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Analysis of sugar chain-binding specificity of tomato lectin using lectin blot: recognition of high mannose-type N-glycans produced by plants and yeast

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The sugar chain-binding specificity of tomato lectin (LEA) against glycoproteins was investigated qualitatively using lectin blot analysis. Glycoproteins containing tri- and tetra-antennary complex-type N-glycans were stained with LEA. Unexpectedly, glycoproteins containing high mannose-type N-glycans and a horseradish peroxidase were stained with LEA. LEA blot analysis of the glycoproteins accompanied by treatment with exoglycosidase revealed that the binding site of LEA for the complex-type N-glycans was the *N*-acetyllactosaminyl side chains, whereas the proximal chitobiose core appeared to be the binding site of LEA for high mannose-type N-glycans. Despite these results, the glycoproteins did not inhibit the hemagglutinating activity of LEA. Among the chitin-binding lectins compared, potato tuber lectin showed specificity similar to LEA on lectin blot analysis, while *Datura stramonium* lectin and wheat germ agglutinin (WGA) did not interact with glycoproteins containing high mannose-type N-glycans, except that RNase B was stained by WGA.

Based on these observations, LEA blot analysis was applied to sugar chain analysis of tomato glycoproteins. The most abundant LEA-reactive glycoprotein was purified from the exocarp of ripe tomato fruits, and was identified as the tomato anionic peroxidase1 (TAP1). These results suggest that LEA interacts with glycoproteins produced by tomatoes, which participate in biological activities in tomato plants.

Keywords: **tomato lectin, tomato anionic peroxidase, lectin blot, sugar chain-binding specificity, glycoprotein**

Abbreviations: **LEA: Tomato lectin, WGA: wheat germ agglutinin, STA: *Solanum tuberosum* lectin, DSA: *Datsura stramonium* lectin, SBA: soybean agglutinin, RNase B: ribonuclease B from bovine pancreas, HRP: horseradish peroxidase, GlcNAc: *N*-acetylglucosamine, Fuc: fucose, Xyl: xylose, Man: mannose, Gal: galactose, TAP1: tomato anionic peroxidase-1.**

Introduction

Tomato (*Lycopersicon esculentum*) lectin (LEA) is a chitin-binding protein contained mainly in the locular fluid of ripe fruit [1–3]. Its hemagglutinating activity is inhibited by β1-4 linked GlcNAc oligomers, but not by the monomer. The inhibitory effects of the GlcNAc-oligomer increase with the chain length. The chitopentaose is approximately 190 fold more potent as an inhibitor of LEA compared to chitobiose (GlcNAc β1-4 GlcNAc) [4]. Not only GlcNAc-oligomers, but *N*-acetyllactosmine (Gal β1-4 GlcNAc) which is the common component of complex-type N-glycans and O-glycans, inhibit the activity of LEA, although its inhibitory effect was lower than that of chitobiose [4]. Immobilized LEA interacts with high affinity with glycopeptides containing three or more poly-*N*-acetyllactosamine structures [5,6], and the presence of a β1-6 linked GlcNAc residue followed by a poly-*N*-acetyllactosamine structure is essential for strong binding with a LEA-Sepharose column [7]. Several chitin-binding lectins from other plants, such as wheat germ agglutinin (WGA), *Datura stramonium* lectin (DSA), and potato (*Solanum tuberosum*) tuber lectin (STA) also bind poly-*N*-acetyllactosamine, with each lectin exhibiting a different specificity for such saccharides [7].

Despite its importance in glycobiology, little is known about the physiological role of LEA. Chitin-binding lectins are thought to be involved in plant defense because several possess antifungal or insecticidal activity [8]. However, LEA has no demonstrated activity against either insects [9] or fungi [10].

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Previous studies have examined the function of LEA through structural analysis [11,12]. To elucidate the physiological role of LEA, information about internal ligands might be helpful, even though chitin is not a component of plants.

To identify the ligands of LEA, several glycoproteins with high mannose-type N-glycans were stained with LEA by lectin blot analysis. The carbohydrate-binding specificity of LEA has been determined from hemagglutination inhibition test results and affinity chromatography of oligosaccharides using a LEA-Sepharose column. However, these observations have not been sufficient to fully understand the results of the LEA blot analysis. Although LEA blot analysis is useful in glycobiology, few studies have characterized the binding specificity of LEA using this technique. The author studied the binding specificity of LEA to glycoproteins containing N-glycans using blot analysis. Based on the results, LEA blot analysis was applied to analyze tomato glycoproteins.

Materials and methods

Materials

Biotin-labeled concanavalin A (Con A), *Datura stramonium* lectin (DSA), wheat germ agglutinin (WGA), and unconjugated soybean agglutinin (SBA) were purchased from Seikagaku Co. (Tokyo, Japan). Biotin-labeled *Solanum tuberosum* lectin (STA) was purchased from Vector Laboratories (Burlingame, CA). A Chitin hydrolyzate was prepared from chitin (Wako Chemical Co.) according to the method of Rupley [13]. TOF-MS analysis revealed that the product was a mixture of chitotriose, chitotetraose, and chitopentaose.

