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Subcellular localization of endo-β-*N*-acetylglucosaminidase and high-mannose type free *N*-glycans in plant cell

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Abstract

Subcellular distribution of plant endo-β-*N*-acetylglucosaminidase (endo-β-GlcNAc-ase) and high-mannose type free *N*-glycans produced by the endoglycosidase has been analyzed using cotyledons of pumpkin seedlings as the model plant cells. Each organelle in the cotyledons was fractionated by ultracentrifugation with the sucrose density gradient system and the endo-β-GlcNAc-ase activity in each fraction was assayed with fluorescence labeled *N*-glycans as substrates. The endoglycosidase activity was exclusively recovered in the soluble fraction (cytosol fraction) but not in other specific organellar fractions, suggesting that the endoglycosidase would reside predominantly in the cytosol. The quantitative analysis of high-mannose type free *N*-glycans occurring in each fraction showed that more than 70% of the free *N*-glycans was recovered from the soluble fraction, suggesting the endoglycosidase would work in the cytosol and the resulting free *N*-glycans would accumulate in the same fraction. The pumpkin endo-β-GlcNAc-ase (endo-CM) partially purified from the cotyledons showed optimum activity around pH 6.5, supporting this enzyme would reside in the cytosol. Furthermore, the detailed analysis of substrate specificity of endo-CM using various high-mannose type *N*-glycans showed that the pumpkin enzyme, as well as other plant endo-β-*N*-acetylglucosaminidases, were highly active toward the high-mannose type glycans bearing the Manα1-2Manα1-3Manβ1-structural unit.

Keywords: Plant *N*-glycan; Free *N*-glycan; Plant endo-β-*N*-acetylglucosaminidase; Substrate specificity; Subcellular distribution; Cotyledon of seedling

1. Introduction

In hypocotyls of seedlings, developing seeds, or matured fruits, free *N*-glycans have been found [1–8], suggesting these free *N*-glycans might play a critical role for plant cell differentiation or maturation [9]. Considering the structural features of such free *N*-glycans, it is reasonable to assume that high-mannose type free *N*-glycans with one GlcNAc residue at the reducing end could be derived by endo-β-*N*-acetylglucosaminidase (endo-β-GlcNAc-ase) and the xylose-containing structures with the chitobiosyl unit could be derived by peptide:*N*-glycanase (PNGase) or glycoamidase, respectively. In our previous reports [5–8], we revealed that the high-mannose type free *N*-glycans ($Man_{9\sim5}GlcNAc_1$) in plant cells have a common core structural unit: Manα1-6Man1-3Manα1-6(Manα1-3)Manβ1-4GlcNAc. This structural feature of the free *N*-glycan ($Man_5GlcNAc_1$) found in plant cells is significantly different from that of the free glycans found in various animal cells [10–14], which have the Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc unit.

Concerning the substrate specificity of several plant endo-β-GlcNAc-ases, we have already revealed that the plant endoglycosidase was highly active towards the high-mannose type *N*-glycans bearing the Manα1-

Abbreviations: PA-, pyridylamino; SF-HPLC, size-fractionation HPLC; RP-HPLC, reverse-phase HPLC; endo-β-GlcNAc-ase, endo-β-*N*-acetylglucosaminidase; endo-CM, endo-β-*N*-acetylglucosaminidase from pumpkin (*Cucurbita moschata*); PNGase, peptide:*N*-glycanase; M6B, Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; M3FX, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; ER, endoplasmic reticulum; IS-MS, ion-spray mass spectrometry; MS/MS, tandem mass spectroscopy

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2Manα1-3Manβ1-structural unit [15–18]. Furthermore, we have assumed that the plant endo-β-GlcNAc-ase would reside in the endoplasmic reticulum (ER) or the cytosol in plant cells, since the plant endo-β-*N*-acetylglucosaminidase shows maximum activity in the neutral pH region (pH 6–7) [15–18]. To date, however, no detailed analysis on the distribution of the endoglycosidase in plant cells has been carried out yet. In this study, therefore, we have analyzed in which subcellular organelle the plant endo-β-GlcNAc-ase resides and the distribution of the high-mannose type free *N*-glycans produced by the endoglycosidase in plant cells.

2. Materials and methods

2.1. Materials

A Cosmosil 5C18-AR column (0.60×25 cm) was purchased from Nacalai Tesque, and a Shodex Asahipak NH2P-50 column (0.46×25 cm) from Showa Denko (Japan). α-Mannosidase (Jack bean) and swainsonine were purchased from Sigma. α-1,2-Mannosidase (*Aspergillus oryzae*) a was generous gift of Dr. T. Yoshida (Hirosaki University, Japan). $Man_{9\sim5}GlcNAc_1$-PA and a Con A Sepharose 4B column were prepared as described in our previous papers [7,8].

2.2. Subcellular fractionation

Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri) seeds were soaked overnight, planted in moist rock fiber and allowed to germinate at 25°C in the dark. The etiolated cotyledons from seedlings that had been grown for 4 days in darkness were used for subcellular fractionation. Cotyledons (2 g) were chopped by a razor with 2 ml of 0.15 M Tricine buffer (pH 7.5) containing 13% sucrose and 1 mM EDTA. The chopped materials were squeezed through three layers of gauze. The resulting filtrate (1.5 ml) was layered on a gradient consisting of a 1 ml cushion of 60% (w/w) sucrose, 13 ml of sucrose solution increasing linearly from 15% to 60% (w/w). The gradient was centrifuged at 87 000×*g* for 2.5 h. After centrifugation, fractions (0.5 ml) were collected and the endo-β-*N*-acetylglucosaminidase activity of each fraction was measured as described below. For separation of the ER and Golgi fractions, 2 mM $MgCl_2$ instead of EDTA was added to the cotyledon extract and then each subcellular fraction was separated by the ultracentrifugation system with the same sucrose density gradient.

