

Research Article

Enzymatic Digestion and Mass Spectroscopies of N-Linked Glycans in Lacquer Stellacyanin from *Rhus vernicifera*

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Lacquer stellacyanin was isolated and purified from lacquer acetone powder by continuous Sephadex column chromatographies using Sephadex C-50, DEAE A-50, and C-50 gels. The purified lacquer stellacyanin had a blue color with one major and three minor bands around 26 k Dain SDS PAGE. Trypsin- and chymotrypsin-treated lacquer stellacyanins were examined by LC/MS/MS to determine three N-glycosylation sites (N28, N60, and N102) and were further analyzed by MALDI TOF MS, indicating that the N-linked glycans were attached to the three asparagine (Asn) sites, respectively. In addition, after trypsin digestion and PNGase A and PNGase F treatments to cleave N-linked glycans from the Asn sites, it was found that lacquer stellacyanin had a xylose containing a biantennary N-linked glycan with core fucosylation consisting of 13 sugar residues (a complex type N-linked glycan) by MALDI TOF MS analysis. This is the first report on the structure of an N-linked glycan in lacquer stellacyanin.

1. Introduction

Lacquer sap is a naturally occurring paint that is polymerized by laccase, an oxidoreductive enzyme of catechols, to give a durable film with a glorious surface. Lacquer sap is a water-in-oil emulsion composed mainly of urushiols, polysaccharides, glycoproteins, laccase, and stellacyanin [1]. Among them, stellacyanin, like laccase, is a copper-containing glycoprotein; it was first characterized as a blue glycoprotein by Keilin and Mann in 1940 [2]. Lacquer stellacyanin is a low molecular weight (approximately 20 kDa) protein with a single polypeptide chain of 107 amino acid residues and a Cu atom. Since the glycoprotein was identified as stellacyanin [3], proteins similar to lacquer stellacyanin have been isolated from cucumber [4], zucchini peel [5], spinach leaf [6], and horseradish root [7]. These glycoproteins are plant specific glycoproteins belonging to the phytocyanin subclass of the cupredoxins. Stellacyanin is an electron transfer protein [8] and is involved in a redox process during primary defence, lignin

formation, and cell-to-cell signalling transmission; however, the exact physiological function is still unclear [9–11]. In addition, N-linked glycans in plant glycoproteins have been shown to affect catalytic activity, thermostability, lignin formation, folding or subcellular localization, and secretion [12, 13]. Recently, it was found that N-linked glycans may play a role in plant pathogen integration and functional pattern recognition receptors [14]. Removal of complex type N-glycans from plant glycoproteins by mutational modification of N-linked glycans in several plants, *Arabidopsis* and tobacco, has demonstrated no or little effect on plant growth [15]. For the structural analysis of the carbohydrate composition of lacquer stellacyanin, no results have been published except a paper on chromatographic separation of monosaccharides of stellacyanin hydrolysate by Peisach et al. in 1967 [16].

We have investigated the structure and specific biological activities of lacquer polysaccharides in Japanese and Chinese

lacquer saps. The lacquer polysaccharides with the number-average molecular weight of $M_n = 10 \times 10^3 - 30 \times 10^3$ were analysed by high resolution NMR measurement, revealing that the lacquer polysaccharides were acidic polysaccharides and had a (1 → 3)- β -D-galactopyranan main chain with complex branches consisting of glucuronic acid in the terminal [17]. The lacquer polysaccharides were found to reduce the growth of sarcoma 180 tumour in mice and had blood coagulation-promoting activity compared to that of control [18]. After sulfation, lacquer polysaccharides showed potent anti-HIV and low blood anticoagulant activities. These specific biological activities should originate from the acidic branched structure [18]. In addition, treatment with diluted alkaline aqueous solution revealed that lacquer polysaccharides were constructed of association of low molecular weight polysaccharides [19].

Glycosylation is one of the most common and important modifications of peptides that changes the functions of proteins. In general, N-linked glycans are located at an asparagine (Asn) residue of the tripeptide sequence, Asn-X-Ser or Thr, where X is any amino acid except proline [20]. There are three possible N-glycosylation sites (Asn-X-Ser or Thr) in the amino acid sequence of stellacyanin (N28, N60, and N102) [21]. Mass spectrometry (MS) is valuable and rapid analytical techniques to analyse structures of glycans in proteomics and proteins by a combination with enzymatic treatments [22]. In this study, we report for the first time the structural analysis of N-linked glycans present in lacquer stellacyanin by both matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) and liquid chromatography mass spectrometry coupled with electrospray ionisation tandem mass spectrometry (LC/MS/MS) after enzymatic digestion for the elucidation of stellacyanin functionality in lacquer sap.

