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Purification and Characterization of Golgi Membrane-Bound Nucleoside Diphosphatase from Suspension-Cultured Cells of Sycamore (*Acer pseudoplatanus* L.)

Satoshi MIKAMI, Ryo SUGANUMA, Hidetaka HORI, and Toshiaki MITSUI*

Laboratories of Molecular Life Science, Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan
**Corresponding author* E-mail address: t.mitsui@agr.niigata-u.ac.jp

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Abstract

Inosine diphosphatase (IDPase) isoforms associated with Golgi membranes were studied in sycamore cell culture. These enzyme isoforms were solubilized with Triton X-100 and purified by chromatography using DEAE-Toyopearl and SOURCE-S columns. The isoforms were separated into two distinguishable fractions (peak 1 and 2) by SOURCE-S column chromatography. Furthermore the peak 1 contained at least two isoform bands detected by native-PAGE analysis. The apparent molecular sizes of these three isoforms were estimated by both gel filtration and SDS-PAGE to be 50 kDa, indicating that the Golgi membrane-bound IDPase has a monomeric structure. These IDPase isoforms required divalent cations (Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}) for their hydrolyzing activity, and were inhibited by ATP. IDP, UDP, and GDP were effective substrates for these enzymes. It is clearly indicated that the sycamore Golgi membrane-bound IDPase is a nucleoside diphosphatase.

Keywords: Cell culture, Golgi membranes, IDPase, NDPase, sycamore.

Introduction

The Golgi complex in plant cells is engaged in the processing and modification of oligosaccharide side chains of glycoproteins and the biosynthesis of complex cell-surface polysaccharides (Hawes and Satiat-Jeunemaitre, 1996; Dupree and Sherrier, 1998; Nebenfuhr and Staehelin, 2001). Recently, Golgi enzyme proteins, such as reversibly glycosylated polypeptide 1 (Dhugga *et al.*, 1997), xyloglucan fucosyltransferase (Faik *et al.*, 2000), GDP-L-Fuc:Asn-linked GlcNAc α1,3-fucosyltransferase (Leiter *et al.*, 1999), β1,2N-acetylglucosaminyltransferase I (Strasser *et al.*, 1999), and β1,2-xylosyltransferase (Strasser *et al.*, 2000) have been molecularly cloned and characterized in plants. In addition, the sugar-nucleotide transporter in pea Golgi complex has been also well-characterized biochemically (Wulff *et al.*, 2000). Nucleoside diphosphatase (NDPase), particularly IDPase, was cytochemically and biochemically identified as a Golgi membrane-bound enzyme in plant cells and has been used as an exclusive marker for Golgi membranes of higher plant cells (Ray *et al.*, 1969; Goff, 1973; Ali *et al.*, 1986; Brummell *et al.*, 1990; White *et al.*, 1993; Baydoun and Brett, 1997; Mikami *et al.*, 2001). Although it is a classically well-known enzyme, there is little information concerning its molecular structure and function. Rice Golgi IDPase has been purified and characterized, indicating that the enzyme is an NDPase, which can hydrolyze IDP, UDP, and GDP (Mitsui *et al.*, 1994). However, it is still obscure whether NDPase commonly exists in plant cells or not. In the present communication, we report the purification and characterization of sycamore Golgi membrane-bound IDPase.

Materials and Methods

Plant materials

Sycamore (*Acer pseudoplatanus* L.) cell culture was carried out using a rotary shaker operated at 150 strokes min^{-1} at 27℃ in darkness as described by Ali *et al.* (1985).

Assays

Activities of IDPase, UDPase, and GDPase were determined according to the methods described

previously (Mitsui *et al.*, 1994). NADPH-cytochrome c reductase was assayed following the procedure of Load *et al.* (1973). Acid phosphatase activity was detected by measuring the liberation of *p*-nitrophenol from *p*-nitrophenylphosphate (NPP) (Boller and Kende, 1979) or Pi (Mitsui *et al.*, 1994). The protein assay was carried out according to the method described by Bradford (1976) with bovine gamma globulin as a standard. The sucrose content was determined with a refractometer (Atago, NAT-1T).