Activities of subcellular marker enzymes were assayed as described in the cited references: catalase activity [19] as a marker enzyme for microbody, NADH-cytochrome *c* reductase [20] for the ER, and triosephosphate isomerase [21] for the soluble fraction (cytosol), respectively.

2.3. Preparation and pyridylamination of free N-glycans

Each subcellular fraction obtained from 16 g cotyledon extract was treated with acetone (20 times volume of each sample) to disrupt the ER or Golgi membrane. After centrifugation (12 000 rpm for 20 min), the supernatant was discarded and the precipitate suspended in water. Each sample was desalted by a Dowex 50×2 column (1.8×20 cm), followed by a Dowex 1×2 column. Desalted sample was applied to a Sephadex G-10 column (2.8×40 cm) to remove sucrose (the elution volume from 40 to 90 ml was collected). After desalting, each fraction was lyophilized and then pyridylaminated as described in the previous paper [8]. After pyridylamination, excess amount of 2-aminopyridine was removed by the phenol/chloroform extraction method [22]. The resulting pyridylamino (PA) derivatives were partially purified by a Sephadex G-25 superfine column (2.8×40 cm) in 50 mM NH_4OH. The pyridylaminated oligosaccharides were monitored by a fluorescence spectrometer (Ex 320 nm, Em 400 nm, Hitachi 650 10S) and by the phenol-sulfuric acid method [23].

2.4. Con A-Sepharose chromatography

The PA-oligosaccharide fraction partially purified by the Sephadex G-25 column was evaporated to dryness. The resulting residue was dissolved in 25 mM Tris–HCl, pH 7.8, containing 0.1 M NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ (5 ml) and applied to a Con A-Sepharose 4B column (2.2×33 cm) equilibrated with the same buffer. After washing the column with about 250 ml of the same buffer, the Con A-bound (Con A (+)) PA-sugar chains were eluted by addition of 0.2 M methyl-α-mannoside. PA-sugar chains were monitored by the fluorescence spectrophotometer. The Con A (+) fraction was concentrated and desalted by gel filtration on a Sephadex G-10 column (2.5×40 cm) in 50 mM NH_4OH. PA derivatives obtained by the gel filtration were further separated by HPLC on a Jasco 880-PU HPLC apparatus equipped with a Jasco 821-FP Intelligent Spectrofluorometer, using the Cosmosil 5C18-AR column (0.6×25 cm) and the Shodex Asahipak NH2P-50 column (0.46×25 cm) as described in our previous paper [7,8].

2.5. α-1,2-Mannosidase digestions

Digestions with *Aspergillus* α-1,2-mannosidase (15 μg enzyme) were done in 50 ml of 0.1 M sodium acetate buffer, pH 5.0, for 4 h at 37°C. The reaction was stopped by boiling for 2 min and a part of the digest was analyzed by size-fractionation (SF)-HPLC using the Shodex Asahipak NH2P-50 column. The PA-sugar chains were eluted by increasing the water content in the water–acetonitrile mixture from 20% to 47% linearly at a flow rate of 0.8 ml/min.

2.6. Ion-spray mass spectrometry (IS-MS)

The mass spectrometer used was a Perkin-Elmer Sciex API-III, triple-quadrupole mass spectrometer with an atmospheric pressure ionization ion source [8]. The mass spectrometer was operated in the positive mode; the ion spray voltage was 4200 V. Samples were typically dissolved in 50% acetonitrile–water (containing 0.05% formic acid) at a concentration of approx. 10 pmol/μl and introduced into the electrospray needle by mechanical infusion through a microsyringe at a flow rate of 10 μl/min. The collisionally activated dissociation (CAD) spectrum was measured with argon as the collision gas. The collision energy was 60–100 eV. The scanning was done with a step size of 0.5 Da and the CAD daughter ion spectrum was recorded from *m/z* 200.

2.7. Preparation of partially purified pumpkin endo-β-N-acetylglucosaminidase

The chopped etiolated cotyledons of pumpkin seedlings (50 g) were homogenized in 200 ml of 50 mM Tris–HCl buffer, pH 7.5, and stirred at 4°C for 2 h. After centrifugation, the supernatant was applied to a DEAE-cellulose column (2.8×20 cm), which had been equilibrated with the same buffer. After the column had been washed with 20 mM Tris–HCl buffer and the same buffer containing 0.1 M NaCl, the enzyme activity was eluted with the same buffer containing 0.2 M NaCl. The resulting enzyme fraction was used as a partially purified endoglycosidase to analyze optimum pH and substrate specificity.

2.8. Assay of endo-β-N-acetylglucosaminidase activity

The endo-β-GlcNAc-ase activity was assayed as described in our previous paper using M6B (Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA) as a substrate and M3FX (Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA) as an internal standard [17]. Briefly, an enzyme solution (10 μl) was mixed with M6B and M3FX (about 100 pmol and 70 pmol) in 0.1 M MES buffer (60 μl), pH 6.5, containing 5 mM DTT and 80 μM swainsonine. After incubation at 37°C for 30 min, the reaction was stopped by heating at 100°C for 3 min. After centrifugation, an aliquot (10 μl) of the resulting supernatant was analyzed by SF-HPLC with the Asahipak NH2P-50 column. The PA-sugar chains (M6B and PA-GlcNAc) were eluted and separated by increasing the water content in the acetonitrile–water mixture from 45% to 50% at a flow rate of 0.8 ml/min.

The substrate specificity of pumpkin endo-β-*N*-acetylglucosaminidase was analyzed using various pyridylaminated *N*-glycans as described in our previous report [15–17].