Glycoproteins

Fetuin from fetal calf serum, human transferrin, human α1-acid glycoprotein, ovalbumin, ovomucoid, and ribonuclease B from bovine pancreas (RNase B) were purchased from Sigma Chemical Co. (St Louis, MO). Horseradish peroxidase (HRP) was obtained from Wako Chemical Co. (Osaka, Japan). Yeast invertase was purchased from Roche Diagnostics (Mannheim, Germany).

Preparation of biotin-labeled tomato lectin

Tomato (*Lycopersicon esculentum* L. cv. Cherry) plants were grown in a greenhouse. Flowers were pollinated by hand, and fruits were harvested on each day after pollination. The lectin was purified from the red ripe fruits (day 40 to 50 after pollination) as described [11]. Purified lectin (1 mg) was biotinylated by incubation with 0.1 mg EZ-link sulfo-NHS-biotin (Pierce, Rockford, IL) according to manufacturer's instructions. After incubation, excess reagent was removed by dialysis against PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The commercially available biotin-labeled tomato lectin was purchased from Vector Laboratories.

Exoglycosidase digestion of glycoproteins

For the preparation of desialylated glycoproteins, glycoproteins (100 μg) were incubated with 100 mU sialidase (neuraminidase from *Arthrobacter ureafaciens*, Nacalai tesque, Kyoto, Japan) in 100 μl 0.4 M sodium acetate buffer (pH 5.0) containing 4 mM $MgCl_2$ at 37°C overnight. For preparation of desialylated and degalactosylated glycoproteins, glycoprotein (100 μg) was incubated with both 100 mU of sialidase and 1 U of β-galactosidase (from *Aspergillus* sp., Toyobo, Tokyo, Japan) in 100 μl 150 mM citrate-phosphate buffer (pH 5.0) at 37°C overnight. To eliminate terminal GlcNAc residues, 100 μg glycoprotein treated with sialidase and β-galactosidase were digested with 250 mU of β-*N*-acetylhexosaminidase (from jack bean, Seikagaku Co.) in 100 μl of 150 mM citrate-phosphate buffer (pH 5.0) at 37°C overnight.

RNase B (50 μg) was digested with 500 mU α-mannosidase (from jack bean, Seikagaku Co.) in 50 μl 100 mM citrate-phosphate buffer (pH 4.5) at 37°C overnight.

Endoglycosidase digestion

Endo-β-*N*-acetylglucosaminidase H (Endo H; New England Bio Lab, Beverly, MA) digestion was conducted according to manufacturer's directions. Glycoprotein (50 μg) was digested with 50 U Endo H at 37°C overnight.

Before *N*-glycosidase F treatment, 100 μg glycoprotein was denatured in 20 μl digestion buffer (50 mM Tris-HCl, pH 7.2 containing 1% mercaptoethanol and 50 mM EDTA) with 1% SDS at 100°C for 3 min. After denaturation, the reaction solution was diluted with 80 μl digestion buffer containing 1.25% n-octylglucoside. Glycoprotein was incubated with 10 U *N*-glycosidase F (Roche Diagnostics) at 37°C overnight. All reactions were terminated by boiling with the SDS-PAGE loading buffer containing dithiothreitol.

Lectin blot analysis

In general, 5 μg glycoprotein was loaded onto the SDS-PAGE gel (12.5% acrylamide) lanes under reducing conditions. A protein molecular weight marker kit (Daiichi Pure Chemicals, Tokyo, Japan) was used for size standards. Biotinylated protein marker (New England Bio labs) was used as the detection standard with the avidin-alkaline phosphatase detection system. After SDS-PAGE, proteins in the gels were stained with Coomassie brilliant blue (CBB) R-250 or transferred to a nitrocellulose membrane *via* electroblot for further lectin blot analysis. Proteins on the membrane were stained transiently with Ponceu S. After blocking with 1% bovine serum albumin in 10 mM Tris-HCl, at pH 7.4, containing 150 mM NaCl (TBS) overnight at 4°C, the membrane was washed three times with TBS containing 0.05% Tween 20 (TBS-T) for 10 min. The membrane was incubated with 5 μg/ml biotin-LEA in TBS-T for 1 h at room temperature. For inhibition of LEA binding, 5 μg/ml of biotin-LEA was pre-incubated with 0.17% (w/v) of chitin hydrolysate for 90 min at room temperature

before application to the membrane. After washing with TBS-T, the membrane was incubated with avidin-conjugated alkaline phosphatase (Vectastein ABC-AP standard kit, Vector) for 1 h at room temperature. The LEA-reactive glycoproteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate disodium salt. Development was stopped when the biotinylated protein marker appeared. The concentrations of biotin-labeled lectin were: WGA and DSA, 2.5 μg/ml; STA, 10 μg/ml. TBS containing 500 mM NaCl was used for the WGA blot analysis. Other blotting conditions were the same as those used for LEA.