Preparation and fractionation of microsomes

Two different sample preparations were used: (1) Sycamore cells (15 g) cultured for 6 days were directly homogenized with a half volume of 50 mM MOPS-NaOH (pH 7.3), 1 mM EDTA, and 0.5 M mannitol by a potter homogenizer. (2) The protoplasts were prepared from sycamore cells and mechanically disrupted in 50 mM MOPS-NaOH (pH 7.3), 1 mM EDTA, and 0.5 M mannitol, according to the procedure described by Ali *et al.* (1985). The homogenate was centrifuged at 200*g* for 10 min, 10,000*g* for 10 min, and 100,000*g* for 1 h, sequentially. The final resulting pellets were used as the microsome preparation.

The microsomal membranes prepared from the protoplasts (procedure 2) were resuspended with 0.8 ml of a buffered solution consisting of 50 mM glycylglycine (pH 7.5), 1 mM EDTA, and 0.5 M mannitol, and layered on a linear sucrose gradient (20-50%, w/w) containing 50 mM glycylglycine (pH 7.5) and 1 mM EDTA. The gradients were centrifuged at 85,000*g* using an SW 50.1 rotor (Beckman) for 3 h at 4℃. After centrifugation, each fraction (0.32 ml) collected from the top of the tube was subjected to the assays.

Enzyme Purification

The microsomal membranes prepared by the direct homogenization (procedure 1) were firstly washed with 50 mM Tris-maleate-NaOH (pH 6.0) containing 20% (w/v) glycerol, 0.3 M NaCl, and solubilized with 50 mM Tris-maleate-NaOH (pH 6.0) containing 20% (w/v) glycerol, 0.15 M NaCl, and 0.5% (w/v) Triton X-100. The suspension was centrifuged at 100,000*g* for 30 min. The resulting supernatant was used as the crude enzyme.

The crude enzyme dialyzed against solution 1 consisting of 50 mM Tris-maleate-NaOH (pH 6.0), 20% (w/v) glycerol, and 0.05% (w/v) Triton X-100 was applied onto a DEAE-Toyopearl 650 M (Tosoh) column (2.5 × 8 cm) equilibrated with solution 1. The pass through fraction was collected and subjected to a SOURCE-S (Pharmacia) column (1.5 × 2 cm) equilibrated with solution 1. The column was washed with 15 ml of solution 1, and then eluted with 20 ml of solution 1 supplemented with 60 mM and 100 mM NaCl at 4℃. Two peak fractions of IDPase were collected and pooled. All procedures were performed at 4℃.

Gel filtration

The peak fractions of IDPase were dialyzed against solution 2 consisting of 50 mM Tris-maleate-NaOH (pH 6.0), 20% (w/v) glycerol, 0.05% (w/v) Triton X-100, and 100 mM NaCl. The dialyzates were applied to a Toyopearl HW 60F gel filtration column (1.5 × 40 cm) equilibrated with solution 2 and eluted at a flow rate of 25 ml h^{-1} at 4℃.

Native- and SDS-PAGE

The procedure of native-PAGE was identical to the method described previously (Mitsui *et al.*, 1994). For detection of the NDPase enzyme bands on a gel after electrophoresis, the gel was incubated with a reaction mixture consisting of 80 mM Tris-HCl (pH 7.0), 4 mM nucleoside diphosphate, 4 mM $MgCl_2$, and lead nitrate for 5 min at 37℃. Then it was rinsed for 1 h in repeated changes of H_2O. The enzyme bands were visualized with 1% (w/v) ammonium sulfite. SDS-PAGE was performed according to the procedure of Laemmli (1970). Protein bands on gels were visualized using a silver-staining kit (Bio-Rad).

Results

In the sycamore cell culture, rapid growth of the cells continued for 10 days. When the microsomal IDPase activity was determined during 10 days of culture, the maximum activity was detected at day-6 (data not shown). Therefore, sycamore cells cultured for 6 days were used for the preparation of microsomal membranes. **Fig. 1** shows the separation profile of microsomal IDPase and NADPH-cytochrome c reductase after sucrose density gradient centrifugation. IDPase and NADPH-cytochrome c reductase (marker for the endoplasmic reticulum) were well separated, strongly suggesting that the microsomal IDPase activity comes from the Golgi IDPase.