3. Results and discussion

3.1. Distribution of endo-β-N-acetylglucosaminidase activity in plant cells

In this study, we used pumpkin cotyledons as a model plant to investigate the distribution of endo-β-*N*-acetylglucosaminidase activity in plant cells, since the analytical system for subcellular fractionation has been established [24–26]. As shown in Fig. 1, almost all the endo-β-GlcNAc-ase activity was recovered in the top four fractions (soluble fraction) and very weak activity was also found in the ER-containing fraction (fraction 6), in which the NADPH-cytochrome *c* reductase activity was detected. The triosephosphate isomerase activity coincided with the endoglycosidase activity, suggesting the plant endo-β-*N*-acetylglucosaminidase should reside in the cytosolic frac-

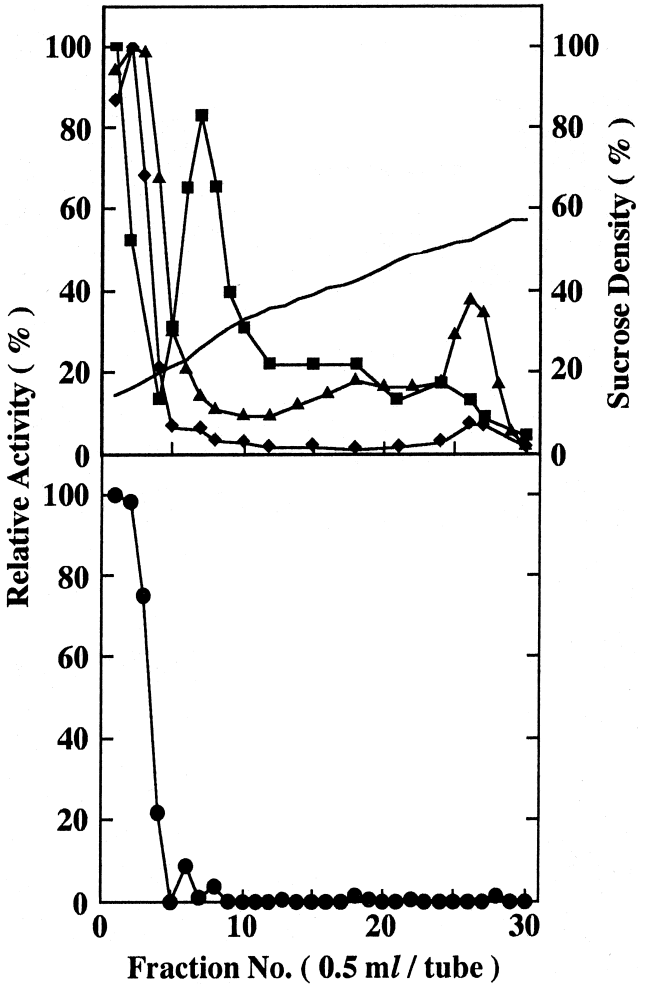

Fig. 1. Distribution of activities of various marker enzymes and the pumpkin endo-β-*N*-acetylglucosaminidase in the presence of EDTA. (Top) The relative activities of marker enzymes; catalase (▲) for microbody, NADPH-cytochrome *c* reductase (■) for the ER, triosephosphate isomerase (♦) for the soluble fraction (cytosolic fraction). (Bottom) The relative activity of the pumpkin endo-β-*N*-acetylglucosaminidase (●). The assay system of endoglycosidase is described in Section 2.

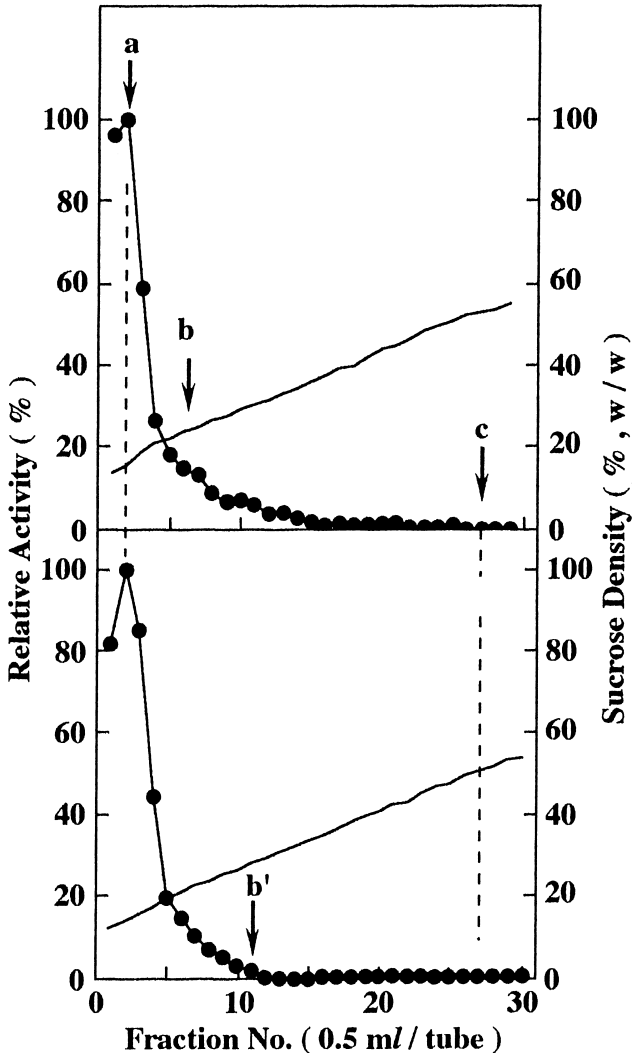

Fig. 2. Distribution of the activities of various marker enzymes and pumpkin endo-β-*N*-acetylglucosaminidase in the presence of EDTA or $MgCl_2$. (Top) In the presence of 1 mM EDTA; (bottom) in the presence of 2 mM $MgCl_2$. Arrow a, peak top of triosephosphate isomerase activity; arrows b (EDTA(−)) and b′ (EDTA(+)), peak top of NADH-cytochrome *c* reductase activity; arrow c, peak top of catalase activity.