Hemagglutination assay

The hemagglutination activity was titrated by serially diluting the sample (20 μl) with PBS, followed by mixing with an equal volume of a 2% suspension of rabbit erythrocytes. For inhibition assays, lectin solutions (titer 8) were incubated with the glycoprotein of interest, which had been serially diluted with PBS [14].

Purification of LEA-reactive 50-kD glycoprotein (GP50)

All procedures were conducted at 4°C. Exocarps were collected from ripe tomato fruits and stored at −30°C. Frozen exocarps (20 g) were ground in 2 vol. of PBS using a grinder. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant was collected and solid ammonium sulfate was added to 80% saturation. The precipitate was collected by centrifugation at 10,000 *g* for 20 min and dissolved in 5 ml PBS. The solution was dialyzed extensively against the column-loading buffer (20 mM Tris-HCl, pH 8.0) and applied to a DEAE-Toyopearl column (18 cm in length, 1 cm i.d; Tosoh, Tokyo, Japan) equilibrated with loading buffer. The column was washed with 100 ml of the loading buffer. Bound protein was eluted with a linear gradient established between 100 ml of loading buffer and 100 ml of the same buffer containing 0.5 M NaCl. The eluted proteins were monitored at absorption at 280 nm. Fractions containing LEA-reactive protein were pooled. The pooled fraction (6 ml) was diluted with 24 ml of the loading buffer and reapplied to the DEAE-Toyopearl column. The proteins bound completely to the column and were eluted by the same method used initially to remove bound protein. The single protein peak was eluted and pooled. The pooled fraction was diluted 5-fold with loading buffer and applied to a Poros-HQ/M column (150 mm in length, 4.6 mm i.d.; Applied Biosystems, Foster City, CA) equilibrated with loading buffer. The proteins bound completely to the column and were eluted with a linear gradient of NaCl (0–0.5 M) in a volume of loading buffer 10 times column volume. Flow rate was 5 ml/min. Eluted protein fractions were collected. The proteins were measured using the BCA protein assay kit -(Pierce), using bovine serum albumin as a standard.

Amino acid sequence analysis of GP50

The amino acid sequences of protease-digested fragments of the proteins were determined by a previously described method [12]. Briefly, purified GP50 (1 nmol) was separated on 12.5% SDS-PAGE under reducing conditions. After SDS-PAGE, the protein was transferred to a PVDF membrane (Pro-blott, Applied Biosystems) by electroblotting, followed by staining with Ponceu S. The GP50 band was cut out, and was subjected to *S*-alkylation following reduction. The protein was then digested with lysylendopeptidase (Wako Chemical) and any fragments released (*K*-fragments) were collected. Peptides remaining on the membrane were incubated with TPCK-trypsin (Promega, Madison, Wis.) and the digests (*T*-fragments) were collected. Each peptide fragment was separated by reverse phase high performance liquid chromatography (Wakosil-II AR 5C18 2.0, 150 mm in length, 2 mm i.d., Wako Chemical) and analyzed with a 477A protein sequencer (Applied Biosystems). The obtained sequences were subjected to BLAST at the National Center for Biotechnology Information (NCBI) for an identity search.

Determination of peroxidase activity

For peroxidase activity staining in polyacrylamide gels, protein samples were resolved by 7.5% PAGE. After electrophoresis, the gel was equilibrated for 30 min in 50 mM sodium phosphate, at pH 7.0, followed by visualization of the peroxidase by incubating the gel in 0.032% guaiacol and 0.012% H_2O_2 in the same buffer [15].

Results

1. Sugar chain-binding specificities of tomato lectin and other chitin-binding lectins

The sugar chain-binding specificities of LEA were determined by lectin blot against nine well-characterized glycoproteins. After SDS-PAGE, 5.0 μg of each glycoprotein was stained with CBB (Figure 1A) or biotin-labeled LEA after transfer onto a nitrocellulose membrane (Figure 1B). The results were compared with those of biotin-labeled chitin-binding lectins from two *Solanaceae* species (*Solanum tuberosum* and *Datura stramonium*) and from wheat germ (Figures 1C–E). Figure 1B shows that LEA markedly stained fetuin, α1-acid glycoprotein, and ovomucoid containing tri- or more highly branched N-glycans (lanes 1, 3, and 5) [16–18]. A faint band was observed for transferrin containing two biantennary complex-type N-glycans per molecule (lane 2) [19]. LEA did not stain ovalbumin containing either a hybrid or a high mannose-type N-glycan per molecule (lane 4) [20,21]. Interestingly, LEA clearly stained glycoproteins containing high mannose-type N-glycans, such as RNase B [21,22], soybean agglutinin (SBA) [23], and yeast invertase [24] (lanes 6, 8, and 9). LEA also stained horseradish peroxidase (HRP) (lane 7). HRP contains N-glycans consisting of the trimannosyl core structure with β1-2 xylose and α1-3 fucose residues, designated as (Xyl)Man_3GlcNAc(Fuc)GlcNAc [25].