2. Materials and Methods

2.1. Materials. Lacquer acetone powder was prepared by addition of acetone to lacquer sap according to the method reported by Reinhammar [23]. CM-Sephadex C-50 and DEAE-Sephadex A-50 were purchased from Healthcare Bio-Sciences AB, Sweden. Trypsin from porcine pancreas ($\geq 10,000$ units/mg), α -chymotrypsin from bovine pancreas (61.75 units/mg), dithiothreitol (DTT), iodoacetamide (IAA), and trifluoroacetic acid (TFA) were obtained from Sigma Aldrich, Japan. Peptide-N-Glycosidase A (PNGase A) from almond and Peptide-N-Glycosidase F (PNGase F) were purchased from Roche. Other reagents, *Arthrobacter ureafaciens* sialidase from Nakalai, a BlotGlyco glycan purification and labeling kit (BS-45603) from Sumitomo Bakelite Co., Ltd., 2,5-dihydroxybenzoic acid (DHB) as a matrix for MALDI-TOF/MS from Bruker Daltonics GmbH, 10–20% gradient gels (E-T1020L), EZ marker standards (AE-1440), and Coomassie blue for SDS-PAGE analysis from ATTO Corporation, Japan, were used.

2.2. Isolation and Purification of Lacquer Stellacyanin from Acetone Powder. Lacquer acetone powder (20 g), which was prepared by the method of Reinhammar [23], was dissolved

in 200 mL of 0.01 M potassium phosphate buffer solution (pH 6.0) and then stirred overnight at 4°C. The mixture was centrifuged and then filtered to remove any insoluble materials. The filtrate was chromatographed on a CM-Sephadex C-50 column (250 mm × 40 mm) to collect lacquer laccase and stellacyanin as blue colorbands eluted with 0.1 M and 0.2 M phosphate buffer solution (pH 6.0), respectively. Lacquer stellacyanin was further purified by DEAE-Sephadex A-50 column chromatography (500 mm × 20 mm) and subsequent CM-Sephadex C-50 column chromatography (500 mm × 20 mm) gradually eluted with 0.005 M, 0.025 M, 0.05 M, and 0.1 M phosphate buffer solutions. The eluent with a blue colour was finally desalted by dialysis with deionized water overnight and then freeze-dried to give 0.233 g (1.17%) and 0.083 g (0.42%) of pure lacquer laccase and stellacyanin, respectively.

2.3. Preparation of Copper-Free Stellacyanin. Blue stellacyanin (56 mg) was dissolved in 20% aqueous trichloroacetic acid solution (10 mL), and the mixture was stirred until the blue color disappeared. After dialysis against deionized water overnight, it was then freeze-dried to give colorless stellacyanin without copper. The purity and molecular weights of the purified stellacyanin were measured by SDS-PAGE and MALDI TOF MS, respectively.

2.4. Enzymatic Digestion and N-Glycan Labeling. Lacquer stellacyanin (1 mg) was suspended in 0.1 M NH_4HCO_3 solution, DTT (5 μL , 120 mM) was added, and then the mixture was incubated for 30 min at 60°C. To the mixture was added 10 μL of IAA (123 mM) and it was incubated in the dark for 1 h at room temperature to give carboxamide-methylated stellacyanin, which was used without purification for the next enzymatic digestion. Trypsin (1 mg) or chymotrypsin (1 mg) solution in 1 mL of 0.1 M NH_4HCO_3 (10 μL) was added to the above carboxamide-methylated stellacyanin solution and incubated overnight at 37°C and 25°C, respectively, to digest stellacyanin, and then the mixture was heated to 95°C to denature trypsin or chymotrypsin.

The trypsin-digested stellacyanin was deglycosylated by adding 0.25 U PNGase A at pH 5.0 (adjusted by 0.1% HCOOH) and 5 U PNGase F at pH 8.5, respectively, overnight at 37°C. The digested N-glycans were labeled with N^α -((aminoxy)acetyl)tryptophanylarginine methyl ester (aoWR) by using a BlotGlyco glycan purification and labeling kit according to the manufacturer's protocol and then the aoWR-labelled glycans were purified by a clean-up column according to the manufacturer's protocol [24]. The chymotrypsin-digested stellacyanin was also treated with 0.25 U PNGase A and 5 U PNGase F, respectively, by the same procedure as above to give aoWR-labelled glycans.

2.5. Sialidase Treatment. Lacquer stellacyanin (0.5 mg) was treated with *Arthrobacter ureafaciens* sialidase (1.0 U in 50 mM Tris/HCl buffer) overnight at pH 5.6 and 37°C to confirm the presence or absence of sialic acid in the glycans.