tion. Since plant endo-β-*N*-acetylglucosaminidase hardly releases high-mannose type *N*-glycans from folded or matured glycoproteins, we presumed that the endogenous substrates might be glycopeptides bearing the high-mannose type glycans derived from misfolded glycoproteins in the quality control system of nascent glycoproteins or another type of glycoconjugate such as dolichol-oligosaccharide intermediates [7,8,27]. This hypothesis led us to propose that the plant endoglycosidase could reside in the cytosol near the ER compartment but not in the compartment. To confirm whether the plant endoglycosidase works in the ER or the cytosol, we tried to separate the cytosol and the ER-rich fraction completely by addition of $MgCl_2$ to the sucrose density solvent. As shown in Fig. 2, the NADPH-cytochrome *c* reductase activity (the ER-rich fraction) shifted to the higher density fraction and was completely separated from the cytosolic fraction. However, the pumpkin endo-β-*N*-acetylglucosaminidase (endo-CM) activity did not shift with the NADPH-cytochrome *c* reductase activity. Furthermore, the significant endoglycosidase activity was hardly detected in the shifted ER-rich fraction. These results indicated that the endo-CM could reside in the cytosolic fraction, suggesting that the free *N*-glycans found in several developing or growing plant cells could also occur in the same fraction.

However, it is noteworthy that a slight increase of the endoglycosidase activity in the ER fraction was observed in Fig. 1 (bottom) (also in Fig. 4). This observation might suggest that the endoglycosidase may have a weak interaction with the ER cytosolic-side membrane and be concentrated in the cytosol near the ER. The concentrated occurrence of the endoglycosidase seems to be convenient for the *N*-glycan turnover mechanism in the ER-associated protein quality control system.

To get further evidence indicating that endo-CM resides in the cytosol, we also analyzed the optimum pH of the enzyme, since several enzymes that localize in the cytosolic fraction usually show their optimum activities in the weak acid or neutral pH region (pH 6–7). As well as several other plant endo-β-*N*-acetylglucosaminidases (from pea seeds, soybean seeds, *Ginkgo* seeds, and tomato fruits) [15–18], endo-CM also showed its optimum activity around pH 6.5 (Fig. 3). This result seems to support that the plant endoglycosidase would reside in the cytosolic fraction but not in an acidic organella such as the

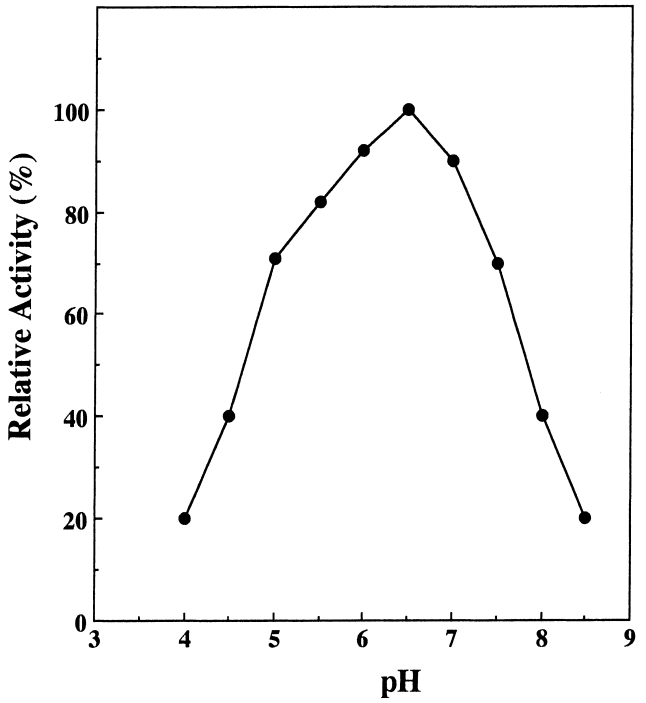

Fig. 3. Effect of pH on the pumpkin endo-β-*N*-acetylglucosaminidase activity. Relative activity was expressed as values relative to the maximum activity at 37°C. The buffers used were: 0.1 M MES (pH 4.0–6.5), and 0.1 M HEPES (pH 7.0–8.5).

vacuole or the cell wall. Furthermore, the absence of endoglycosidase activity in the vacuoles was suggested, since the enzyme activity was not detected in the high sucrose density region (~40%), in which the proglobulin processing enzyme specific for the vacuole is specifically detected [26].

3.2. Substrate specificity of the pumpkin endo-β-N-acetylglucosaminidase

Concerning substrate specificity of the plant endo-β-*N*-acetylglucosaminidases (endo-β-GlcNAc-ase), we have already revealed that, in general, the plant endoglycosidase can hydrolyze effectively the chitobiosyl linkage of various high-mannose type *N*-glycans bearing a Manα1-2Manα1-3Manβ1-structural unit [15–18]. The pumpkin endo-β-GlcNAc-ase (endo-CM) was partially purified by a DEAE-cellulose column from the extract of the cotyledons (50 g fresh weight cotyledons) by the same method as described in our previous papers [17]. The same as other plant endo-β-GlcNAc-ase, the pumpkin endoglycosidase activity was recovered in the 0.2 M NaCl fraction on the DEAE-cellulose column. The pumpkin endoglycosidase was purified about 25-fold. Using the partially purified pumpkin enzyme and various pyridylaminated *N*-glycans, the detailed substrate specificity was analyzed. As shown in Table 1, endo-CM could also hydrolyze the chitobiose linkage of high-mannose type *N*-glycans. However, as well as other plant endoglycosidases, the relative reaction rate for the $Man_5GlcNAc_2$ structure having no α-1,2-mannosyl residue was only 30% of that for $Man_6GlcNAc_2$, suggesting that endo-CM would also have a subsite specific for the α-1,2-mannosyl residue in the Manα1-2Manα1-3Manβ1-structural unit. The plant complex type *N*-glycan bearing an α1-3-fucosyl residue at the proximal GlcNAc residue could not be a substrate for endo-CM.