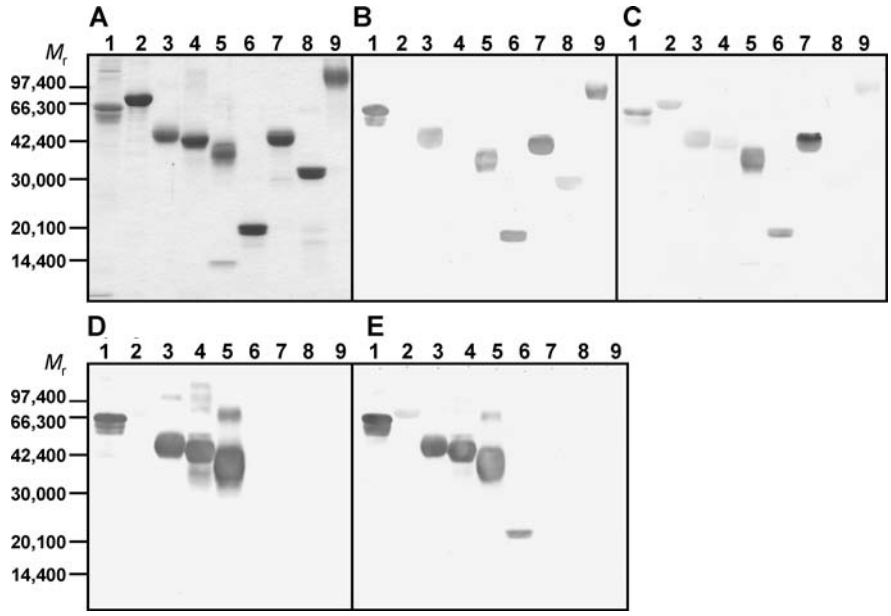

Figure 1. Comparison of the staining pattern of glycoproteins by lectin blot using chitin-binding lectins. Glycoproteins (5 μg each) were applied to SDS-PAGE gel (12.5%) and subjected to electrophoresis. (A) Glycoproteins were stained with CBB R-250. (B–E) After electrophoresis, the glycoproteins were transferred to a nitrocellulose membrane and stained with each lectin: **B**, LEA; **C**, STA; **D**, DSA; **E**, WGA. *Lanes:* 1, fetuin; 2, transferrin; 3, α1-acid glycoprotein; 4, ovalbumin; 5, ovomucoid; 6, RNase B; 7, HRP; 8, SBA; 9, yeast invertase.

No glycoproteins were stained with LEA in the presence of chitin hydrolyzate, a potent hapten sugar of LEA, indicating that these signals resulted from specific binding of LEA to the sugar chains (data not shown). The same result was obtained from lectin blot analysis using purchased LEA samples (data not shown). STA produced a staining pattern similar to that produced by LEA (Figure 1C), except that the staining intensities of SBA and yeast invertase were faint (lanes 8 and 9). In contrast, DSA did not react with glycoproteins containing high mannose-type N-glycans and HRP (Figure 1D, lanes 6–9). WGA gave a staining pattern similar to that produced by DSA, except that RNase B was stained (Figure 1E, lane 6).

To examine whether the glycoproteins with LEA reactivity inhibit the hemagglutinating activity of LEA, an LEA solution (titer 8) was incubated with some glycoproteins. Glycoproteins that did not inhibit activity up to 25 mg/ml included fetuin, ovomucoid, RNaseB, HRP, and yeast invertase.

2. Recognition of complex-type N-glycans by LEA

The structural requirements for interaction between complex-type *N*-glycans and LEA were investigated using LEA blot analysis (Figure 2). Fetuin and transferrin were sequentially digested with exoglycosidases and subjected to LEA blot analysis. Desialylation did not affect the LEA-reactivity of fetuin (Figure 2B, lane 2), while the treatment increased the reactivity of transferrin (Figure 2B, lane 7). The LEA-reactivities of both desialylated glycoproteins were greatly reduced following the elimination of galactosyl residues (Figure 2B, lanes 3 and 8), indicating that the *N*-acetyllactosaminyl structure is the binding site of LEA for the complex-type N-glycans. Further elimination of the GlcNAc residues resulted in the significant increase of LEA-reactivities of both glycoproteins (Figure 2B, lanes 4 and 9). The bands of M_r 120,000 detected in lanes 3, 4, 8, and 9 were derived from galactosidase. Fetuin and transferrin lost their LEA-reactivities on *N*-glycosidase F treatment (Figure 2B, lanes 5 and 10), indicating that LEA interacts with N-glycans on both glycoproteins.

