Doctoral Thesis

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

酵素処理と質量分析による漆ステラシアニンと ラッカーゼ中の N-型糖鎖の解析

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

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GENERAL ABSTRACT

Lacquer sap is water-in-oil emulsion consisted of urushiols, polysaccharides, glycoproteins, laccase, and stellacyanin that have been used as natural paint in Asian countries. Laccase and stellacyanin are copper–containing glycoproteins, which are heavily glycosylated proteins containing 3 and 15 possible N-glycosylation sites (Asn-x-Thr/Ser), respectively. Structural studies on their carbohydrate structures have not been accomplished yet.

In present study, we isolated laccase and stellacyanin from lacquer acetone powder by continuous ion exchange chromatography using Sephadex CM-50 and DEAE A-50. Protein glycosylarion was characterized using enzymatic digestion, chemical labeling techniques of released N-linked glycan and subsequent mass spectrometric analysis MALDI TOF MS an LC/MS/MS, respectively. We found that three N-glycosylation sites (Asn28, 60 and 102) in lacquer stellacyanin all glycosylated with same complex-type N-linked glycan, constituted with $GlcNAc_4Man_3Gal_2Fuc_3Xyl_1$.

Also lacquer laccase glycosylated with complex- and also hybride-type N-linked glycans that are common plant-specific structural units of β-xylose, core α-fucose and Lewis epitope (GlcNAc-Gal-Fuc) were observed by MALDI TOF MS. LC/MS/MS analysis of glycopeptides revealed that 13 N-glycosylation sites in laccase were glycosylated frequently with complex type N-glycan, $GlcNAc_4Man_3Gal_2Fuc_3Xyl_1$, and among them Asn5, 233 and 381 were also found to be glycosylated with hybride–type N-glycan, mainly $GlcNAc_{2\text{-}3}Man_{4\text{-}5}Fuc_1Xyl_1$. Glycopeptides which carry possible N-glycosylation sites at Asn 364 and 519 were not confirmed by LC/MS/MS analysis.

Chapter 1

General Introduction

1.1 Lacquer

1.1.1 Lacquer tree and lacquer sap

Lacquer is a valuable material that is used as a natural paint in Japan and China in thousands of years ago, which has been a closely related theme to Japan, especially Japanese culture. Lacquer sap is a natural product collected from lacquer tree, *Toxicodendron vernicifluum* contributed in East Asian countries, but origin was China and contemporary historical research indicates that lacquerware technology was introduced from China to Korea, and from to Japan.

Toxicodendron vernicifluum is one of the familiar species belongs to genus of poison oak of Anacardiaceae family. Lacquer tree is usually called as *Urushi* and *Rhus vernicifera,* which are synonym of *Toxicodendron vernicifluum* in Japan and China. Taxonomy of lacquer tree presented in **Table 1.1** was provided online plants database (http://plants.usda.gov/).

Table1.1: Scientific classification of lacquer tree

Family	*Anacardiaceae*	Sumac
Genus	*Toxidendron Mill.*	Poison oak
Species	*Toxicodentron vernicifluum*	Chinese lacquer
	Toxicodendron vernicifera DC.	Japanese Lacquer
	Synonym Rhus vernicifera*	

Lacquer sap has a long history as valuable and durable coating materials. It has been demonstrated by many cultural treasures coated with lacquer have still preserved without losing their original elegant beauty [Snyder, 1989 and Hu Jiago, 1980]. Oriental lacquer has been expected an environment friendly paint for the next generation, because lacquer sap is polymerized only with an enzyme laccase, and the resuting film is a very hard, durable and plastic like substance [Kumanotani, 1995].

Raw lacquer is the secondary metabolite function has protecting from harmful insects, animals andhas ability to cure itself when the surface has been broken. It secreted by laticiferous canals and the main part of secreting and storing raw lacquer are a phloem of each organ of *Toxicodendron vernicifluum* [Zhao, 2015]. After the maturation of lacquer tree, laticiferous canals synthesized. In summar and early autumn, laticiferous canals cavity of the phloem are fully filled with raw lacquer [Shuonan *et al*., 2009]. Thus, harvest season begins from June to late of October by cutting the phloem at 5 day intervals and the yield of the sap is 120-150g per tree, the properties of the sap depends on the time and place collected [Kumanotani, 1995].

1.1.2 Studies on lacquer and its constituents

The chemical challenge of lacquers was begun in 1882 by Ishimatsu who was separated the constituents of the sap of *Rhus vernicifera* using ethanol and water as solvents [Ishimatsu, 1882]. Next year, Yoshida discovered an oxidoreductase enzyme with blue color in the sap, which is now known as laccase [Yoshida, 1883]. Nowadays it is clear that raw lacquer sap is constituted of water (20-30%), urushiol (60-65%), polysaccharides (6.5-10%), water insoluble glycoprotein (3-5%) and enzymes (1%) namely laccase, stellacyanin and peroxidase.

The main component of lacquer is a phenolic lipid that found by Miyama in 1907 and named urushiol [Miyama, 1907]. Urushiol is a catechol derivative having a linear alkyl side chain of 15-17 carbon atoms and known as a dermatitis allergen [Ma *et al.*,

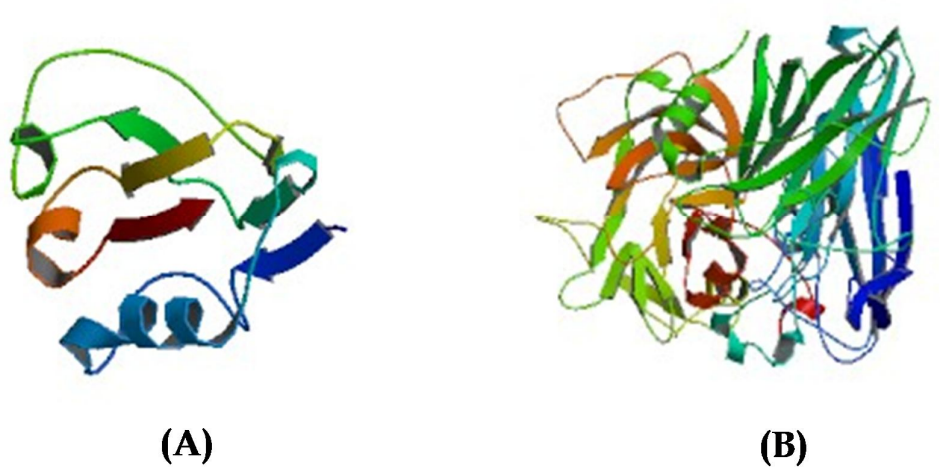
2011]. Despite an allergic reaction, biological activities of lacquer urushiol such as anti-microbial and antioxidant activities were investigated by Korean researches in vitro on *Helicobacter pylori* [Suk *et al*., 2011].