3.3. Distribution and structural analysis of high-mannose type free N-glycans in plant cells

The high-mannose type free *N*-glycans were partially purified by Con A affinity chromatography from each fraction (F-I–F-V) shown in Fig. 4A after subcellular fractionation using the ultracentrifugation method. The Con A bound (Con A (+)) fraction was eluted by addition of 0.2 M methyl α-mannoside. The SF-HPLC pattern of the total Con A (+) fraction obtained from each subcellular fraction is shown in Fig. 4B. As shown in Fig. 4B, various high-mannose type *N*-glycans were recovered mainly from F-I, -II, and -III, but trace amounts from F-IV and -V. To purify each high-mannose type oligosaccharide, each Con A (+) fraction was first applied to reverse-phase (RP)-HPLC. Since we have already confirmed that the high-mannose type *N*-glycans bearing only one GlcNAc residue ($Man_{9-5}GlcNAc_1$-PA) are eluted in the run-through fraction or the slightly retained fraction in our previous reports [5–8], the run-though fraction (5–20 min), designated A, was further fractionated on the Asahipak NH_2 column as shown in Fig. 5B. IS-MS and tandem mass spectroscopy (MS/MS) analyses showed that the molecular mass of these PA-sugar chains obtained in Fig. 5B were *m/z* 1758.5 ($[M+H]^+$) for $Man_9GlcNAc_1$-PA (M9′), *m/z* 1597.0 ($[M+H]^+$) for $Man_8GlcNAc_1$-PA (M8′), *m/z* 1435.0 ($[M+H]^+$) for $Man_7GlcNAc_1$-PA (M7′), *m/z* 1272.5

Table 1
Comparison of substrate specificities of various plant endo-β-*N*-acetylglucosaminidases

Glycan Structures	Relative Hydrolysis[a] Endo-CM[b] (%)	Endo-LE[c] (%)	Endo-GM[c] (%)
Manα1-6, Manα1-6, Manα1-3′ Manβ1-4GlcNAcβ1-4GlcNAc-PA, Manα1-2Manα1-3′ **M6B**	100	100	100
Manα1-6, Manα1-6, Manα1-3′ Manβ1-4GlcNAcβ1-4GlcNAc-PA, Manα1-2Manα1-2Manα1-3′	89	80	86
Manα1-2Manα1-6, Manα1-6, Manα1-3′ Manβ1-4GlcNAcβ1-4GlcNAc-PA, Manα1-2Manα1-2Manα1-3′	83	90	86
Manα1-2Manα1-6, Manα1-6, Manα1-2Manα1-3′ Manβ1-4GlcNAcβ1-4GlcNAc-PA, Manα1-2Manα1-2Manα1-3′	80	100	91
Manα1-6, Manα1-6, Manα1-3′ Manβ1-4GlcNAcβ1-4GlcNAc-PA, Manα1-3′	30	32	43
Manα1-6, Manβ1-4GlcNAcβ1-4GlcNAc-PA, Manα1-3′	-	16	9
Manα1-6, Manβ1-4GlcNAcβ1-4GlcNAc-PA, Manα1-3′ 2, Xylβ1 3 Fucα1 **[M3FX]**	-	-	-

Substrates (50–100 pmol each) were incubated with the plant endo-β-*N*-acetylglucosaminidase and M3FX (70 pmol) in MES buffer (pH 6.5, 5 mM DTT) at 37°C for 30 min.

[a]Relative hydrolysis (%) was estimated by taking the hydrolysis rate of M6B (80 pmol hydrolyzed/30 min) as 100.

[b]Endo-CM, pumpkin (*Cucurbita* sp.) enzyme.

[c]Endo-LE, *Lycopersicon esculentum* enzyme; Endo-GM, *Glycine max* enzyme [17].