3. Recognition of high mannose-type N-glycans by LEA

To confirm the specific interaction of LEA with high mannose-type N-glycans, the reactivity of LEA against Endo H-treated glycoproteins was examined. RNase B, SBA, and yeast invertase lost the LEA-reactivity upon Endo H treatment, indicating that LEA binds to high mannose-type N-glycans on these glycoproteins (Figures 3B, lanes 1–6). The HRP band did not shift after digestion with either Endo H or *N*-glycosidase F, and it maintained the LEA-reactivity (Figures 3A and B, lanes 7–9), indicating that LEA binds to sugar chains which are resistant to these enzymes.

Structural requirements for the interaction of high mannose-type N-glycans with LEA were investigated using LEA blot analysis. RNaseB (5 μg) was treated with various glycosidases and subjected to LEA blot analysis. Glycosidase treatment resulted in the decrease of RNaseB molecular weight at each lane on SDS-PAGE (Figure 4A). The α-mannosidase-treated RNaseB was stained with LEA as well as the intact RNaseB (Figure 4B, lane 2). The elimination of α-mannose residues from the sugar chain of RNaseB was also confirmed by the loss

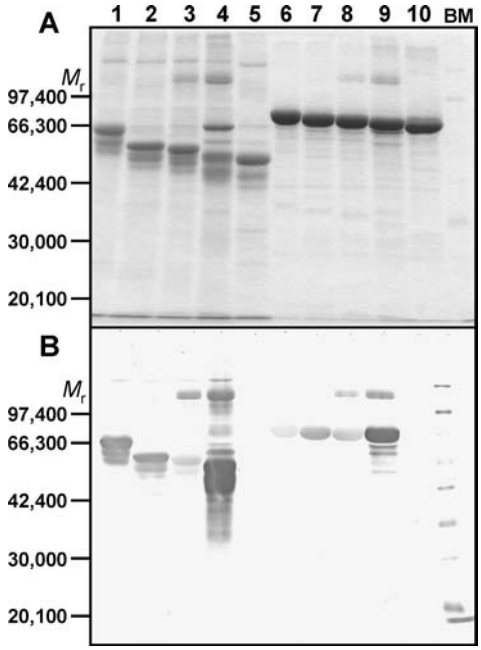

Figure 2. LEA blot analysis of fetuin and transferrin treated with various glycosidases. Fetuin (5 μg, lanes 1–5) and transferrin (lanes 6–10) treated with various glycosidases were applied to SDS-PAGE gel (10%) and subjected to electrophoresis. (A) Samples were stained with CBB R-250. (B) After electrophoresis, the proteins were transferred to a nitrocellulose membrane and stained with LEA. *Lanes:* 1, 6, without glycosidase treatment; 2, 7, with sialidase treatment; 3, 8, with sialidase and β-galactosidase treatment; 4, 9, with sialidase, β-galactosidase, and β-*N*-acetylhexosaminidase treatment; 5, 10, with *N*-glycosidase F treatment; BM, biotin-labeled size standards.

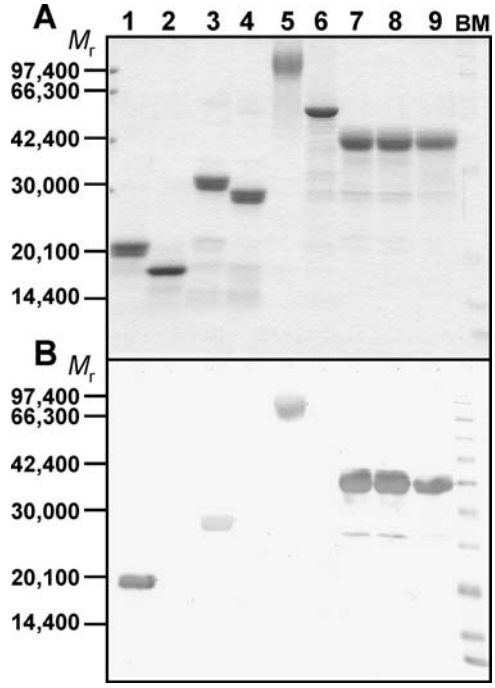

Figure 3. LEA blot analysis of glycoproteins with high mannose-type N-glycans. Each glycoprotein (5 μg) treated with Endo H was applied to SDS-PAGE gel (15%) and subjected to electrophoresis. (A) Samples were stained with CBB R-250. (B) After electrophoresis, the proteins were transferred to a nitrocellulose membrane and stained with LEA. *Lanes:* 1, RNase B; 2, RNase B with Endo H treatment; 3, SBA; 4, SBA with Endo H treatment; 5, Yeast invertase; 6, Yeast invertase with Endo H treatment; 7, HRP; 8, HRP with Endo H treatment; 9, HRP with *N*-glycosidase F treatment; BM, biotin-labeled size standards.

of the concanavalin A reactivity (data not shown). RNaseB lost the LEA-reactivity upon EndoH treatment (Figure 4B, lane 3). These results indicate that the binding site of LEA for the high mannose-type N-glycans is restricted within the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc in the trimannosyl core structure, and the chitobiose core structure is essential for LEA-binding. The bands around M_r 60,000 detected in lanes 2 and 3 were derived from α-mannosidase (Figures 4A and B).