2.6. MALDI TOF MS. The MALDI TOF MS spectrum was recorded on an Ultraflex II instrument (Bruker Daltonics) in reflection mode with an acceleration voltage of 25 kV in

the positive ion mode (positive voltage polarity). The MS spectrum was automatically provided by using FlexControl software 3.0 version. External calibration was carried out with singly charged monoisotopic peaks of peptide standards (1046.5 Da angiotensin II, 1296.6 Da angiotensin I, 1347.7 Da substance P, 1619.8 Da bombesin, and 2093.0, 3474.5 ACTH CLIP peptides). DHB matrix in acetonitrile/water (3:7, v/v) was used for the MALDI TOF MS measurements of the aoWR-labelled N-glycans, trypsin-digested peptide, and glycopeptide mixture. The aoWR-labelled N-glycans were mainly detected as protonated molecular ions $[M+H]^+$. Sialic acid residues in glycan were esterified by a methyl group ($-\text{COOH} \rightarrow -\text{COOCH}_3$; mass signal increases +14.02), and the exact masses of glycans were calculated as glycan exact mass $[M] = [\text{observed } m/z] - 447.21 + 18.01 - (14.02 \times n) - 1.00$, where 447.21 is the mass of the aoWR labelling reagent and n is the number of sialic acids.

2.7. LC/MS/MS. LC/MS/MS was performed using a liquid chromatography (Shimadzu LC system) tandem mass spectrometry (AB SCIEX API 4000 Q TRAP) system with Turbo spray source and electrospray ionization (ESI). An ODS column (TSK-Gel ODS-80Ts (5 μm) with ID 2.5 mm \times 15 cm and a TSK guard cartridge (5 μm) with ID 3.2 mm \times 1.5 cm) was used at 30°C. The eluents used were (A) 0.1% TFA in 90% acetonitrile and (B) 0.1% TFA in 10% acetonitrile with a flow rate of 0.2 mL/min in a gradient program for 120 min. And MS/MS spectra were acquired in the positive ionization mode with an acquisition time of 0.63 spectra per second.

Also, the following MS data were acquired by enhanced MS (EMS), in which the scan rate was 4000 Da/s, scan range was 100–2800 Da, fixed linear ion trap (LIT) full time was 10 ms, and Q0 was off check. The enhanced resolution MS (ER), in which scan rate was 250 Da/s, fixed LIT full time was 10 ms, and Q0 was on, and information dependent acquisition (IDA) criteria were inserted to select the 1–3 most intense peaks; ions greater than 400 (m/z) and smaller than 1500 (m/z) and peak intensity which exceeds 50000 cps were chosen. Finally, two enhanced product ion (EPI) experiments were performed, in which scan rate was 4000 Da/s, scan range was 100–2500, fixed LIT full time was 50 ms, and Q0 was trapping on.

MS data were processed with DataAnalyst 4.0 by using a default setting for glycopeptide analysis. Peptide sequences in glycopeptides obtained by proteases were annotated by BioAnalyst (peptide sequence), and MS/MS analyses of attached glycans were interpreted manually. Protein information is available in UniProt with ID number P00302 and entry name STEL_TOXVR (<http://www.uniprot.org/uniprot/P00302>).

3. Results and Discussion

3.1. Purification and Molecular Weight of Lacquer Stellacyanin. Scheme 1 shows the analytical scheme of the lacquer stellacyanin by enzymatic hydrolysis. The lacquer stellacyanin was isolated and purified from lacquer acetone powder, which was obtained by addition of acetone to lacquer sap, by continuous column chromatographies of Sephadex C50 and DEAE A50 gels. Figure 1 shows the (a) SDS-PAGE profile,

(b) MALDI TOF MS profile, and (c) amino acid sequence of lacquer stellacyanin. The purified lacquer stellacyanin was confirmed by SDS-PAGE, which revealed that the lacquer stellacyanin had one major and three minor bands around 26 kDa. The bands were very close to each other as shown in Figure 1(a). We used this lacquer stellacyanin without further purification. Figure 1(b) shows the MALDI TOF MS spectrum of the lacquer stellacyanin using DHB as the matrix, in which several signals, at least four peaks, appeared around 17000–19000 Da with one strong and three weak signals. The strongest signal appeared around 18700 Da. These results suggested that the lacquer stellacyanin is not a single protein but consists of isoenzymes of the same protein. The molecular weights by the SDS-PAGE electrophoresis were larger than those of MALDI TOF MS, probably due to the linear structure by SDS. Figure 1(c) exhibits the amino acid sequence of the lacquer stellacyanin according to the literature [21]. The lacquer stellacyanin had 107 amino acid residues and the molecular weight of the amino acid residues was 12296 Da. Therefore, the molecular weight of the carbohydrate portions of the lacquer stellacyanin is around 7000 Da.