The structure and specific biological activities of lacquer polysaccharides in Japanese and Chinese lacquer saps were investigated by Lu *et al*. The lacquer polysaccharides with the number-average molecular weight of $\overline{M}_n$= 10x 10^3- 30x 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 [Lu and Yoshida, 2003]. 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. After sulfation, lacquer polysaccharides showed potent anti-HIV and low blood anticoagulant activities. These specific biological activities should originate from the acidic branched structure [Lu *et al*., 2000]. In addition, treatment with diluted alkaline aqueous solution revealed that lacquer polysaccharides were constructed of association of low molecular weight polysaccharides [Bai and Yoshida, 2013].

However lacquer glycoprotein that contained in water insoluble part of acetone powder has not been investigated chemically.

Lacquer stellacyanin and laccase are kind of a “blue” copper containing glycoproteins belongs to metalloprotein that contains a metal ion as cofactor. Metalloproteins have been known numerous biological functions, such as enzymes, transport and storage proteins, and the most of their functions are still not completely understood yet [Lu *et al*., 2009]. Lacquer laccase is glycoprotein has moleculer weight of 100kDa, which consisited 533 amino acid and 4 copper atoms in molecule as active center [Nitta *et al*., 2002] that is the most important for polymerization of urushiol. Iso-enzyme of lacquer laccase named as Laccase2 (Lac2)

also identified that consisted of 530 amino acid residue and registered Q8H979 in protein database [Sakurai *et al*., 2002]. Lacquer stellacyanin is plant specific glycoprotein has moleculer weight around 20kDa, which consisited 107 amino acid and 1 copper atom in molecule [Bergman, 1977]. Lacquer stellacyanin has no methionine residue and some specific properties of stellacyanin such as a low redox potential and a fast electron transfer rate are described [Alejandro and Claudio, 1996]. The 3D structure of lacquer glycoproteins was not completed, because crystallization of proteins have not obtained yet, however the predicted 3D model of proteins was also available on protein data, Uniprot.



(A) **(B)**

Figure 1.1: 3D model of lacquer stellacyanin and lassace. Protein data of lacquer stellacyanin (A) and laccase (B) are available with ID: P00302 (entry name: STEL-TOXVR) and Q94IDO (Q94IDO-TOXVR), respectively at http://www.uniprot.org.

1.1.3 Stellacyanin

Stellacyanin is a small sized (20kDa) single copper containing glycoprotein belongs to the phytocyanin family that was first found in lacquer sap of Japanese lacquer tree, Rhus vernicifera by Keilin and Mann in 1940 [Keilin and Mann, 1940]. Similar proteins to stellacyanin such as cucumber stellacyanin, umecyanin from horseradish, plantacyanin from spinach mavicyanin from *Cucumbita pepo* and uclacyanin 1 from *Arabidopsis* have been repeoted [Claudio *et al*., 1997]. Occurrence

of stellacyanins and similar proteins has not been confirmed in any other organisms except of plants currently, therefore these cupredoxins are also called plant-specific mononuclear blue protein. The physiological role of these proteins was not fully understood, but preliminary studies suggest that they participate in redox processes during the primary defense and/or lignin formation in plants [Nersissian *et al*., 2011]. Generally, cupredoxins serve as mobile electron carriers in a variety of charge-transport systems and they may involve in redox reactions with small molecular weight compounds, rather than with protein electron mediators [Nersissian *et al*., 1998].

1.1.4 Laccase

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2), a multicopper oxidase (MCOs) enzyme has an ability to catalyze oxidation of various phenolic and non-phenolic substrates, as well as diamines and aromatic amines with the concomitant four electron reduction of oxygen to water [Madhavi and Lele, 2009]. More than 2200 protein to belong MCOs family have been reported, where laccases is one of the largest subgroup of MCOs, it consisted 350 representatives according to Laccase Engineering Database [Sirim *et al*., 2011].

1.1.4.1 Natural occurence and functions of laccase

Laccase and laccase-like enzymes are widely distributed in higher plant, fungi, some insect, and bacteria. Laccase have been identified in trees, various vegetables, some fruits and herbes. Laccase from *Rhus vernicirefa* is the oldest one and a best-studied representative of multi-copperoxidases among of plants resources. Plant laccases are found in xylem, where they involved in lignification, and participate in the radical-based mechanisms of lignin polymer formation. In addition, laccases have been shown to be involved in the first steps of healing in wounded leaves [McCaig *et al*., 2005].

However, laccase is distributed in fungi rather than in higher plants. In 1986 laccase was found to be presented in fungi, now laccases more than 100 kinds have been found in fungi. These laccase were produced in ascomycetes, basidomycetes, deuteromycetes and other wood rooting fingi. Fungal laccase implicated in degradation of lignin, detoxification of pathogenesis, pigment production and also in fungal morphogenesis including of sporulation and fruiting body [Madhavi *et al*., 2009].

A bacterial laccase was described for the first time in the plant root-associated bacterium *Azospirillum lipoferum* [Givaudan *et al*., 1993] and laccase activity was later reported in *Marinomonas mediterranea*, *Bacillus subtilis, Streptomyces cyaneus* and *Streptomyces lavendulae*. Their functions have key role in pigment production, protection from UV light and the hydrogen peroxide, and detoxification of phenolic acids [Dwivedi *et al*., 2011].

In addition, laccases have also been found in insects, such as *Bombyx, Calliphora, Drasophilia* etc [Madhavi *et al*., 2009]. Insect laccase plays an important role in cuticle sclerotization with its ability to catalyze the oxidation of phenolic compounds to their corresponding quinones [Yatsu and Asano, 2009].

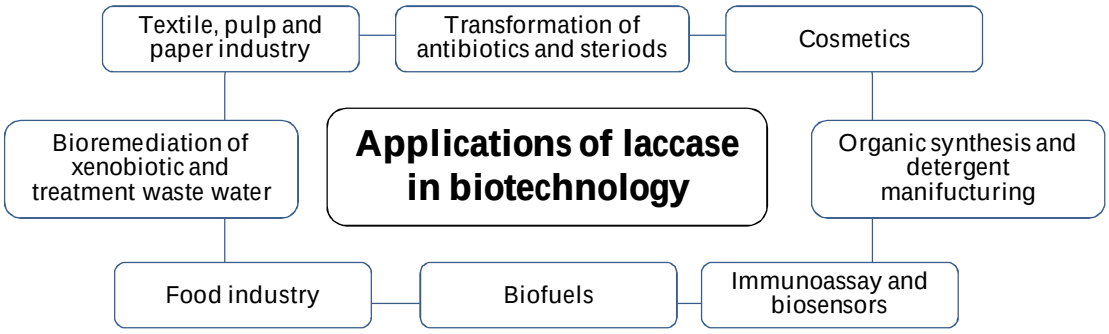
1.1.4.2 Laccase activity and substrate specificity

Substrate specificity and optimium pH and temperature of laccases from different resources are distinguished. For example, most of the fungal laccase has pH optima between pH 3.6 and 5.2, while lacquer laccase have maximum activity under neutral and weakly alkaline condition of pH 7-9 [Madhavi *et al*., 2009]. The optimum temperature of laccase is between of 50°C and 70°C, however some of fungal laccases exhibites a high enzyme activity below 30°C. Such differences of physicochemical properties of laccases from various sources should be conducted to their biological functions in individual organisms. In spite of the enzyme origin, laccase activity is

usually measured by spectrophotometric assay using broad spectrum of substrates. Several compounds, such as ABTS, syringaldazine, o-dianisidine, DMP and guaicol are commonly used as substrates [Li *et al*., 2008].