For solubilization of the IDPase from sycamore microsomal membranes 0.5% Triton X-100 solution containing 50 mM Tris-maleate-NaOH (pH 6.0), 0.15 M NaCl, and 20% glycerol was most effective. Glycerol was necessary for stabilizing the solubilized IDPase. The solubilized crude enzyme was applied to a DEAE-Toyopearl column, and the pass through fraction was subjected to SOURCE-S

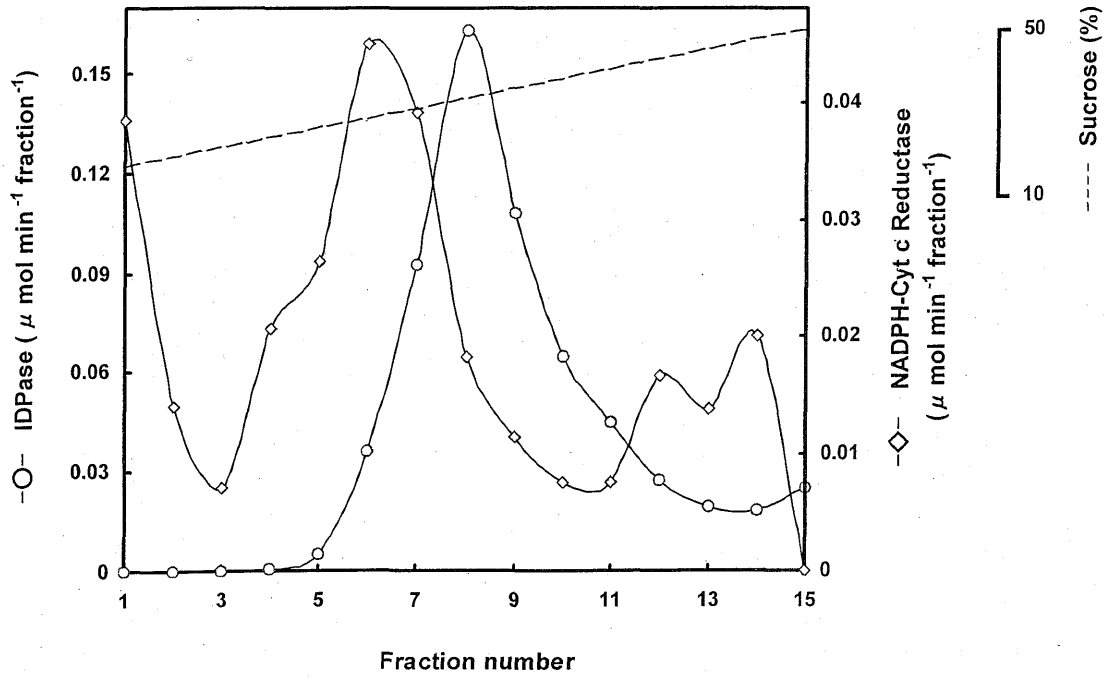

Fig. 1 Separation profile of sycamore microsomal IDPase and NADPH-cytochrome c reductase activities after linear sucrose density gradient centrifugation.

The microsomal membranes were prepared from the protoplasts of sycamore cells as described in the text. Open circle and diamond represent IDPase and NADPH-cytochrome c reductase, respectively. Broken line shows sucrose concentration.