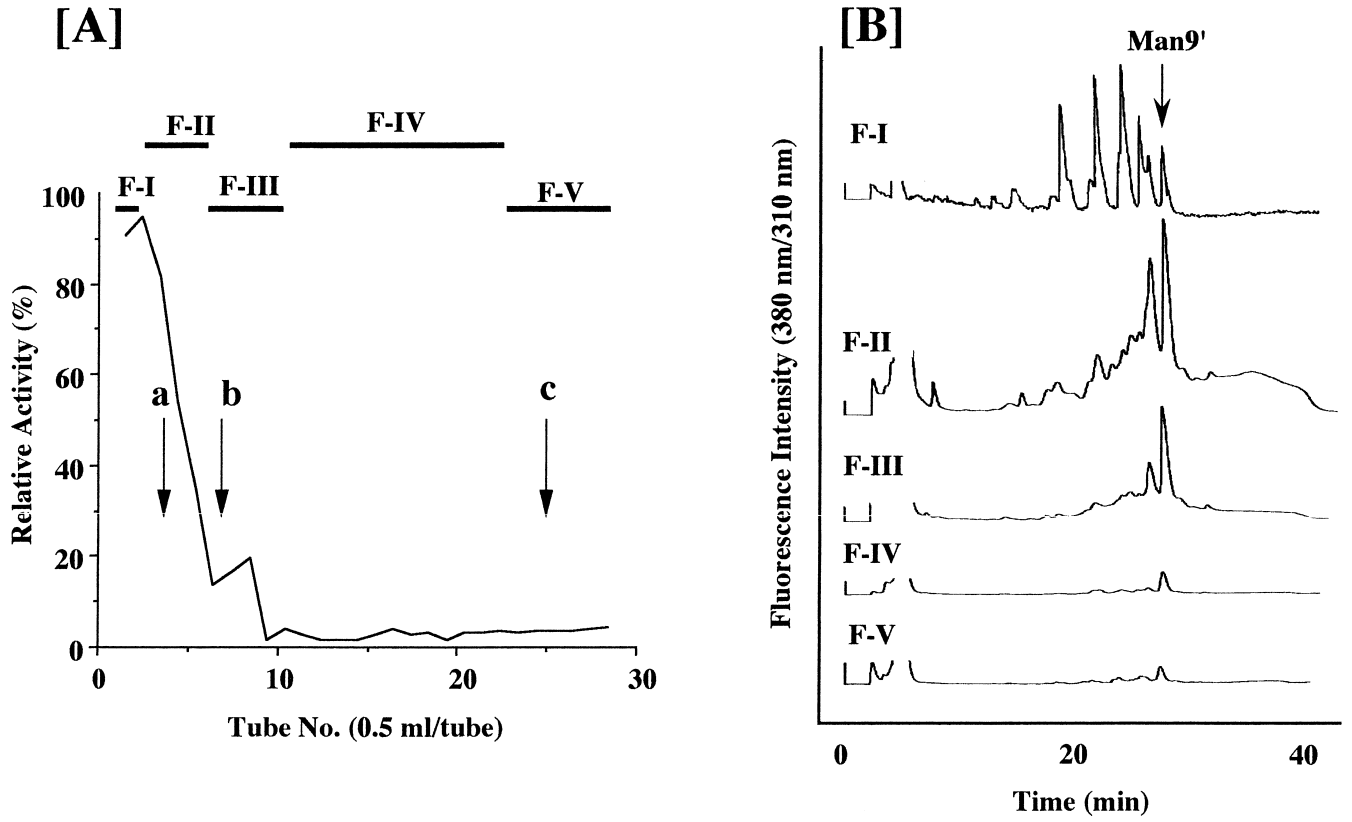

Fig. 4. Distribution of high-mannose type free *N*-glycans in subcellular fraction prepared from pumpkin cotyledons. (A) Subcellular fractionation by sucrose density gradient ultracentrifugation. Five fractions were pooled as indicated by the bars (F-I, -II, -III, -IV, and -V). The solid line indicates the endoglycosidase activity. Arrow a, peak top of triosephosphate isomerase activity; arrow b, the second peak top of NADH-cytochrome *c* reductase activity; arrow c, the second peak top of catalase activity. (B) SF-HPLC profiles of PA derivatives recovered in the Con A (+) fraction obtained from each fraction (F-I–F-V) shown in A. This fraction contained the two species of high-mannose type free *N*-glycans, oligosaccharide-$GlcNAc_2$ and oligosaccharide-$GlcNAc_1$. M9′ shows the elution position of the authentic $Man_9GlcNAc_1$-PA.

($[M+H]^+$) for $Man_6GlcNAc_1$-PA (M6′), *m/z* 1110.5 ($[M+H]^+$) for $Man_5GlcNAc_1$-PA (M5′). The pyridylaminated high-mannose type free *N*-glycans obtained from F-I to F-III in Fig. 4 were converted to be $Man_5GlcNAc_1$-PA by α-1,2-mannosidase digestion, suggesting that these PA-sugar chains have the common core structure, Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc (Fig. 5B,C). As shown in Fig. 5C, a shoulder peak just after the main product peak ($Man_5GlcNAc_1$-PA) was always observed. The structural difference between the main peak and the shoulder peak is obscure at this time; however, IS-MS analysis gave the same mass number (*m/z* 1110.5), suggesting that this shoulder also has the $Hex_5HexNAc_1$-PA structure. Considering these results of structural analysis together with the substrate specificity of endo-CM, these high-mannose type free *N*-glycans must be produced by the endoglycosidase in the soluble (cytosol) fraction of plant cells.

The amounts of high-mannose type free *N*-glycans in the cotyledons of pumpkin seedlings are summarized in Table 2. More than 70% of total free *N*-glycans was recovered in the cytosolic fraction (F-I and -II), suggesting that the free oligosaccharides may be produced and accumulated in the cytosol.

It is noteworthy that the compositions of the high-mannose type free *N*-glycans in F-I, -II, and -III were different from each other. $Man_{7\sim5}GlcNAc_1$ were predominant species in F-I, $Man_9GlcNAc_1$ and $Man_8GlcNAc_1$ in F-II, and $Man_9GlcNAc_1$ in F-III, respectively. It is not clear that this compositional difference in the free *N*-glycans occurring in the cotyledon of seedlings suggests what kind of biological significance exists; however, this observation seems to suggest that not all structural species ($Man_{9\sim5}GlcNAc_1$) of free *N*-glycans produced by the endo-β-GlcNAc-ase are distributed uniformly in the cytosolic fraction of plant cells.