1.1.4.3 Application of laccase

Currently, laccases have been investigated as economical important and industrially relevant enzymes in biotechnology because of their broad substrate specificity and catalytic activity. Among them, fungal laccase has been getting a considerable attention for its production of high potential and low-cost enzymes [Rodgers *et al*., 2010]. Also fungal laccases, as well as bacterial laccases possess a high activity and much stable in envorimental criteria than plant. In the present biotechnology, an effective utilization of laccase has been reported that summarized in **Scheme 1.1** [Morozova *et al*., 2007].



Scheme1.1: Potential applications of laccases in Biotechnology [Morozova, 2007]

Industrial application of lacquer laccase is very limited. Lu *et al* investigated enzyme activity and compared the stability of free and immobilized lacquer laccase on zirconium chloride result revealed that immobilized laccase has more stable and it suggest a repeated use of enzymes [Lu *et al*., 2012].

1.1.4.4 N-glycosylation of laccase

Current understanding, plant and fungal laccases are glycosylated protein.

Laccase have a molecular weight of 50-100kDa, in which sugars have been contained in 10-40%. Carbohydrate moiety of laccase and their structure are quite different depending on laccase resource and plant laccase has higher extent of glycosylation 20-40% of their molecular weight. The glycosylation of laccase is affective for the protein secretion, stability the active centre, protecting against proteolysis, termostability and enzyme activity [Dwivedi *et al*., 2011 and Rodgers *et al*., 2010]

N-linked glycan of plant laccase was studied in sycorome, *Acer pseudoplatanus* L and the xylose-containing biantennary complex type were identified [Tezuka *et al*., 1993]. Structure of N-linked glycan and their function of fungal laccas have frequently studied in recent years. Laccases in fungi are often glycosylated by mannose, HexNAc2 and Man5-8. The function of N-glycosylation on enzymatic properties of laccase from *Pycnoporus sanguineus* was investigated using enzymatic cleavage of N-glycans in laccase by Vita-Vallejo et al. Deglycosylation of high mannose glycan present in *P.sanguineu* laccase has a significant effect on enzyme activity, especially at low temperature and also protein stability, but provoked slight changes in kinetic parameters compare to native form [Vite-Vallejo *et al*., 2009].

1.2 Protein glycosylation

The post-translational modifications (PTMs) including phosphorylation, methylation, glycosylation and acetylation etc that are greatly increases impact of proteomes. These modifications are the result of complex enzymatic reaction and significantly influence proteins function by influence on their chemical and physical properties [Schwarz and Aebi, 2011].

Glycosylation is one of the most common post-translational modifications and the attachment of monosaccharides or extended sugar chain to proteins is catalysed by enzymes. Glycoproteins are known to exhibit multiple biological functions and carbohydrates have been found to participate in molecular recognition, inter- and

extracellular signaling, embryonic development, immune defence, cell adhesion and division processes, viral replication and parasitic infections. [Geyer *et al.*, 2006 and Roth *et al.*, 2012].

There are two major types of protein glycosylation, N- and O-glycosylation that are both directly involved in protein conformation and their secretion. The all N-glycans share a $GluNAc_2Man_3$ pentasaccharide core and N-acetylglucosamine (GluNAc) is attached to the amide group of asparagines within an Asn-X-Ser/Thr motif, where X is any amino acid except of proline and due to their common biosynthetic pathway. Regarding O-glycosylation, carbohydrate unit, usually N-acetylgalactosamine (GalNAc) is α-glycosidically linked to serine or threonine residues.

1.2.1 N-glycosylation in plant

Almost all of the secreted proteins produced in eukaryotes are modified with N-glycosylation. In plant, N-linked glycans are generally classified in two general groups, that is, the high mannose type N-glycan having the general structure $Man_{5-9}GlcNAc_2$ and the complex type N-glycans, in which pentasaccharide core modified with α(1,3) fucose and β(1,2) xylose that called paucemannosidic type. This paucemannosidic type N-glycan is further modified by biantennary structure that constituted of Gal-Fuc-GlcNAc and has identified by complex type N-linked glycan in plant [Rayon *et al.*, 1998]. Biosynthesis of plant N-linked glycans is carried in the endoplasmic reticulum (ER) and then modified in Golgi apparatus to complex type, after their maturation the complex type N-glycans can be modified either during glycoprotein transport or in compartment of its final location. Biosynthesis and secretory pathway of N-glycoprotein in plant are represented in **Figure1.2** [Ruiz-May *et al.*, 2012].

Hybride-type N-glycan structure modification of $GlcNAc_2(Fuc)XylMan_4$ and $GlcNAc_2(Fuc)XylMan_5$ is occur in the Golgi by xylose transferase and fucosyltransferase from high mannose structure [Soll, 2012].