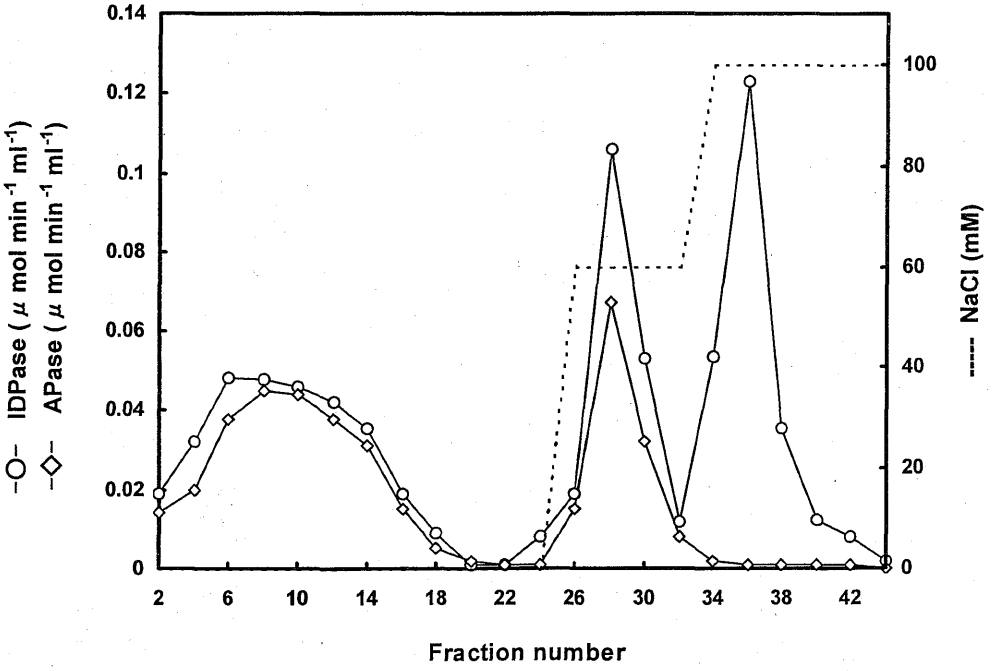

Fig. 2 Elution profile of IDPase from SOURCE-S ion exchange column chromatography.

The pass through fractions collected from DEAE-Toyopearl 650 M column chromatography were applied to a SOURCE-S column and eluted with 60 mM and 100 mM NaCl. Open circle and diamond represent IDPase and acid phosphatase, respectively.

column chromatography. As shown in **Fig. 2**, two peaks of IDPase activity were eluted and detected at 60 mM and 100 mM NaCl. The contamination of acid phosphatase was not detectable in the latter peak (Peak 2), although the former peak (Peak 1) contained some acid phosphatase activity. A summary of the stepwise purification of sycamore Golgi membrane-bound IDPase is given in **Table 1**. The specific activities of Peak 1 and Peak 2 were 2.2 and 2.8 μmol min^{-1} (mg of protein)$^{-1}$, respectively. The sample preparation of Peak 2 was subjected to native-PAGE. After electrophoresis, the IDPase

Table 1 Purification of sycamore Golgi membrane-bound IDPase

	Total activity (μmol min^{-1})	Total protein (mg)	Specific activity (μmol min^{-1}mg^{-1})	Purification (fold)	Acid phosphatase (μmol min^{-1})
1. Crude extract	3.50	38.6	0.09	1	2.77
2. DEAE-Toyopearl 650M	3.24	20.5	0.16	1.8	2.25
3. SOURCE-S					
Peak1	0.87	0.39	2.2	24.4	0.46
Peak2	0.96	0.34	2.8	31.0	N.D.

N.D., Not detected.

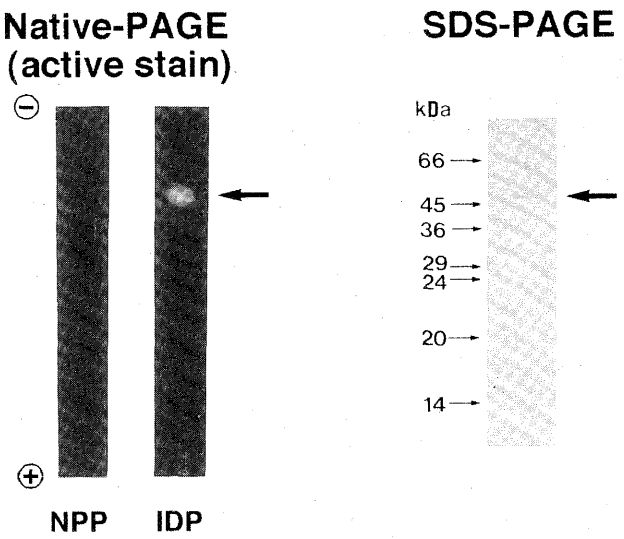

Fig. 3 Native- and SDS-PAGE analyses of Peak 2 IDPase.