3.2. LC/MS/MS Analysis of Glycopeptides. For identification of the N-glycosylation sites in the lacquer stellacyanin, trypsin-digested peptides and glycopeptides were directly analysed by LC/MS/MS without any separation and purification because LC/MS/MS is a high resolution tool for analysis of mixed samples with the liquid chromatography (LC) system. We used two different proteases, trypsin and chymotrypsin, to obtain three possible N-glycosylation sites that contain Asn with an N-linked glycan from the amino acid sequence of stellacyanin. For identification of glycopeptides, the mixture of the trypsin-digested peptides and glycopeptides was selectively detected by extracted ion chromatography (XIC) to reveal that it contains a glycan marker ion of m/z 204.1 due to glucosamine, GlcNAc^+ . The marker ion was extracted by total ion chromatography (TIC) in the LC/MS profile, and then it appears in several separated areas in the XIC. We identified the three glycopeptides with an N-linked glycan at Asn28, Asn60, and Asn102, based on the peptides' theoretical masses; these were WASN_{28}K ($m/z = 605.2$), $\text{NYQSCN}_{60}\text{DTTPIASYNTGBBR}$ ($m/z = 2291.9$), and $\text{VHIN}_{102}\text{VTVR}$ ($m/z = 937.5$) as shown in Table 1. The MS/MS spectrum of the glycopeptides was analysed, and then the signals at $m/z = 204.1$ (GlcNAc_1), $m/z = 366.1$ ($\text{Gal}+\text{GlcNAc}$), and $m/z = 512.1$ ($\text{Fuc}+\text{Gal}+\text{GlcNAc}$) due to fragment ions of N-linked glycans as well as the b and y type fragment ions of oligopeptides cleaved at the glycosidic bond were very frequently observed. After confirmation of the N-glycopeptides derived by trypsin in the MS/MS, the characterization of the attached N-linked glycans was based on their product ion spectra.

The LC/MS/MS spectra of trypsin-digested lacquer stellacyanin are shown in Figure 2. The TIC and XIC containing the $m/z = 204.1$ ion signal due to GlcNAc are presented in Figures 2(a) and 2(b). A glycopeptide WASN_{28}K with Asn at Asn28 was observed around 13–15 min in the TIC, and the most intense ion signal at $m/z = 605.2$ corresponded to the molecular weight of the oligopeptide part.