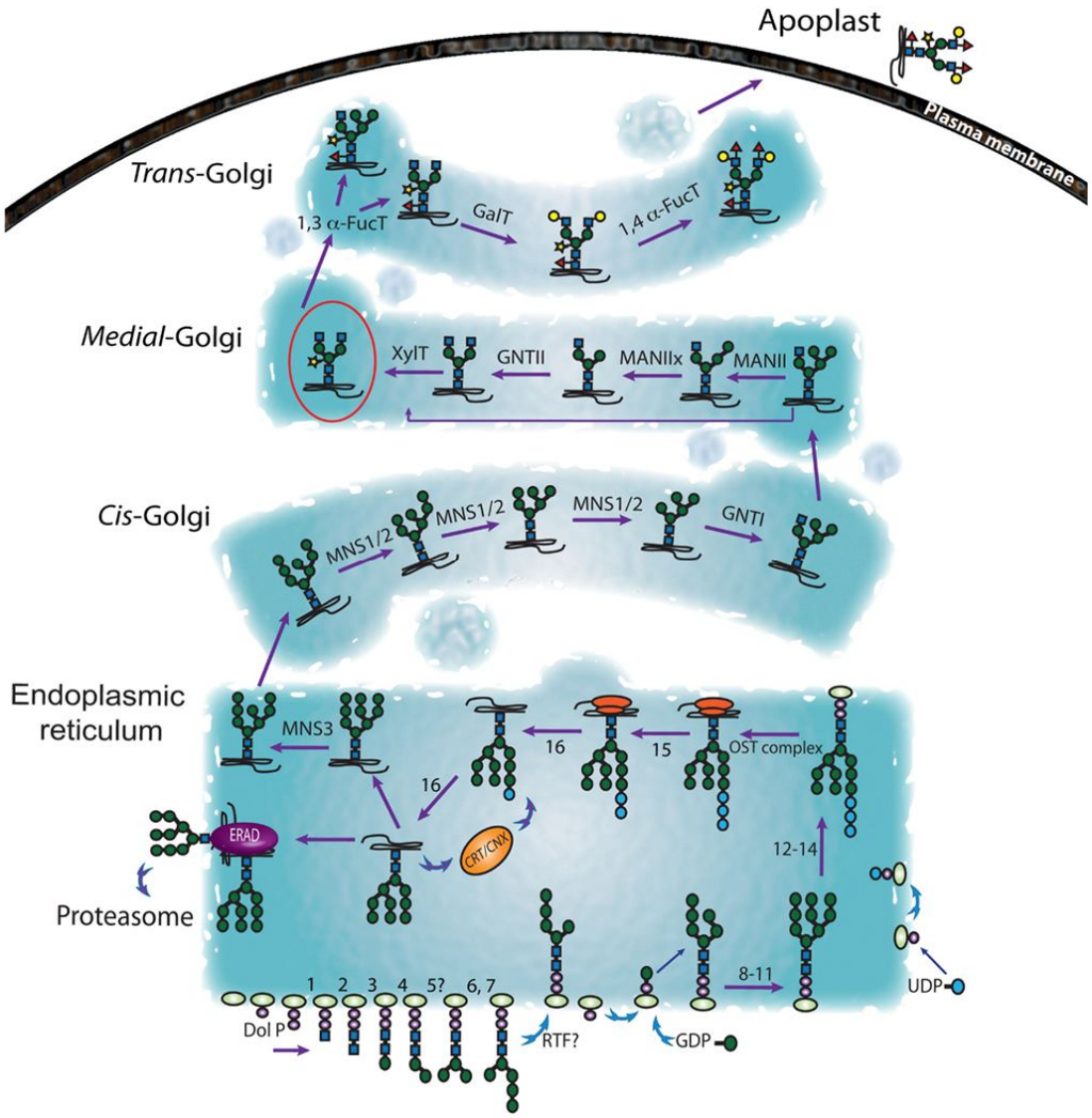


Figure 1.2: Biosynthesis of N-glycoproteins and secretory pathway [Ruiz-May *et al.*, 2012]

1.2.2 Functions of plant N-linked glycans

Protein N-glycosylation in plants has an impact on the glycoprotein conformation, stability and biological activity [Song *et al.*, 2011]. Numerous studies have demonstrated that N-linked glycanls can protect the protein from proteolytic degradation as well as thermal stability, solubility and biological activity of glycoproteins [Spiro, 2004]. For example, the mannose type N-glycan in ER supposedly aids the protein folding process, because the misfolded protein is retained in ER or when it transported to the Golgi apparatus may be recycled back to the ER, untill correctly folded.

1.3 Mass Spectrometry in proteomics

Mass spectrometry (MS) is a powerful and valuable modern analytical technique that has become more developed and equipped for resolving of difficulty on characterization of biological analyst. Recently, MS based proteomics studies has rapidly become a popular analytical method for complete structural characterization and identification of proteins [Settle, 1997].

MS allows the analysis of ionized components samples and their subsequent separation followed detection their mass to charge ratio (m/z). There are three major components of a typical mass instruments are the ion source, the mass analyzer and the detector system. There are two ionization techniques: the electrospray ionization (ESI) and MALDI that are widely used in present proteomics.

Matrix Assisted Laser Desorption Ionization Time of Flight Mass (MALDI TOF MS). MALDI is one of the recent introduced techniques with a time-of-flight (TOF) mass analyzer in 1989 [Karas and Hillenkamp, 1988]. The TOF analyzer has a large working mass range (100Da~250,000Da) and matrix compounds such as CHCA, DHB, Sinapic acid are used to co-crystallization of analyte in the MALDI TOF analysis. On laser radiation of matrix-analyte, the matrix is desorbed as plume, thus

the analyte flight along with it into gas phase.

Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC ESI MS). **E**lectrospray ionization technique was first introduced in 1990 [Fenn *et al.*, 1990]. The on-line combination of ESI/MS with HPLC is able to solve problems dealing with high-mass peptides, proteins, and other biopolymers not only single but also in the biological mixture samples. ESI is atmospheric pressure ionization (API) technique in which production of ions occurs by electrospraying the solution of an analyte into a chamber maintained at nearly atmospheric pressure. In LC-ESI/MS analysis, molecular weight and structure of the individual components of the mixture are resolved by the LC column and data provide in the form of ion chromatogram and mass spectrum. A major benefit of ESI/MS is applicability to determination of the molecular weight of macromolecules, which is providing of the multiple-charging phenomenon.

1.4 Recent statement and research purpose

Lacquer sap is a naturally occurring paint that is polymerized by laccase, anoxidoreductive 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. Lacquer constituents, except of glycoprotein, have been frequently investigated chemically, biologically and enzymatically.

Wan *et al* isolated hydrosoluble components of Chinese lacquer and their structural properties was evaluated including two laccase isoenzymes (L1 and L2) having molecular masses of 120kDa and 103kD, respectively [Wan *et al*., 2006]. Authors also studied the effect of other lacquer components such as polysaccharides, stellacyanin and laccase isoenzymes on enzyme activity of free and immobilised laccase, result releaved that polysaccharides and stellacyanin have a negative effect,

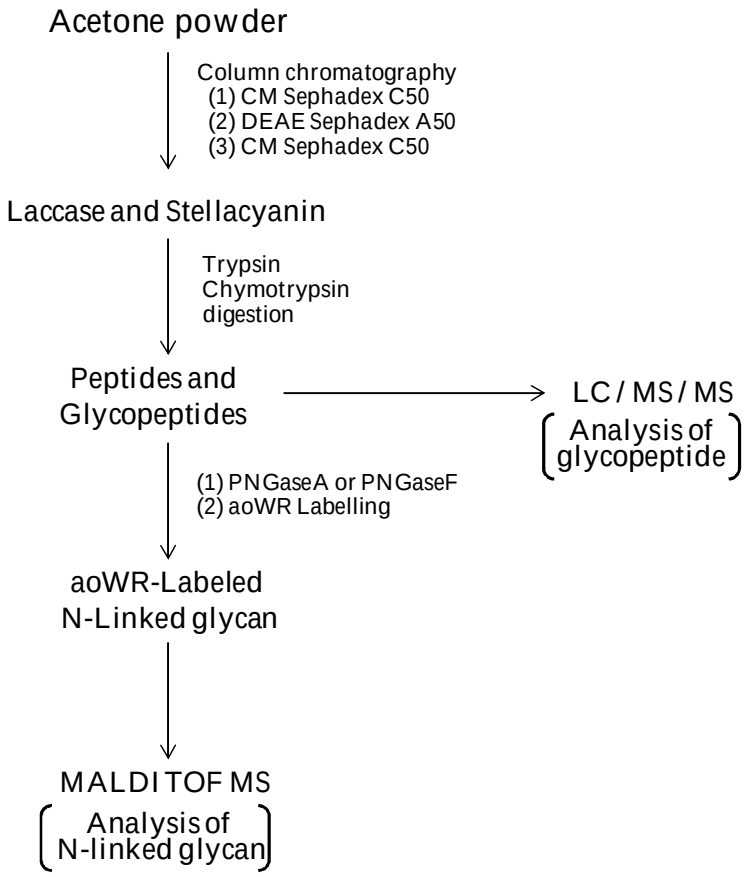
but isoenzymes have synergenic effect on laccase activity [Wan *et al*., 2010]. Lu *et al* investigated enzyme activity and stability of free and immobilized lacquer laccase result releaved that immobilized laccase has more stable and it suggest a repeated use of enzymes [Lu *et al*., 2012].