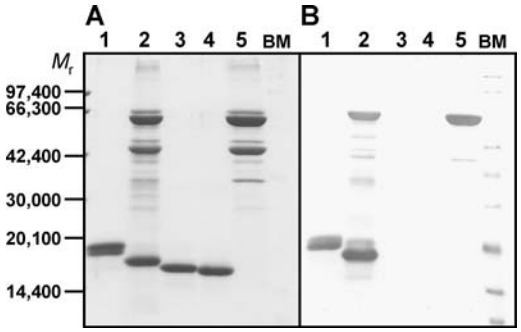

Figure 4. LEA blot analysis of RNaseB treated with various glycosidases. RNaseB (5 μg) treated with various glycosidases was applied to SDS-PAGE gel (15%) and subjected to electrophoresis. (A) Samples were stained with CBB R-250. (B) After electrophoresis, the proteins were transferred to a nitrocellulose membrane and stained with LEA. *Lanes:* 1, without glycosidase treatment; 2, with a α-mannosidase treatment; 3, with Endo H treatment; 4, with *N*-glycosidase F treatment. In *lane 5*, only α-mannosidase was applied. Biotin-labeled size standards are shown in *lane BM*.

4. Identification of tomato glycoproteins recognized by LEA

Based on these observations, LEA blot analysis was applied to the sugar chain analysis of tomato glycoproteins to identify the glycoproteins interacting with LEA in tomato fruits. By LEA blot analysis, some glycoproteins were detected at each development stage of the tomato fruit (Figure 5A). Among them, the glycoprotein with a M_r value of 50,000 (designated GP50) was dominant. It appeared at day 30 after pollination, and the content gradually increased during ripening. Figures 5B and C

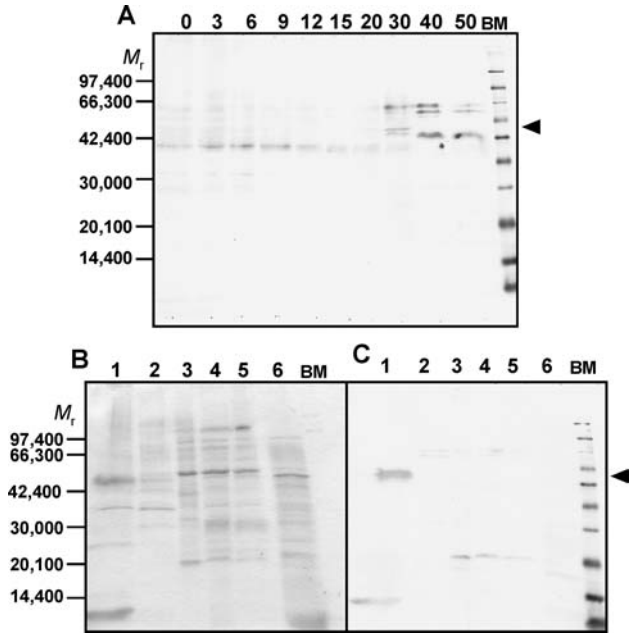

Figure 5. LEA blot analysis of proteins extracted from tomato fruits. (A) LEA blot analysis of glycoproteins from developing tomato fruits. Crude extracts (20 μg of protein equivalent) prepared from whole tomato fruits were analyzed by SDS-PAGE (12.5%). After blotting, the membrane was incubated with LEA. Lane numbers indicate the number of days after pollination. (B) and (C) LEA blot analysis of glycoproteins from ripe tomato fruit tissues. Crude extracts (20 μg of protein equivalent) prepared from ripe tomato fruit tissues were analyzed by SDS-PAGE (12.5%). Proteins were stained with CBB R-250 (B). After blotting, the membrane was stained with LEA (C). *Lanes:* 1, Exocarp; 2, Mesocarp; 3, Placental tissue; 4, Locular gel; 5, Locular fluid; 6, Seeds; BM, biotin-labeled size standards. *Arrow heads* indicate the position of GP 50.

show that GP50 is localized to the exocarp of ripe fruit (lane 1). These findings suggest the possibility that GP50 may interact with LEA in tomato fruits, since the exocarp from ripe tomatoe is known to contain hemagglutinating activities [2]. GP50 was purified from the exocarp of ripe tomatoes. After ammonium precipitation, the protein fraction was loaded on a DEAE-Toyopearl column and bound proteins were eluted with a linear NaCl gradient. The fraction containing GP50 was collected and further purified using a Poros HQ/M anion exchange column (Figure 6A and B). The purified GP50 was subjected to internal sequence analysis. As shown in Table 1, the database homology search (NCBI blastp) indicated that the sequences of peptides derived from the protease digestion of GP50 agreed with the protein sequence deduced from the tomato anionic peroxidase1 (TAP1) gene [26]. Furthermore, purified GP50 possessed peroxidase activity (Figure 6C, lane 1). From these results, GP50 was identified as TAP1. The TAP1 is predicted to encode a protein with an M_r of 30,600, and has seven putative N-glycosylation sites [26]. The purified GP50 fraction gave multiple bands on PAGE after peroxidase activity staining

Table 1. Comparison of the amino acid sequences of GP50 and tomato anionic peroxidase-1 (TAP1). The peptide sequence derived from lysylendopeptidase digest (K-peptide) and trypsin digests (T-peptides) of GP50 are aligned with sequence from TAP1 [26].