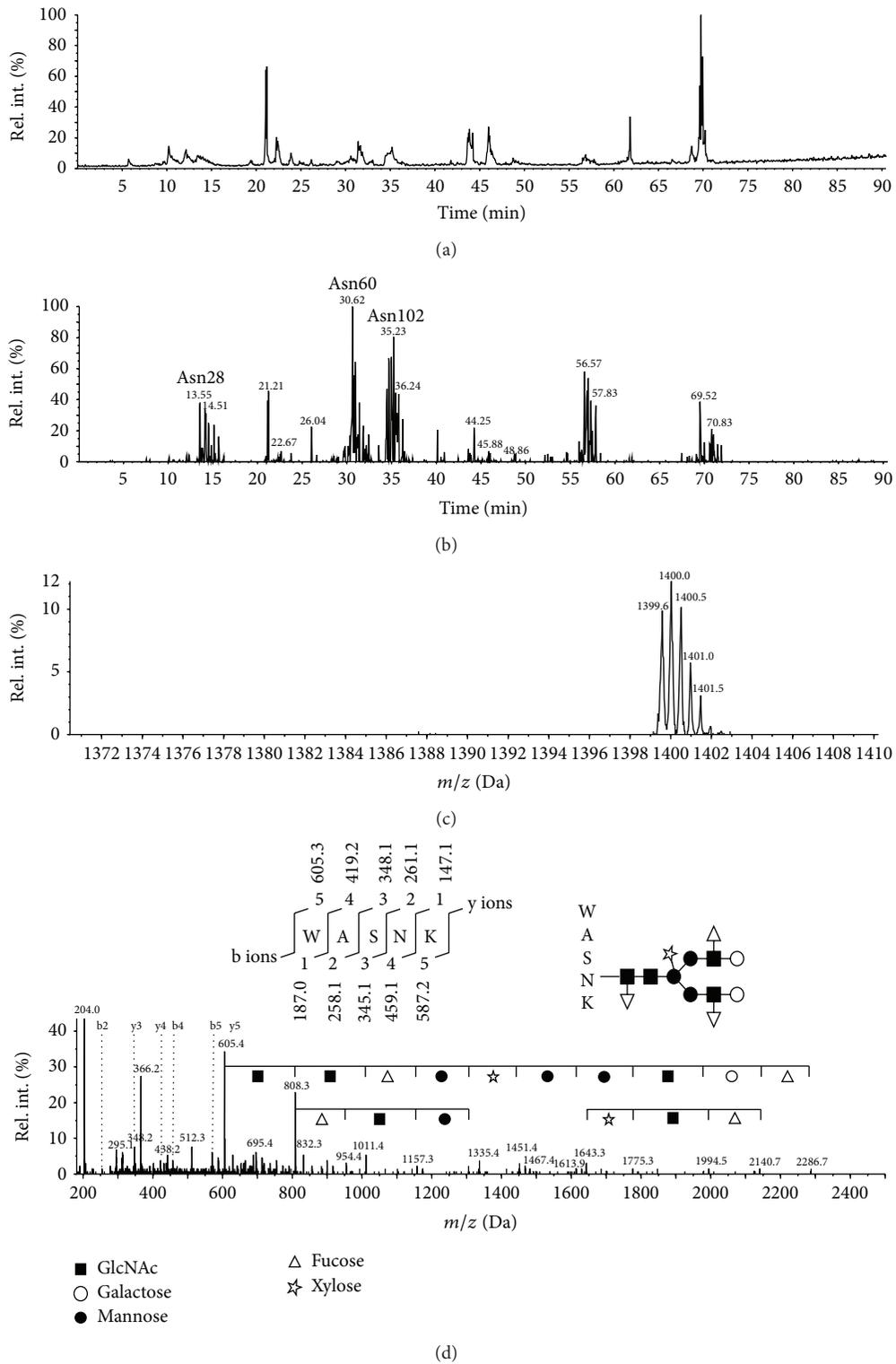


FIGURE 2: LC/MS/MS analysis of trypsin-derived N-glycopeptide WASNK (Asn28) of lacquer stellacyanin. (a) Total ion chromatography, (b) extracted ion chromatography, (c) enhanced mass spectrum of m/z 1399.6 (2+) acquired at 13.53 min, and (d) MS/MS spectrum of 1399.6 (2+) as a precursor.

TABLE 1: Observed N-glycopeptides derived enzymatic digestion of lacquer stellacyanin.

Glycosylation Site	Trypsin-derived glycopeptides		Chymotrypsin-derived glycopeptides			N-glycan Composition ^a
	Peptide (mass)	Obs. m/z	Peptide (mass)	Obs. m/z	Mass	
Asn 28	WAS <u>N</u> K (604.29)	888.12/2	AS <u>N</u> KTF (666.34)	919.19/2	1188.42	GlcNAc2 Hex3 Fuc1 Xyl1
		1070.85/2		1101.88/2	1553.56	GlcNAc3 Hex4 Fuc1 Xyl1
		1144.03/2		1175.03/2	1699.61	GlcNAc3 Hex4 Fuc2 Xyl1
		1326.56/2		1357.50/2	2064.75	GlcNAc4 Hex5 Fuc2 Xyl1
		1399.88/2		1430.63/2	2210.80	GlcNAc4 Hex5 Fuc3 Xyl1
Asn 60	NYQSC <u>N</u> DTTPIASYNGTBBR (2290.93)	1154.68/3	QSC <u>N</u> DTTPIASY (1356.57)	1264.51/2	1188.42	GlcNAc2 Hex3 Fuc1 Xyl1
		1276.85/3		nd ^b	1553.56	GlcNAc3 Hex4 Fuc1 Xyl1
		1324.24/3		1014.33/2	1699.61	GlcNAc3 Hex4 Fuc2 Xyl1
		1496.33/3		1184.74/3	2210.80	GlcNAc4 Hex5 Fuc3 Xyl1
Asn 102	VH <u>I</u> NVTVR (936.55)	981.40/2	H <u>I</u> NVTV (681.38)	nd	1042.37	GlcNAc2 Hex3 Xyl1
		703.41/3		926.74/2	1188.42	GlcNAc2 Hex3 Fuc1 Xyl1
		776.31/3		nd	1407.50	GlcNAc3 Hex4 Xyl1
		825.35/3		nd	1553.56	GlcNAc3 Hex4 Fuc1 Xyl1
		1237.38/2			1553.56	GlcNAc3 Hex4 Fuc1 Xyl1
		873.47/3		1182.87/2	1699.61	GlcNAc3 Hex4 Fuc2 Xyl1
		1310.40/2			1699.61	GlcNAc3 Hex4 Fuc2 Xyl1
1044.45/3	1438.56/2	2210.80	GlcNAc4 Hex5 Fuc3 Xyl1			

^aMonosaccharides represented as GlcNAc, N-acetylglucosamine; Hex, mannose or galactose; Fuc, fucose; Xyl, xylose.