The thermodynamics and kinetics of electron transfer of electrode-immobilized *Rhus* stellacyanin was carried out at different pH and temperature conditions. In immobilized stellacyanin, electrode-SAM-protein construct was observed, that is interesting finding should be an application in protein based molecular electronic devices [Ranieri *et al*, 2012].

Lacquer laccase and stellacyanin are heavely glycosylated proteins, however their glycosylation analysis of these blue copper-containing glycoproteins have not been investigated yet. From their amino acid sequences of laccase and stellacyanin, N-glycosylation sites were contained 15 and 3, respectively [Nitta *et al*., 2002 and Bergman, 1977] should be responsible for the glycoprotein conformation, stability and biological activity, in particular enzyme activity of laccase.

Research purpose:

In the present study, we isolated lacquer glycoproteins, namely stellacyanin and laccase from lacquer acetone powder and to characterize the N-linked glycans structures. We used enzymatic digestions and N-glycan labeling methods and then measured the mass using MALDI-TOF/MS and LC/MS/MS, respectively. Our experimental strategy for N-glycosylation analysis of lacquer glycoproteins represented in **Scheme1.2**.



Scheme1.2: General approach of isolation of lacquer glycoproteins and structure characterization of N-linked glycan by MS analysis.

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Chapter 2

Isolation and Purification of Lacquer Glycoproteins

2.1 Abstract

Lacquer stellacyanin and laccase were isolated and purified from lacquer acetone powder by continuous Sephadex column chromatographies using Sephadex C-50, DEAE A-50, and C-50 gels. Laccase and stellacyanin were clearly seperated on CM-sephadex C-50 as two blue bands while column was eluting 0.1M phosphate buffer, stellacyanin remain at the top of column was later eluted with 0.2M buffer. The purified lacquer stellacyanin and laccase had molecular weight of approximately 26kDa and 100kDa, respectively by SDS PAGE. Amino acid sequences of proteins are already available and existing of possible N-glycosylation sites have also known. These results suggest that carbohydrate moieties presented in lacquer glycoproteins were predictable 30-40% of molecular weight of each protein.

2.2 Introduction

The chemical challenge of oriental lacquers was begun in 1880s. Ishimatsu introduced a separation method of the constituents of the sap of *Rhus vernicifera* using ethanol and water as solvents in 1882 [Kumanotani, 1995]. One year later Yoshida discovered an oxidoreductase enzyme with blue color as known laccase in the sap [Yoshida, 1883] that was the oldest multi-copper oxidase. Bertrand was partially separated lacquer laccase in 1894 [Bertrand, 1945]. Since a simple and efficient chromatographic separation method was discovered by Reinhammar that have been used for separation and purification of lacquer components, frequently [Reinhammar, 1970]. Nowadays it is clear that raw lacquer sap is constituted from water (20-30%), urushiol (60-65%), polysaccharides (6.5-10%), water insoluble glycoprotein (3-5%) and enzymes (1%) namely laccase, stellacyanin and peroxidase.

2.3 Experimental

2.3.1 Materials

Lacquer acetone powder was prepared by addition of acetone to lacquer sap according to the method reported by Reinhammar. CM Sephadex C-50 and DEAE Sephadex A-50 were purchased from Healthcare Bio-Sciences AB, Sweden. 10-20% gradient gels (E-T1020L), EZ marker standards (AE-1440), and Coomassie blue for SDS-PAGE analysis from ATTO Corporation, Japan, were used.

2.3.2 Isolation and purification of lacquer stellacyanin and laccase from acetone powder

Preparation of Acetone powder: Lacquer acetone powder prepared by the method of Reinhammar, briefly 1000 ml of acetone was added to the lacquer sap (300mg) and stirred for 30 min at room temperature, then filtered. Obtained acetone powder also washed acetone several times to recover acetone soluble materials, it

resulted the acetone powder become silver grey color and dried at room temperature under vacuum. The yield of acetone powder was 28.2 g (9.4%).

Lacquer acetone powder (20g) was dissolved in 200mL of 0.01M potassium phosphate buffer solution (pH 6.0) and then stirred overnight at 4ºC. The mixture was centrifuged and then supernatant filtered to remove any insoluble materials. The filtrate was chromatographed on a CM-Sephadex C-50 column (250mm x 40mm), gradually eluted with 0.01M, 0.05M, 0.1M and 0.2M phosphate buffer for initial separation of lacquer components. Lacquer polysaccharides and peroxidase fractions were collected 0.01M and 0.05M buffer eluent, respectively. When the CM-Sephadex C-50 column was eluented with 0.1M buffer, blue bands separated each other, and there are two blue bands, separated and remained on the top of column, are corresponding to laccase and stellacyanin, eluted with 0.1M and 0.2Mphosphate buffer solution (pH 6.0), respectively. Lacquer laccase and stellacyanin were further purified by DEAE-Sephadex A-50 column chromatography (500 mm x 20mm) to remove some yellow impurities and subsequent CM-Sephadex C-50 column chromatography (500 mm x 20mm) gradually eluted with 0.005 M, 0.025 M, 0.05 M, and 0.1M phosphate buffer for laccase. In case of purification of stellacyanin, the column also eluted with 0.15M and 0.2M buffer additionally. The obtained eluent with a blue colour were finally desalted by dialysis with deionized water overnight and then freeze-dried to give 0.233g (1.17%) and 0.083g (0.42%) of pure lacquer laccase and stellacyanin, respectively.

Preparation of copper-free glycoproteins

Blue stellacyanin (56 mg) and laccase (100 mg) were 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 glycoproteins without copper. The purity and molecular weights of the purified glycoproteins were measured by SDS PAGE and

lacquer stellacyanin was also subjected to MALDI TOF MS.