As well as the high-mannose type free *N*-glycans found in other developing plant cells [1–8], the dominant structure always bears only one GlcNAc residue at its reducing end. As shown in Fig. 6, however, in this report, small but comparable amounts (~25%) of the high-mannose type free *N*-glycans bearing the *N*-acetyl chitobiosyl unit (oligomannoside-$GlcNAc_2$) were found in the absorbed fraction on RP-HPLC (B fraction indicated by the horizontal bars in Fig. 5A). The IS-MS and MS/MS analyses of these free *N*-glycans were as follows: *m/z* 981 ($[M+2H]^{2+}$) for $Man_9GlcNAc_2$-PA, *m/z* 1801 ($[M+H]^+$) for $Man_8GlcNAc_2$-PA, *m/z* 1638 ($[M+H]^+$) for $Man_7GlcNAc_2$-

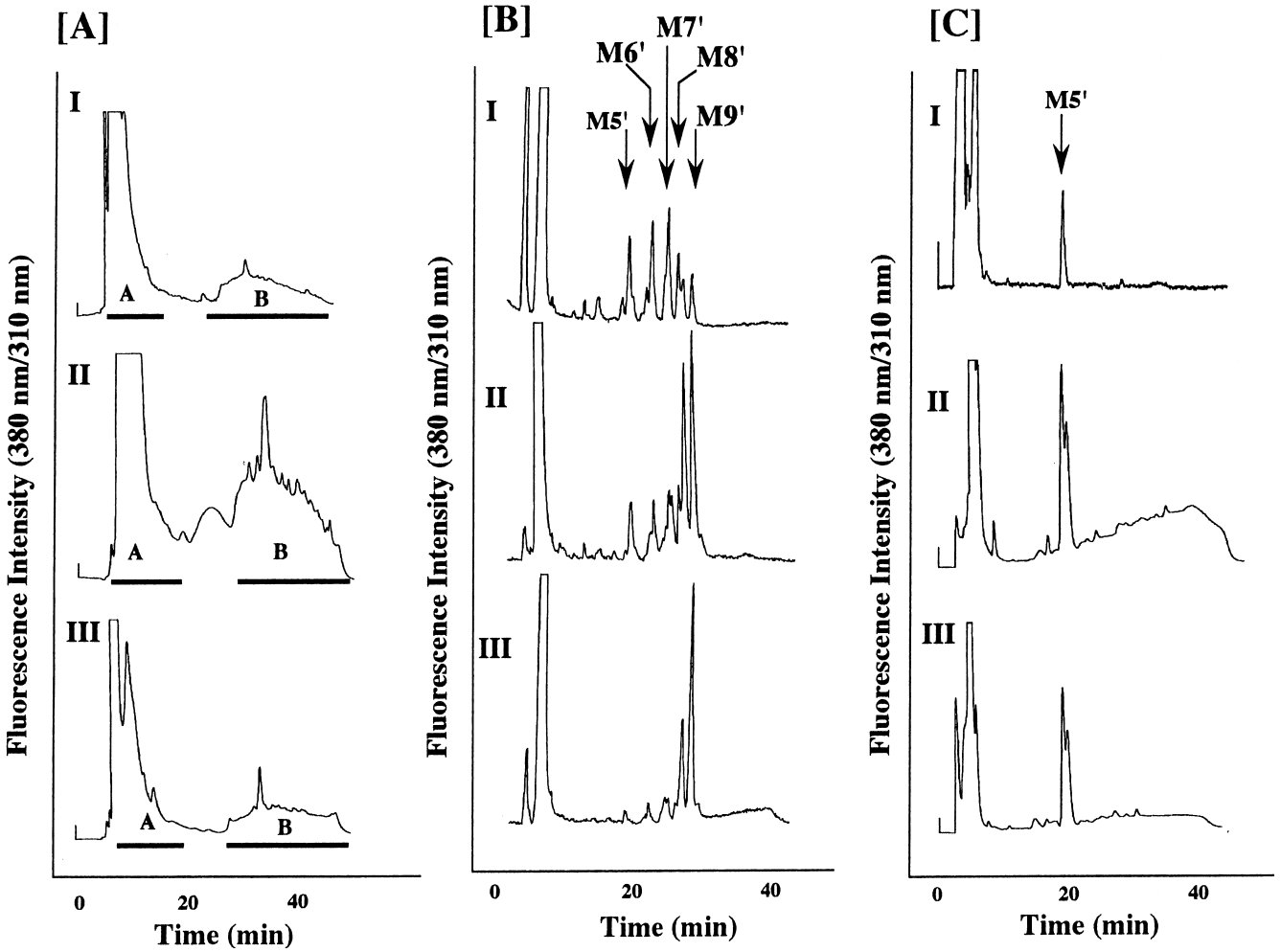

Fig. 5. Structural analysis of the high-mannose type free *N*-glycans by RP-HPLC and SF-HPLC. I, fraction I obtained in Fig. 4; II, fraction II obtained in Fig. 4; III, fraction III obtained in Fig. 4. (A) RP-HPLC profiles of the Con A (+) fractions. PA derivatives were separated on the Cosmosil 5C18-AR column using the water–acetonitrile solvent system described in our report [8]. (B) SF-HPLC profiles of the run-through fractions on the Cosmosil 5C18 column obtained in A. PA derivatives were separated on the Shodex Asahipak NH2P-50 column using the water–acetonitrile solvent system as described in Section 2. (C) SF-HPLC profiles of α-1,2-mannosidase digests of PA-sugar chains obtained in B. M9′, $Man_9GlcNAc_1$-PA; M8′, $Man_8GlcNAc_1$-PA; M7′, $Man_7GlcNAc_1$-PA; M6′, $Man_6GlcNAc_1$-PA; M5′, $Man_5GlcNAc_1$-PA.