^bnd: not detected.

TABLE 2: MALDI TOF MS assignment of the N-linked glycans from lacquer stellacyanin.

Peak number	Observed m/z ^a		Theoretical m/z ^b		N-linked glycan ^c
	PNGase A	PNGase F	N-glycan	aoWR-glycan	
1	2642.08	2641.60	2192.27	2640.48	(HexNAc)4 (Hex)5 (Fuc)3 Xyl
2	2495.98	2495.83	2046.74	2494.95	(HexNAc)4 (Hex)5 (Fuc)2 Xyl
3	—	2349.64	1900.50	2347.71	(HexNAc)4 (Hex)5 (Fuc) Xyl
4	2130.84	2130.08	1681.60	2129.81	(HexNAc)3 (Hex)4 (Fuc)2 Xyl
5	1984.78	1984.60	1535.55	1983.76	(HexNAc)2 (Hex)4 (Fuc) Xyl
6	1838.68	1838.63	1389.49	1837.70	(HexNAc)3 (Hex)4 Xyl
7	1619.5	—	1170.41	1618.62	(HexNAc)2 (Hex)3 (Fuc) Xyl

^aMALDI TOF MS signal digested with PNGase A and PNGase F.

^bTheoretical molecular weight of N-linked glycan.

^cHexNAc: N-acetylglucosamine, Hex: mannose or glucose, Fuc: fucose, and Xyl: xylose.

From the observed molecular weight in Figure 2(c) and the MS/MS profile of the glycopeptides between 605.4 and 2286.7 [8], the N-linked glycan at Asn28 was characterized as (HexNAc)4(Hex)5(Fuc)3Xyl, a complex type glycan obtained from the product ion spectrum with doubly charged $m/z = 1399.87$. Other forms of the complex type glycans were observed in Asn28, as shown in Table 2.

The trypsin-derived N-glycopeptide NYQSCNDTTPIASYNGTBBR ($m/z = 2291.7$) bearing Asn60 was observed as a long peptide with only one GlcNAc residue attached to the peptide in the MS/MS spectrum as presented in Figure 3(a). However, when we used chymotrypsin for the digestion of lacquer stellacyanin, an N-linked glycan was obtained in an oligopeptide, QSCNDTTPIASY ($m/z = 1356.5$)

by LC/MS/MS analysis as shown in Figure 3. As a result, the same N-linked glycan structure of HexNAc₄Hex₅Fuc₃Xyl₁ was also identified at Asn60. We also found that the same complex type glycan was connected at Asn102 by both trypsin and chymotrypsin digestions and subsequent LC/MS/MS analyses. These results are summarized in Table 1.

Trypsin-digested oligopeptides and oligoglycosyl peptides were also applied to MALDI TOF MS without any further purification. Signals between $m/z = 700$ and 4500 were observed from both trypsin-digested oligopeptides and oligoglycosyl peptides (data are shown in supporting information). The strongest signal appeared at $m/z = 4487.4$ due to the glycopeptides at Asn60 bearing a complex type N-linked glycan with the molecular weight of 2192. From the MALDI