2.3.3 SDS-polyacrylamide-gel electrophoresis

The purified protein solution (2mg/ml) were diluted 1:1 with SDS-PAGE buffer (50mM Tis-HCl, 2% SDS, 20% sucrose, 100mM DTT, Bromphenolblue), denaturated for 10 min at 95 °C and after cooled 10 μl sample was loaded to 10–20% SDS-PAGE gel. In this system, proteins denatured in the presence of SDS and DTT as reducing agent of disulfide bonds that allow a linear structure of protein and a consistent charge-to-mass ratio proportional to their molecular weights by binding SDS with the same affinity. Separation was carried out under at 20 mA for 80 min at room temperature using 100mM tris, 50mM glycine and 0.1% SDS then gels stained by colloidal Coomassie staining (ATTO). For protein MW determination, 5 µl of protein standard protein marker (Protein-Marker, EzStandard AE-1440, ATTO, Japan) was runned and the protein size were compared by migration of the standard bands.

2.4 Result and Discussion

We obtained 28.2 g acetone powder which in yield 9.4% from 300 g lacquer sap and water soluble parts (greenish color solution) was further separated by continuous Sephadex column chromatographies using Sephadex C-50, DEAE A-50, and C-50 gels. Main components such as polysaccharides, peroxidase, laccase and stellacyanin were gradually eluted with phosphate buffer and yielded at 22.5%, 1.26%, 1.16%, and 0.41%, respectively, as summarized in **Table 2.1**.

Table2.1: Yeild of the constituents from the water soluble acetone powder

Constituents of acetone powder	Eluent /phosphate buffer,pH=6.0	Yield in weight (g)	Yield in percentage (%)
Polysaccharides	0.01M	4.31	22.5
Peroxidase	0.05M	0.25	1.26
Laccase	0.1M	0.23	1.16
Stellacyanin	0.2M	0.083	0.41

Purified lacquer stellacyanin and laccase were confirmed by SDS PAGE and bands had molecular weight of approximately 26kDa and 100kDa, respectively by comparing protein marker. Lacquer stellacyanin had one major and three minor bands around 26kDa and bands were clearly separated but very close each other as shown in Figure 2.1 (A). Figure 2.1 (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. We couldn't obtain the clear MALDI TOF spectrum of lacquer laccase because it has relatively larger molecular weight than stellacyanin.

Amino acid sequence of lacquer stellacyanin and laccase are already determined and they consisted of 107 and 533 amino acid residues with one and four copper atoms per molecules, respectively [Bergman, 1977 and Nitta *et al*., 2002]. We calculated corresponding molecular weights of each protein from their one dimensional structure were 12.2kDa and 59.0kDa. Perhaps there is still no detailed investigation on carbohydrate portion and their function on lacquer blue glycoproteins namely, stellacyanin and laccase.

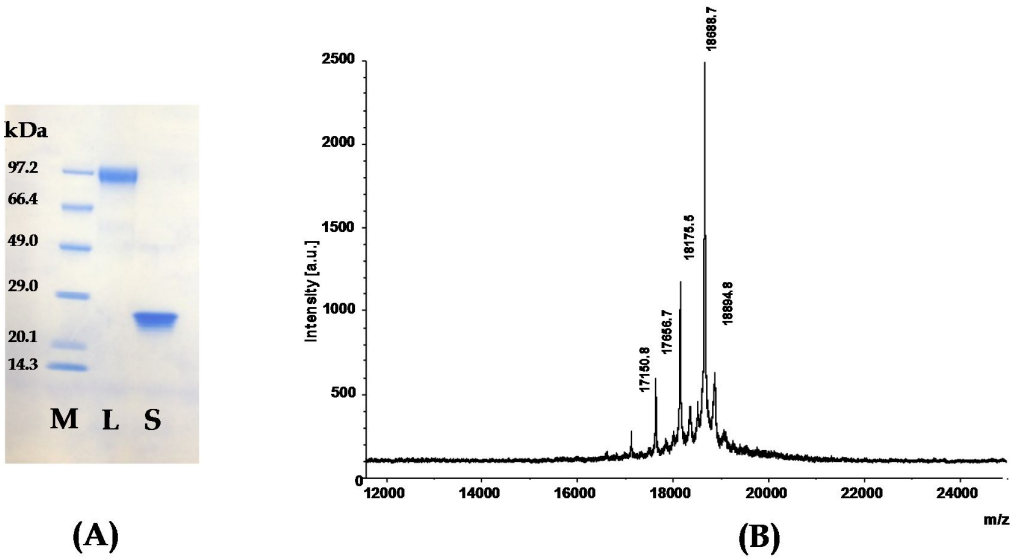

Figure 2.1: SDS-PAGE profile and MALDI TOF MS spectrum of lacquer glycoprotein. (A) SDS-PAGE of lacquer laccase and stellayanin; M-marker, L-laccase, S-stellacyanin. and (B) MALDI TOF MS spectrum of stellacyanin; matrix was DHB.

Lacquer stellacyanin and laccase are known to heavily glycosylated proteins that they have 3 and 15 possible N-glycosylation sites from their amino acid sequences. These results suggest that carbohydrate moieties presented in lacquer glycoproteins were predictable 30-40% of molecular weight of each protein.

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Chapter 3

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

3.1 Abstract

Lacquer stellacyanin was isolated and purified from lacquer acetone powder by continuous Sephadex column chromatographies, that had a blue color with one major and three minor bands around 26k 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 then 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.

3.2 Introduction

Lacquer sap is a naturally occurring paint that is polymerized by laccase, anoxidoreductive 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 [Kumanotani, 1995]. Among them, stellacyanin, like laccase,is a copper-containing glycoprotein; it was first characterized as a blue glycoprotein by Keilin and Mann in 1940 [Keilin and Mann, 1940].

Lacquer stellacyanin is a low molecular weight (approximately 20k Da) protein with a single polypeptide chain of 107 amino acid residues and a Cu atom.Since the glycoprotein was identified as stellacyanin [Omura, 1961], proteins similar to lacquer stellacyanin have been isolated from cucumber [Aikazyan *et al*., 1979], zucchini peel [Marchesini *et al*., 1979], spinach leaf [Sarkissian and Nalbandyan, 1983], and horseradish root [Paul and Stigbrand, 1970]. These glycoproteins are plant specific glycoprotein belonging to the phytocyanin subclass of the cupredoxins. Stellacyanin plays a role in an electron transfer protein [Ikeda and Sakurai, 1993] and is involved in a redox process during primary defence, lignin formation, cell-to-cell signalling transmission, however, the exact physiological function is still unclear [Ranieri *et al*., 2012, Nersissian *et al*., 2011, Borner *et al*., 2003]. The thermodynamics and kinetics of electron transfer of electrode-immobilized *Rhus* stellacyanin was carried out at different pH and temperature conditions. In immobilized stellacyanin, electrode-SAM-protein construct was observed, that is interesting finding in protein based molecular electronic devices in application [Ranieri *et al*, 2012]

N-linked glycans in plant glycoproteins have been shown to affect catalytic activity, thermostability, lignin formation, folding or subcellular localization, and secretion [Song *et al*., 2011 and Ceriotti *et al*., 1998]. Recently it was found that N-linked glycans may play a role in plant pathogen integration and functional

pattern recognition receptors [Häweker *et al*., 2010]. Removal of complex type N-glycans from plant glycoproteins by mutational modification of N-linked glycans inseveral plants, Arabidopsis and tobacco, has demonstrated no or little effect on plant growth [Strasser, 2014]. For the structural analysis of the carbohydrate composition of lacquer stellacyanin, no results have been published excepta paper chromatographic separation of monosaccharides of stellacyanin hydrolysate by Peisach et al. in 1967 [Peisach *et al*., 1967].