The peak 2 IDPase was subjected to native- and SDS-PAGE as described in the text. After native-PAGE, the gel was incubated with IDP and lead nitrate. Then the IDPase band was visualized with ammonium sulfite (left panel). The IDPase band in the SDS-gel was visualized by silver staining (right panel).

band on the gel was visualized by active staining (**Fig. 3, left panel**). Then, the IDPase band was cut and subjected to SDS-PAGE (**Fig. 3, right panel**). The molecular size of the IDPase subunit was estimated to be 50 kDa (**Fig. 3, right panel** and **Fig. 4B**). Furthermore, the size of native IDPase was also estimated by Toyopearl HW 60F gel filtration column chromatography to be 50 kDa (**Fig. 4A**). While, in Peak 1, two IDPase bands were detected on the native gel (**Fig. 5, left lane**). However, the SDS-PAGE and gel filtration analyses for the sample preparation of Peak 1 exhibited results similar to those of Peak 2 (data not shown).

The sycamore Golgi membrane-bound IDPase isoforms were divalent-dependent enzymes. The catalytic activities of enzymes in both Peak 1 and Peak 2 were activated by Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, and weakly activated by Zn^{2+}. The enzyme acti-

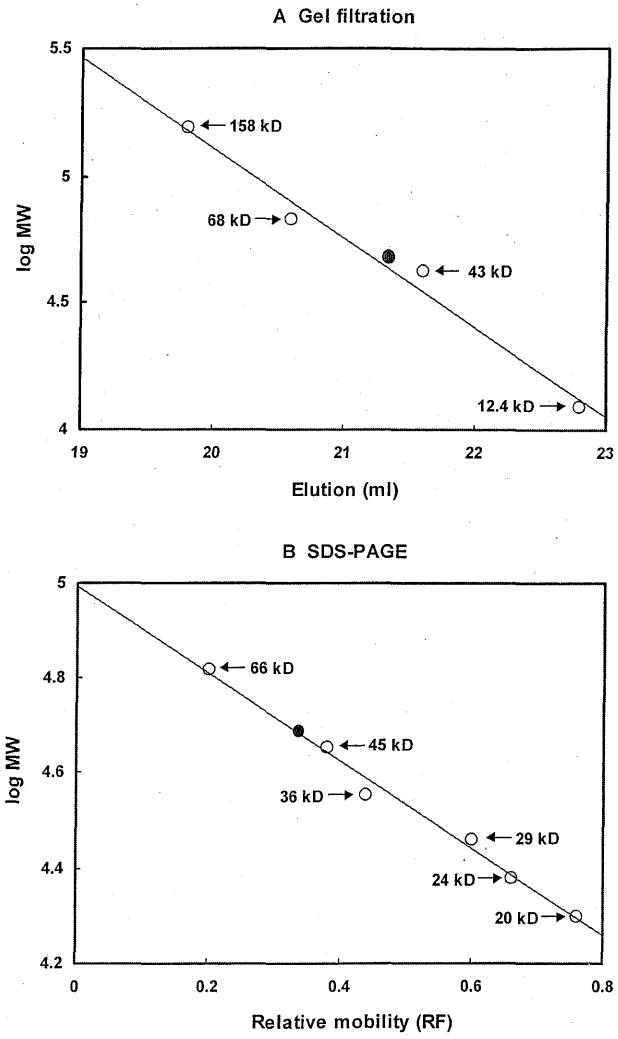

Fig. 4 Estimation of molecular size of Peak 2 IDPase. (A) Gel filtration; aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), cytochrome c (12.4 kDa) were used as molecular mass standards. (B) SDS-PAGE; bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactoalbumin (14 kDa) were used as standards. Open and closed circles represent molecular mass standards and Peak 2 IDPase, respectively.