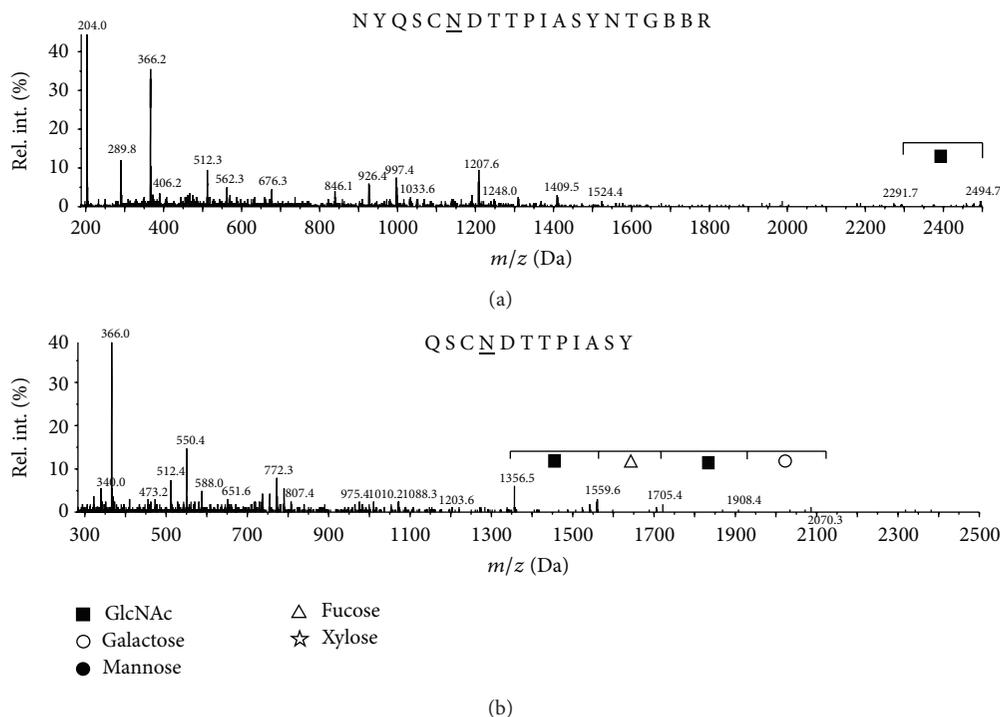


FIGURE 3: LC/MS/MS spectra of (a) trypsin- and (b) chymotrypsin-derived N-glycopeptide from lacquer stellacyanin. Observed mass numbers at $m/z = 2291.7$ (a) and $m/z = 1356.5$ (b) were due to oligopeptides, NYQSCNDTTPIASYNTGBBR and QSCNDTTPIASY at Asn60 site, respectively.

TOF MS analysis, the fucose and xylose-containing complex type biantennary glycan was linked at the three Asn sites in lacquer stellacyanin. Fucose and xylose should be attached to the GlcNAc next to Asn and at the central mannose residues, respectively.

3.3. MALDI TOF MS Analysis of N-Linked Glycans in Lacquer Stellacyanin. After isolation and purification of the lacquer stellacyanin, trypsin and chymotrypsin digestions were carried out, respectively, to give digested peptides and glycopeptides. Each product was further treated with PNGase A and PNGase F, respectively, to cleave N-linked glycans from Asn to afford N-linked glycans, which were labelled by using the BlotGlyco glycan purification and labeling kit to give the aoWR-labelled N-linked glycans. The aoWR-labelled N-linked glycans were mixed with the matrix (DHB) and then subjected to MALDI TOF MS to provide initial information on the relative quantities of N-linked glycans presented in lacquer stellacyanin. Figure 4 shows the MALDI TOF MS spectra of the aoWR-labelled N-linked glycans after (a) PNGase A and (b) PNGase F digestion of the lacquer stellacyanin. The observed mass numbers increased 447.21 due to the aoWR labelling reagent compared to the underived N-linked glycans. Although the signals appeared at the same m/z positions in Figures 4(a) and 4(b), the intensity was slightly different. These results suggest that the N-linked glycan in the lacquer stellacyanin has fucose at the glucosamine next to Asn because PNGase A releases N-linked glycans with and

without α -1,3-fucosylated GlcNAc next to Asn and PNGase F cleaves N-linked glycans with α -1,6-fucosylated GlcNAc. Table 2 shows the structure of N-linked glycans based on the m/z in MALDI TOF MS spectra in Figure 4. The most intense peak (number 1) at the m/z of 2642 was assigned to a complex type N-linked glycan consisting of four glucosamines (HexNAc), five hexoses (Hex), three fucoses (Fuc), and one xylose (Xyl), that is, (HexNAc)₄(Hex)₅(Fuc)₃Xyl (those that follow have the same description) [2], suggesting that the major oligosaccharide in lacquer stellacyanin was the complex type N-linked glycan. This type of N-linked glycan is the most common structure in plants, which has an α -1,3 linked fucosylated GlcNAc next to Asn, and a β -1,2 linked xylose should be attached to the central mannose residue [20]. Other signal peaks, numbers 2–7 in Figure 4, decreased the mass numbers from the m/z of 2642, indicating that one or more sugar residues were cleaved from the N-linked glycan (HexNAc)₄(Hex)₅(Fuc)₃Xyl as illustrated in Figure 4. These results suggest that lacquer stellacyanin has the same complex type N-linked glycan in the three Asn sites.

In the MALDI TOF MS spectra of the lacquer stellacyanin treated and untreated with sialidase, the two spectra were identical. In addition, the signals at $m/z = 292.1$ due to NeuAc, $m/z = 274$ due to NeuAc-H₂O, and $m/z = 657.1$ due to Hex+HexNAc+NeuAc are diagnostic or marker ions originating from sialylated glycopeptides. However, none of these signals appeared in the LC/MS/MS spectra. These results suggest that the N-linked glycan in lacquer stellacyanin had no sialic acids.