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 [Rayon *et al*., 1998].There are three possible N-glycosylation sites (Asn-X-Ser or Thr) in the amino acid sequence of stellacyanin (N28, N60, and N102) [Bergman *et al*., 1977]. Mass spectrometry (MS) is valuable and rapid analytical techniques to analyse structures of glycans in proteomics and proteins by a combination of enzymatic treatments [Geyer and Geyer, 2006].

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.

3.3 Experimental

3.3.1 Materials

Lacquer stellacyanin was isolated from acetone powder according to the method reported by Reinhammar [20]. Trypsin from porcine pancreas (≥10,000 units/mg), α-chymotrypsin from bovine pancreas (61.75 units/mg), 1, 4-dithiothreitol (DTT),

iodoacetamide (IAA), and trifluoroacetic acid (TFA) were obtained from Sigma Aldrich, Japan. Peptide-N-Glycosidase A (PNGaseA) from almond and peptide-N-Glycosidase F (PNGaseF) 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 Daltonik GmbH,10-20% gradient gels (E-T1020L), EZ marker standards (AE-1440), and Coomassie blue for SDS-PAGE analysis fromAtto Corporation, Japan, were used.

3.3.2 Enzymatic digestion and N-glycan labeling

Lacquer stellacyanin (1mg) was suspended in 0.1M NH_4HCO_3 solution, DTT (5μl, 120mM) was added, and then the mixture was incubated for 30 min at 60°C. To the mixture was added 10μl of IAA (123mM) and incubated in the darkfor 1h at room temperature to give carboxyamidemethylated stellacyanin, which was used without purification for the next enzymatic digestion. Trypsin(1mg) or chymotrypsin(1mg) solution in 1ml of 0.1M NH_4HCO_3(10 μl)was added to the above carboxyamidemethylated 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 0.25U PNGaseAat pH 5.0 (adjusted by 0.1%HCOOH) and 5U PNGase F at pH 8.5, respectively, overnight at 37°C. The digested N-glycols were labeled with N^{α}-((aminooxy)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 bya clean-up column according to the manufacturer's protocol [24].The chymotrypsin-digested stellacyanin was also treated with 0.25 U PNGaseA and 5 UPNGase F, respectively, by the same procedure as above to give aoWR-labelled glycans.

Sialidase treatment

Lacquer stellacyanin (0.5mg) was treated with *Arthrobacterureafaciens*sialidase (1.0U in 50mM Tris/HCI buffer) overnight at pH5.6 and 37ºC to confirm the presence or absence of sialic acid in the glycans.

3.3.3 MALDI TOF MS measurement

The MALDI TOF MS spectrum was recorded on an Ultraflex II instrument (Bruker Daltonics) in reflection mode with an acceleration voltage of 25kV in the positive ion mode (positive voltage polarity). The MS spectrum was automatically provided by using Flex Control software 3.0 version. External calibration was carried out with singly charged monoisotopic peaks of peptide standards (1046.5Da angiotensin II, 1296.6Da angiotensin I, 1347.7Da substance P, 1619.8 Da bombesin, and 2093.0, 3474.5 ACTH CLIP peptides). DHBmatrix in acetonitrile/water (3:7, v/v) was used for the MALDITOFMS measurements of the aoWR-labelledN-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 (-COOH→-$COOCH_3$; mass signal increases +14.02), and the exact masses of glycans were calculated as glycan exact mass [M]=[observed *m/z*] – 447.21 + 18.01- (14.02×n) – 1.00, where 447.21 is themass of the aoWR labelling reagent and n is the number of sialic acids.

3.3.4 LC/MS/MS measurement

LC/MS/MS was performed using aliquid 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 (5μm) with ID 2.5mmx15cm anda TSK guard cartridge (5μm) with ID 3.2mmx1.5cm) were used at 30ºC. The eluents used were (A) 0.1% TFA in 90% acetonitrile and (B) 0.1% TFA in 10% acetonitrile with a flow rate of 0.2ml/min in a

gradient programfor 120min. 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 100-2800 Da, fixed linear ion trap (LIT) full time 10ms,Q0 off check. The enhanced resolution MS (ER), in which scan rate was 250 Da/s, fixed LIT full time 10ms, 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 100-2500, fixed LIT full time 50ms, and Q0 was trapping on.

MS data were processed with DataAnalyst 4.0 by using a default setting for glycopeptide analysis. Peptide sequences inglycopeptides 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).

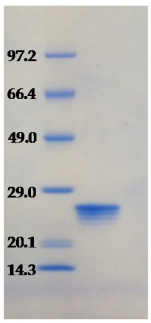
3.4 Results and Discussion

3.4.1 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 3.1** shows the (A) SDS PAGE profile, (B) 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 26kDa. The bands were very closeeach other as shown in **Figure 3.1A**. We used this lacquer stellacyanin

without further purification. MALDI TOF MS spectrum (Figure 2.1 B) 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 1C exhibits the amino acid sequence of the lacquer stellacyanin according to the literature [Bergman *et al*., 1977]. The lacquer stellacyanin had 107 amino acid residues and the molecular weight of the amino acid residueswas12296Da. Therefore, the molecular weight of the carbohydrate portionsofthe lacquer stellacyanin is around 7000 Da.



(A)

TVYTVGDSAG WKVPFFGDVD YDWKWASNKT
FHIGDVLVFK YDRRFHNVDK VTQKNYQSCN
DTTPIASYNT GBBRINLKTV GQKYYICGVP
KHCDLGQKVH INVTVRS

(B)

Figure 3.1: SDS-PAGE profile (A) and and amino lacquer stellacyanin (B). N-glycosylation sites in blue and copper binding sites are underlined, respectively

3.4.2 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 ofmixed 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 glycopeptideswas selectively detected byextracted ion chromatography (XIC) to reveal that it containsa glycan marker ionof *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), $NYQSCN_{60}DTTPIASYNTGBBR$ (*m/z*=2291.9), and $VHIN_{102}VTVR$ (*m/z*=937.5) as shown in **Table 3.1**

Table3.1: Observed N-glycopeptides derived enzymatic digestion of lacquer stellacyanin.