PA, *m/z* 1476 ($[M+H]^+$) for $Man_6GlcNAc_2$-PA, *m/z* 1314 ($[M+H]^+$) for $Man_5GlcNAc_2$-PA. Since these high-mannose type *N*-glycans were converted to $Man_1GlcNAc_2$-PA by jack bean α-mannosidase digestions, these sugar chains should be typical high-mannose type (data not shown).

At the present time it is obscure what kind of enzyme is involved in the production of these free high-mannose type

Table 2
Amount of high-mannose type free *N*-glycans in subcellular fractions prepared from pumpkin cotyledons

Fraction A on RP-HPLC (pmol/g fresh weight)						
	$Man_5GlcNAc_1$	$Man_6GlcNAc_1$	$Man_7GlcNAc_1$	$Man_8GlcNAc_1$	$Man_9GlcNAc_1$	Total
F-I	18	21	24	9	10	82
F-II	11	12	13	37	44	117
F-III	2	3	4	18	43	70
Fraction B on RP-HPLC (pmol/g fresh weight)						
	$Man_5GlcNAc_2$	$Man_6GlcNAc_2$	$Man_7GlcNAc_2$	$Man_8GlcNAc_2$	$Man_9GlcNAc_2$	Total
F-I					5	5
F-II		13	9	19	30	71
F-III	2	1	3	5	15	26

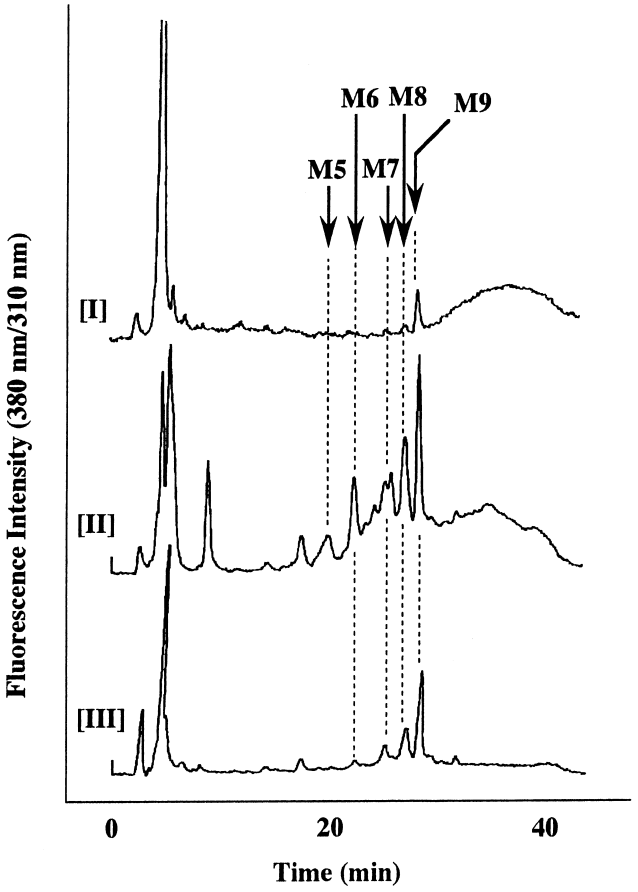

Fig. 6. SF-HPLC of the high-mannose type free *N*-glycans bearing the chitobiosyl residue at their reducing end. [I], fraction I obtained in Fig. 4; [II], fraction II obtained in Fig. 4; [III], fraction III obtained in Fig. 4. PA derivatives were separated on the Shodex Asahipak NH2P-50 column. M9, $Man_9GlcNAc_2$-PA; M8, $Man_8GlcNAc_2$-PA; M7, $Man_7GlcNAc_2$-PA; M6, $Man_6GlcNAc_2$-PA; M5, $Man_5GlcNAc_2$-PA.

N-glycans; however, the involvement of the glycoamidase activity toward high-mannose type glycopeptides or the hydrolytic action of an oligosaccharyltransferase or a phosphatase toward the dolichol-oligosaccharide intermediates may be considered. In animal cells, involvement of the PNGase or glycoamidase in the production of free *N*-glycans bearing the *N*-acetyl chitobiosyl unit has been postulated [28,29], since animal glycoamidase activity was recovered in the ER-associated fraction and such animal glycoamidase showed its optimum activity at the neutral pH region. On the contrary, it has been reported that the plant glycoamidase showed its optimum activity at the acidic pH region (pH 4–5) [30–32], suggesting that the plant enzyme would localize in an acidic organelle such as the vacuole (protein body) or the cell wall but not in the cytosol, although the detailed subcellular distribution of the plant glycoamidase has not been established so far. In our previous reports [2,5–8] and other reports [1,3,4], the plant complex type free *N*-glycans bearing xylose/fucose residue(s) ($GlcNAc_{2\sim0}Man_3Xyl_1Fuc_{1\sim0}GlcNAc_2$) have been found in various plant cells. Since it has been established that the plant complex type *N*-glycans are formed in the Golgi apparatus during the late processing process of *N*-glycosylated proteins, the plant complex type free *N*-glycans [1–8] must be formed in certain organelles or cellular sites other than the ER or the ER-associated cytosol. Therefore, distribution analysis of the plant glycoamidase should be necessary to clarify whether this enzyme is involved in the production of the oligomannoside-$GlcNAc_2$ species found in this report, in addition to the production of plant complex type free *N*-glycan.

Concerning the physiological function of these high-mannose type free *N*-glycans, we have recently reported that the high-mannose type free *N*-glycans could induce relevant reconstruction of the quaternary structure of the denatured and deglycosylated plant α-mannosidase and the recovery of the enzymatic activity [18,33]. This may suggest that the free *N*-glycans occurring ubiquitously in plant cells could play an important role as a 'go-between' during protein oligomerization or the assembly of oligomeric proteins, besides their putative function as signaling molecules [9].

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