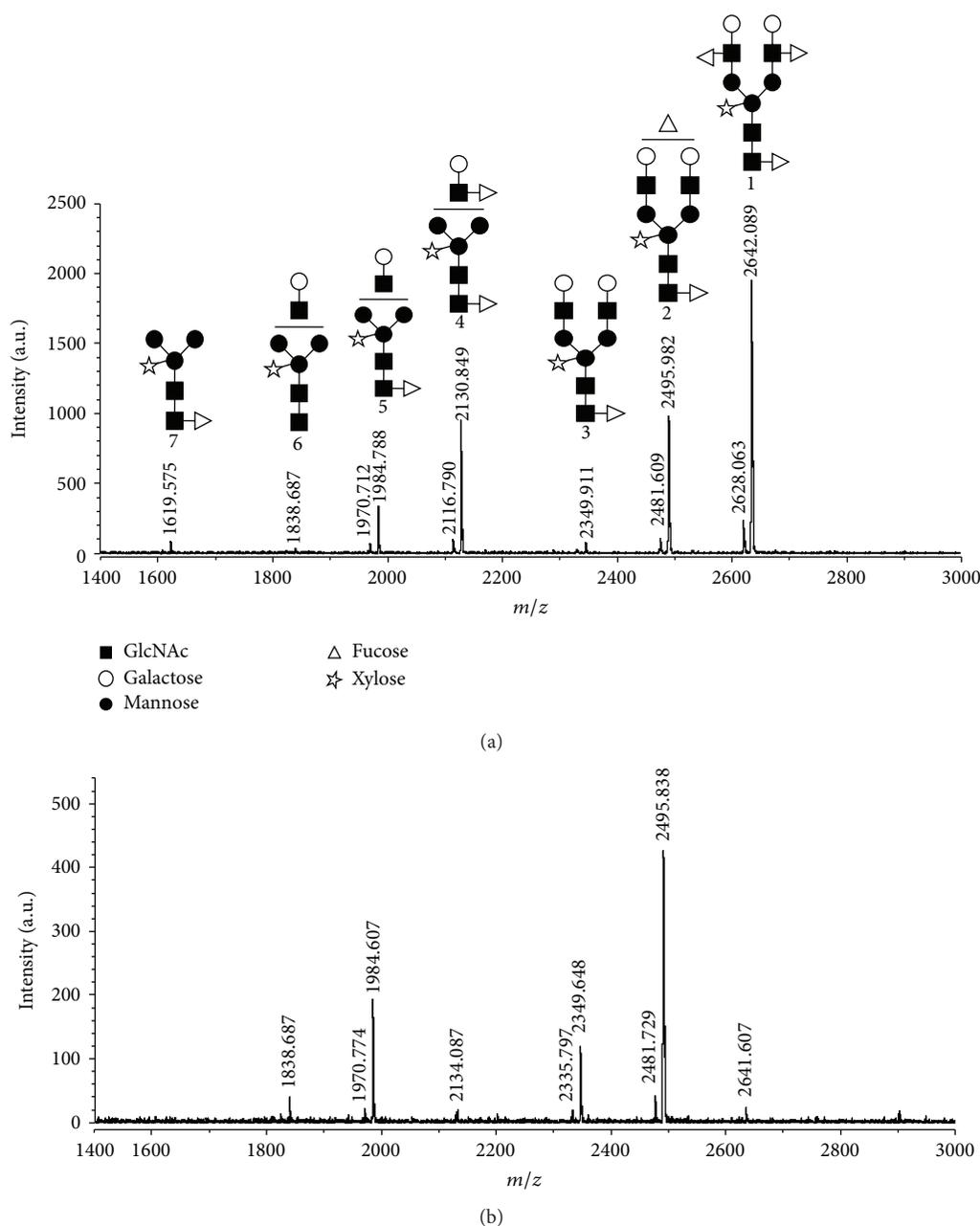


FIGURE 4: MALDI TOF MS profiles of aoWR-labelled N-linked glycans after (a) PNGase A and (b) PNGase F treatment of lacquer stellacyanin. N-glycan mass = [observed mass - 447.21 + 17].

4. Conclusion

By enzymatic digestions and a combination of MALDI TOF MS and LC/MS/MS analyses, we revealed the structures of the N-linked glycan and oligoglycopeptides at the three Asn residues (Asn28, Asn60, and Asn102) in lacquer stellacyanin, indicating that the N-linked glycan had the same structure at the three Asn sites and was a biantennary complex type N-linked glycan containing fucose at GlcNAc next to Asn and xylose at the central mannose residues. The complex type N-linked glycan confirmed in lacquer stellacyanin is the

most common N-linked glycan in plants. No sialic acids were identified in the lacquer stellacyanin. This is the first report on the structural characterization of N-linked glycans in lacquer stellacyanin from *Rhus vernicifera*, a phytolectin family of plant glycoproteins. Characterization of N-linked glycans in lacquer laccase is also under investigation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding to the publication of this paper.

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