Peptides[a]	*Sequence*
T2	AVVDSAIDAET
TAP1 (88–98)	AVVDSAIDAET
T1	GYEVIAQAK
TAP1 (145–153)	GYEVIAQAK
T3	EMVALAGAHTVGFAR
TAP1 (231–245)	EMVALAGAHTVGFAR
K1	MGDLPPSAGA
TAP1 (336–345)	MGDLPPSAGA

[a]Numbers in parentheses are residue numbers of the sequence.

(Figure 6C, lane 1). Further analysis indicated that they were isozymes derived from the TAP1 gene as a result of processing (author's unpublished experiments).

The binding of LEA to GP50 (TAP1) was investigated by LEA blot analysis. As observed in HRP (Figure 3), the TAP1 band did not shift upon digestion with EndoH or *N*-glycosidase F, and it retained LEA reactivity (Figures 7A and B). These results indicate that LEA binds to sugar chains on TAP1, which are resistant to EndoH or *N*-glycosidase F.

Discussion

This report describes the sugar chain binding-specificity of LEA using lectin blot analysis. Glycoproteins containing tri- or more-highly branched complex-type N-glycans were clearly stained with LEA, while transferrin containing biantennary complex-type N-glycans showed a faint staining reaction with LEA, indicating that the tri-antennary structure with the C-2,4 branch may be essential for binding to LEA. Lee *et al*. reported that glycopeptides with tri-antennary complex-type N-glycans lacking the poly-*N*-acetyllactosaminyl repeat are not retained on an LEA-Sepharose column [6], which suggests that LEA blot analysis detects the relatively weak interactions between LEA and glycoproteins. The lack of inhibition of the hemagglutination activity of LEA by LEA-reactive glycoproteins supports this suggestion. Further analysis is needed to estimate the affinity of LEA for these glycoproteins.

LEA recognized different sugar chain units in complex-type and high mannose-type N-glycans. The *N*-acetyllactosamine structure is the primary binding site of LEA for complex-type N-glycans. In contrast, the chitobiose core appears to be the binding site of LEA for high mannose-type N-glycans. N-glycans of HRP have been reported to contain an α1-3 fucose residue attached to the proximal GlcNAc of the chitobiose core, which are resistant to *N*-glycosidase F [27]. The fucose substituent must not affect LEA binding, since *N*-glycosidase F-

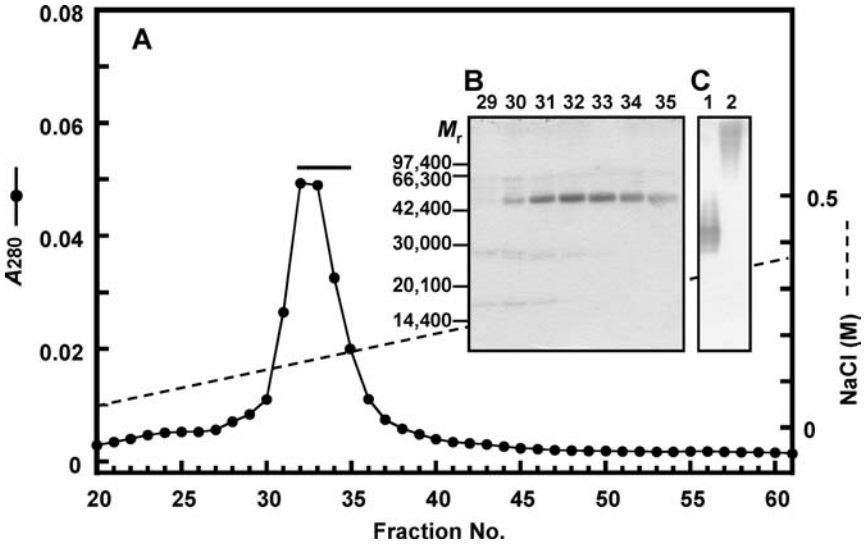

Figure 6. Anion exchange chromatography of GP50. The LEA-reactive protein fraction from DEAE - Toyopearl chromatography was applied to a Poros HQ/M column. (A) The column was eluted with a linear gradient of NaCl (0 – 0.5 M). (B) Purity of each fraction (fractions 29-35) was confirmed by SDS-PAGE. Fractions indicated by a bar were collected. (C) An aliquot of the pooled fraction (lane 1) and 5 μg of HRP as a control (lane 2) were subjected to PAGE. After electrophoresis, the gel was stained for peroxidase activity using the substrate guaiacol and H_2O_2.