Table 2 Effects of various divalent cations on the activity of sycamore Golgi membrane-bound IDPases

	Relative activity (%)	
	Peak 1	Peak2
None	12	10
4 mM Mg^{2+}	100*	100*
4 mM Ca^{2+}	105	109
4 mM Co^{2+}	98	102
4 mM Mn^{2+}	90	92
4 mM Zn^{2+}	24	20
1 mM EDTA	0	0

*The enzyme activity with 4 mM Mg^{2+} (Peak 1, 2.2 μmol min^{-1} mg^{-1}, Peak 2, 2.8 μmol min^{-1} mg^{-1}) were normalized to 100%.

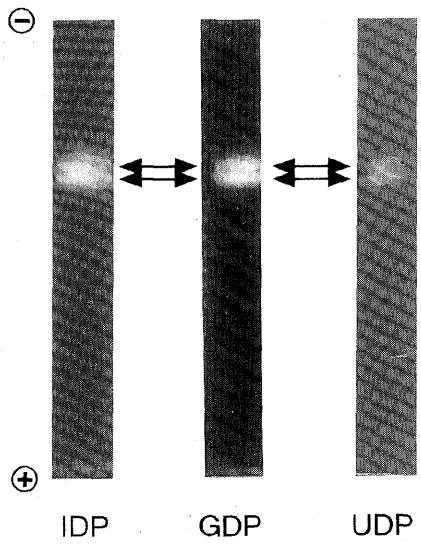

Fig. 5 Substrate specificity of Peak 1 NDPase.
Peak 1 NDPases were subjected to native-PAGE, followed by active staining with IDP, GDP and UDP. Experimental details are described in the text.

vities were completely inhibited by EDTA (**Table 2**). The optimum pH for both enzyme activities were approximately at a neutral pH, and these enzymes were stable at 0 to 40℃. The phosphatase inhibitors, ascorbate (10 mM), molybdate (80 mM), KF (20 mM), and vanadate (10 mM) did not exhibit an inhibitory effect. In addition, the effects of ATP on the IDPase isoforms were examined. ATP (10 mM) reduced the enzyme activities of Peak 1 and Peak 2 to 30% and 20%, respectively (**Fig. 6**).

The substrate specificities of sycamore Golgi IDPase isoforms were determined. IDP, UDP, and GDP were effective substrates for all these enzyme isoforms tested (**Fig. 5, Table 3**). In Peak 2, the values of *Km* for IDP, UDP, and GDP were calcu-

Table 3 Substrate specificity of Peak 2 sycamore Golgi IDPase

Substrate (4mM)	Relative activity (%)
AMP	0
CMP	0
GMP	0
IMP	0
dTMP	0
UMP	0
ADP	0
CDP	0
GDP	110
IDP	100*
TDP	0
UDP	90
ATP	0
GTP	3
ITP	3
TTP	0
UTP	0
NPP	0

*The enzyme activity with 4 mM IDP (2.8 μmol min^{-1} mg^{-1}) was normalized to 100%.

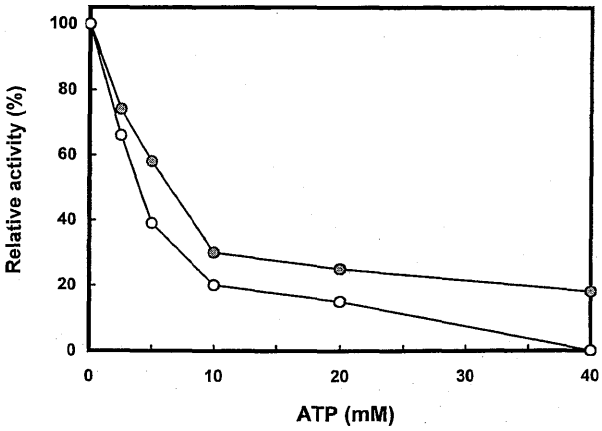

Fig. 6 Inhibitory effects of ATP on Peak 1 (●) and Peak 2 (○) IDPases.
Enzyme assays were performed in the reaction mixture consisting of 4 mM IDP and various concentration of ATP. The enzyme activities without ATP (Peak 1, 2.2 μmol min^{-1} mg^{-1}; Peak 2, 2.8 μmol min^{-1} mg^{-1}) were normalized to 100%.

lated by double reciprocal plots to be 0.68, 0.53, and 0.77 mM, respectively (data not shown).

