exchange chromatography, Con A affinity chromatography and gel filtration through Sephadex G-200 column (Table 1). Con A affinity chromatography in the purification procedures represented a good increase in the specific activity. Surprising increase of specific activity may be attributed to the removal of endogenous extracellular invertase inhibitor through Con A-Sepharose 4B column. The possibility is also shown in some reports that extracellular invertase of *Arabidopsis* binds to the Con A matrix because of the glycosylation of the enzyme whereas endogenous inhibitor activity is found in the unbound fractions (Weil *et al.*, 1994). The finally purified extracellular invertase exhibited a specific activity of about 113 μmol of glucose produced mg^{-1} protein min^{-1} at pH 5.0, which was lower than intracellular acid form of the same tissues (Lee and Kim, 1995) and higher than extracellular form of melon fruits (Iwatsubo *et al.*, 1992). The specific activity of the enzyme could differ from different plant species as well as even the same plant, as shown in some report (Bracho and Whitaker, 1990).

Acid invertase had showed the diverse types in the enzyme configuration. The enzyme was monomer in spruce and tobacco (Salzer and Hanger, 1993; Weil *et al.*, 1994), dimer in barley (Karuppiah *et al.*, 1989), tetramer in wheat (Krishnam *et al.*, 1985), and heptamer in *Ricinus communis* (Prado *et al.*, 1985). The purified extracellular invertase from mung bean hypocotyls, which shown a single band in nondenaturing gel (data not shown), had a native molecular mass of 134 kD, as determined by gel filtration through Sephadex G-200 column (Fig. 5). And the enzyme, which showed a single band in the denaturing gel, had an estimated molecular weight of about 67 kD (Fig. 1), indicating that extracellular invertase was composed of two polypeptide chains. Therefore the enzyme has been identified to be homodimeric proteins. The fact agrees with the observations that the cell wall bound invertase of barley elongation stem tissues was composed of two subunits of 60 kD and the native molecular weight is estimated to be about 120 kD, thus the enzyme is dimers (Karuppiah *et al.*, 1989). On the other hand, intracellular acid invertase in the same tissues was monomeric proteins of 70 kD (Lee and Kim, 1995). Of interest is the surprising diversity in their native molecular mass ranging from 48.5 kD for radish seedlings (Faye *et al.*, 1981) to 450 kD for Lillium pollens (Singh and Knox, 1984). The variability may be due to glycosylated nature of the enzyme or different degrees of oligomerization of the enzyme subunits, though accurate reason for the diversity in their native from are not clear yet. Therefore, it is apparent that further studies should be directed toward an understanding of accurate molecular configuration and glycosylation status of extracellular invertase of mung bean hypocotyls.

The extracellular invertase from the hypocotyls of mung bean may be a glycoprotein containing mannosyl residues on the basis of the strong binding of both enzyme onto the immobilized Con A and the separability of invertase-Con A complex by 0.2 M metyl-α-D-mannopyranoside, as shown in the intracellular acid invertase of the same tissues as well as in the extracellular form of tobacco crown-gall cells (Weil and Rausch, 1994; Lee and Kim, 1995). The glycosylation of the enzyme may be enable to be located in the extracellular matrix of cell wall via Golgi complex, and necessory for its transport across the tonoplast or the plasmamembrane to a final location in apoplast (Avigad, 1982; Lauriere *et al.*,

1988). Also, higher degree of glycosylation may protect against digestion of protease (Faye and Chrispeels, 1989). However, we could not describe the glycan characterization of the enzyme and whether the enzyme contained N-linked or O-linked carbohydrate chains. Therefore, in order to elucidate the glycosylation status of the enzyme, further studies should be directed toward the deglycosylation of the enzyme with endo-β-N-acetylglucosaminidase H (Endo H, removal of high-mannose glycan), trifluoromethanesulfonic acid (TFMS, complete deglycosylation) and peptide-N-glycosidase F (PNGase F, removal of high-mannose and complex glycans).

The extracellular invertase of mung bean hypocotyls had maximum activity around pH 4.0 (Fig. 6), whereas intracellular acid invertase in the same tissue showed highest activity around pH 5.0 (Lee and Kim, 1995). These results are consistent with the proposal that extracellular invertases show optimal activity at a more acidic pH than do the intracellular form (Doehlert and Felker, 1987; Fahrendorf and Beck, 1990). The response versus sucrose concentration of the enzyme fit Michaelis-Menten kinetics and the K_m value for sucrose obtained from Lineweaver-Burk double reciprocal plots was about 3.4 mM (Fig. 7). This K_m value was similarly low with that of extracellular invertase from radish seedlings (Faye and Ghorbel, 1983). On the other hand, the enzyme displayed non-Michaelis-Menten kinetics in some plants because of the impurity of enzyme preparation (Chen and Black, 1992). The enzyme was more specific for sucrose with less activity for raffinose and cellobiose, and with no activity for maltose and lactose (Table 2), as shown in intracellular acid invertase in the same tissues. These results indicate, therefore, that the extracellular invertase from the hypocotyls of mung bean is a β-fructofuranosidase. It will be great interest to elucidate the accurate configuration and glycosylation status of the extracellular invertase. Therefore, further studies are needed on the molecular configuration, such as gene structure and glycan characterization in the extracellular invertase from the hypocotyls of mung bean.

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