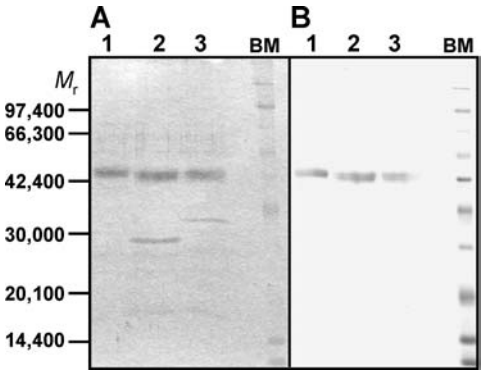

Figure 7. LEA blot analysis of GP50 and HRP. GP50 (2 μg) treated with endoglycosidases was applied to SDS-PAGE gel (12.5%) and subjected to electrophoresis. (A) Samples were stained with CBB R-250. (B) After electrophoresis, the proteins were transferred to a nitrocellulose membrane and stained with LEA. Lanes: 1, GP50; 2, GP50 treated with Endo H; 3, GP-50 treated with N-glycosidase F; BM, biotin-labeled size standards.

treated HRP was stained with LEA. It is unclear why LEA was not able to access the chitobiose core in complex-type N-glycans. One possible explanation is the influence of both GlcNAc residues attached on the Manα1-3 and Manα1-6 arms in the trimannosyl core structure, since the elimination of the two terminal GlcNAc residues from the desialylated and degalactosylated forms of transferrin resulted in higher reactivity toward LEA. Ovalbumin was not stained with LEA. Ovalbumin is a glycoprotein with a single glycosylation site, and the N-glycans are either a hybrid or a high mannose-type [20,21]. It is unlikely that all glycoproteins containing high mannose-type N-glycans are stained with LEA, but this remains to be determined.

Based on the preference for high mannose-type N-glycans, LEA and STA were distinguished from DSA and WGA on blot analysis. The lectin-blot analysis used here is an effective method for characterizing the chitin-binding lectins that share similar specificities. The chitin-binding lectins contain common structural motifs of 30–43 amino acids known as the hevein domain [8]. The mature WGA polypeptide consists of four tandem arrayed homologous hevein domains [28]. Although STA also contains four hevein domains, its domain construction is different from WGA. STA consists of two homologous chitin-binding modules, built up of two in-tandem arrayed hevein domains that are interconnected by a hydroxyproline-rich glycoprotein-like domain [29]. From this molecular feature, STA adopts an elongated structure with a chitin-binding module at both ends. It appears that this domain construction enables STA to approach the chitobiose core of high mannose-type N-glycans. Since the genes for LEA and DSA have not yet been isolated, further research is needed to reveal the relationships between the sugar chain-binding specificities and the structures of chitin-binding lectins.

TAP1 was identified as a possible ligand glycoprotein of LEA in tomato fruits. TAP1 is a tomato anionic peroxidase expressed in the exocarp of green tomato fruits. This gene is induced by the wounding of tomato fruits and by elicitor treatment in cell suspension cultures [26,30], indicating TAP1 is probably involved in suberization of the cell wall during the maturation of tomato fruits and wound-healing [31]. Recently, TAP1 was identified as a possible allergen, recog-

nized by IgE in food-allergic patients [32]. Since the N-glycans of TAP1 were tolerant to both Endo H and N-glycosidase F treatment, the structures must be similar to those of HRP. Zenley *et al*. reported that (Xyl)Man_3GlcNAc(Fuc)GlcNAc and (Xyl)Man_2GlcNAc(Fuc)GlcNAc are abundant structures in the total N-glycan pool released from glycoproteins obtained from ripe tomato fruits [33]. Since TAP1 is a major glycoprotein in ripe tomatoes, the majority of these N-glycans are likely derived from TAP1. It remains unknown whether LEA interacts with TAP1 in tomato fruits. Some researchers have reported that the activity of plant peroxidases decreased upon the *in vitro* addition of lectins such as WGA and concanavalin A [34]. It is of interest to determine the ability of LEA to inhibit TAP1 activity.

LEA blot analysis indicated that LEA interacts with high mannose-type N-glycans of yeast invertase. Basse *et al*. reported that the glycopeptides of yeast invertase with high mannose-type N-glycans elicited high activity against tomato cells at nanomolar concentrations [35]. It has been reported that free N-glycans contained in plant tissues have biological activities. Gross *et al*. revealed that the free N-glycans (Xyl)Man_3GlcNAc(Fuc)GlcNAc and Man_5GlcNAc stimulated and delayed the ripening of mature and green tomato fruit, respectively, when these sugar chains were applied to tomato pericarp discs. Furthermore, Man_5GlcNAc lost biological activity in the presence of concanavalin A and LEA [36], although the mechanism for this activity loss has not yet been elucidated. It is likely that LEA plays a role in the interaction with biologically active glycans in tomato plants. The interaction of LEA with N-glycans may lead to the discovery of novel functions of LEA, which was believed previously to act through interaction with chitin.

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