Discussion

Proteins and lipids are glycosylated and cell wall polysaccharides are synthesized in the lumen of the

Table 4 Enzymatic characteristics of sycamore and rice Golgi membrane-bound NDPase

	Sycamore Peak 1 NDPase	Sycamore Peak 2 NDPase	Rice NDPase*
Molecular mass			
Gel filtration	50 kDa	50 kDa	200 kDa
SDS-PAGE	50 kDa	50 kDa	55 kDa
Substrate specificity	GDP, IDP, UDP	GDP, IDP, UDP	IDP, GDP, UDP
Km (IDP)	0.65 mM	0.68 mM	0.48 mM
Km (GDP)	0.45 mM	0.52 mM	0.67 mM
Km (UDP)	0.80 mM	0.77 mM	0.50 mM
Vmax (IDP)	2.20	2.80	1.85
		μmol min^{-1} (mg protein)$^{-1}$	
Inhibition by ATP (IC_{50})	6.5 mM	4.0 mM	12.0 mM
Cation requirement	Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}	Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}	Mg^{2+}, Mn^{2+}>Co^{2+}, Ca^{2+}
Optimum pH	7	7	7
Thermal Stability	0-40℃	0-40℃	0-40℃

*Data are from a previous report (Mitsui *et al.*, 1994).

Golgi complex. UDP- and GDP-sugars are sugar donors in the glycosylation. After transfer of sugar residues to protein, lipid and polysaccharide by glycosyltransferases, UDPase and GDPase hydrolyze the resulting UDP and GDP to UMP and GMP, respectively. UDP and GDP have been reported to be highly inhibitory to the Golgi glycosyltransferases (Abeijon *et al.*, 1993; Mitsui *et al.*, 1994; Wang and Guidotti, 1998). Thus, UDPase and GDPase play an important role in the regulation of glycosylation in the Golgi complex. There is evidence that an IDPase is present on the luminal side of the Golgi complex of plant cells (Dauwalder *et al.*, 1969; Ray *et al.*, 1969). However, the role of Golgi IDPase in plant cells is still not well understood. In the present study, we purified and characterized the Golgi membrane-bound IDPase isoforms from suspension-cultured cells of sycamore. The membrane-bound IDPase isoforms were effectively solubilized by 0.5% Triton X-100, and separated by chromatography using DEAE-Toyopearl and SOURCE-S columns. Native-PAGE analyses showed that there exist at least three IDPase isoforms in sycamore microsomal membranes (**Fig. 3** and **5**). The apparent molecular mass of the Peak 2 IDPase isoform was estimated by gel filtration column chromatography and SDS-PAGE to be 50 kDa (**Fig. 4**), indicating that the enzyme exists as a monomeric protein. The sizes of the other two isoforms were also analyzed; the results obtained were similar to those of the Peak 2 IDPase (data not shown). Previously, we purified the Golgi membrane-bound IDPase (NDPase) from rice suspension-cultured cells. The rice Golgi IDPase was shown to form an oligomeric structure, composed of four 55 kDa subunits (Mitsui *et al.*, 1994). It has been reported that a yeast GDPase functions as a homodimer in the Golgi membranes (Berninsone *et al.*, 1995). These findings suggested that the functional oligomeric structure of nucleoside diphosphatase might vary among the species.

The enzymatic characteristics of sycamore and rice Golgi membrane-bound IDPase are summarized in **Table 4**. The determined substrate specificity of sycamore Golgi IDPase indicated that the enzyme is an NDPase. The values of *Km* for IDP, GDP, and UDP of sycamore Golgi NDPase were similar to those of rice Golgi NDPase (Mitsui *et al.*, 1994). We could not detect any UDP-specific or GDP-specific NDPase in either sycamore or rice suspension-cultured cells, although a GDPase has been identified in yeast (Yanagisawa *et al.*, 1990). Yeast Golgi GDPase and human Golgi UDPase have been classified to the E-ATPase protein family (Wang and Guidotti, 1998). In plants, potato apyrase (Handa and Guidotti, 1996) and pea NTPase (Hsieh *et al.*, 1996) have been identified as members of the E-ATPase protein family. Identification of plant Golgi NDPase genes is in progress in our laboratory.

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