Glycosylation	Trypsin derived glycopeptides		Chymotrypsin derived glycopeptides		N-glycan	
site	peptide (mass)	obs. m/z	peptide (mass)	obs. m/z	mass	composition[a]
		888.12 / 2		919.19 / 2	1188.42	GlcNAc2 Hex3 Fuc1 Xyl1
		1070.85 / 2		1101.88 / 2	1553.56	GlcNAc3 Hex4 Fuc1 Xyl1
Asn 28	WASNK (604.29)	1144.03 / 2	ASNKTF (666.34)	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
		1154.68 / 3		1264.51 / 2	1188.42	GlcNAc2 Hex3 Fuc1 Xyl1
Asn 60	NYQSCNDTTPIASYNGTBBR	1276.85 / 3	QSCNDTTPIASY	nd[b]	1553.56	GlcNAc3 Hex4 Fuc1 Xyl1
	(2290.93)	1324.24 / 3	(1356.57)	1014.33 / 2	1699.61	GlcNAc3 Hex4 Fuc2 Xyl1
		1496.33 / 3		1184.74 / 3	2210.80	GlcNAc4 Hex5 Fuc3 Xyl1
		981.40 / 2		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
Asn102	VHINVTVR (936.55)	825.35 / 3	HINVTV (681.38)	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

[a]Monosaccharides represented as GlcNAc, N-acetylglucoseamine; Hex, mannose or galactose; Fuc, fucose; Xyl, Xylose.

[b]nd: Not detected.

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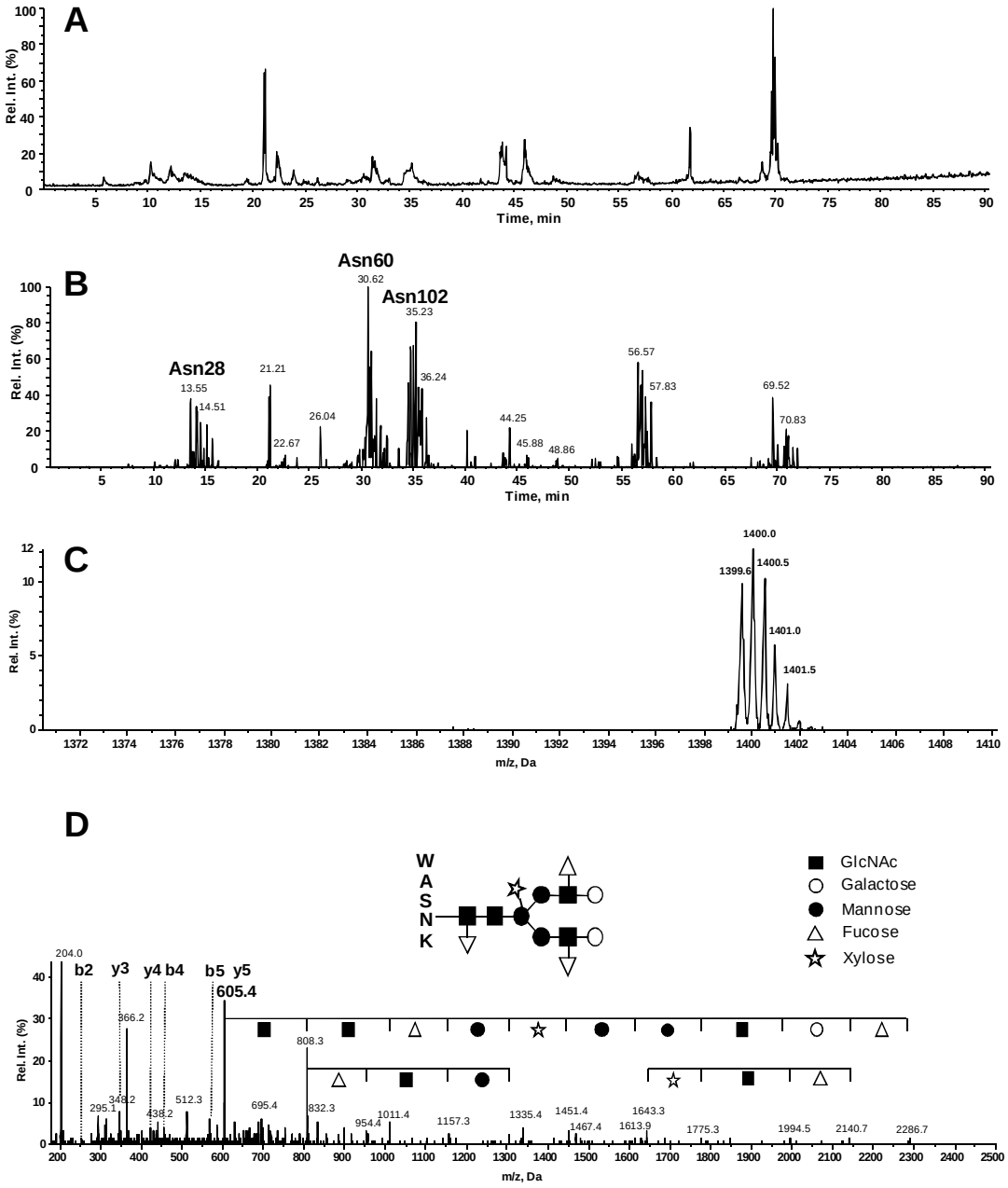

Figure3.2: LC/MS/MS analysis of trypsin derived N-glycopeptide WASNK (Asn28) of lacquer stellacyanin. (A)Total ion chromatography, (B) extracted ion chromatography, (C) enhanced mass spectrum of m/z 1399.6 (2+) acquired at 13.53 min, and (D) MS/MS spectrum of 1399.6 (2+) as a precursor.

The MS/MS spectrum of the glycopeptides was analysed, and then the signals at *m/z*=204.1 ($GlcNAc_1$), *m/z*=366.1 (Gal+GlcNAc), and *m/z*=512.1 (Fuc+Gal+GlcNAc) due to fragment ions of N-linked glycansas 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-glycopeptidesderived 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 **Figure3.2**. The TIC and XIC containing the m/z=204.1 ion signal due to GlcNAc are presented in **Figure**s **3.2A** and **3.2B**. A glycopeptide $WASN_{28}K$ withAsnat Asn28 was observed around 13-15min in the TIC, and the most intense ion signal at *m/z*=605.2 corresponded to the molecular weight of the oligopeptide part. From the observed molecular weight in **Figure 3.2C** 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 **Table3.1**.

The trypsin-derived N-glycopeptide NYQSCNDTTPIASYNTGBBR (*m/z*=2291.7) bearing Asn 60 was observed as a long peptide with only one GlcNAc residue attached to the peptide in the MS/MS spectrum as presented in **Figure 3.3A**. 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) byLC/MS/MS analysis as shown in **Figure 3.3**. As a result, the same N-linked glycan structure of$HexNAc_4$ $Hex_5Fuc_3Xyl_1$was also identified at Asn60. In analysis of the N-linked glycan at Asn102, we 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 also summarized in **Table3.1**.

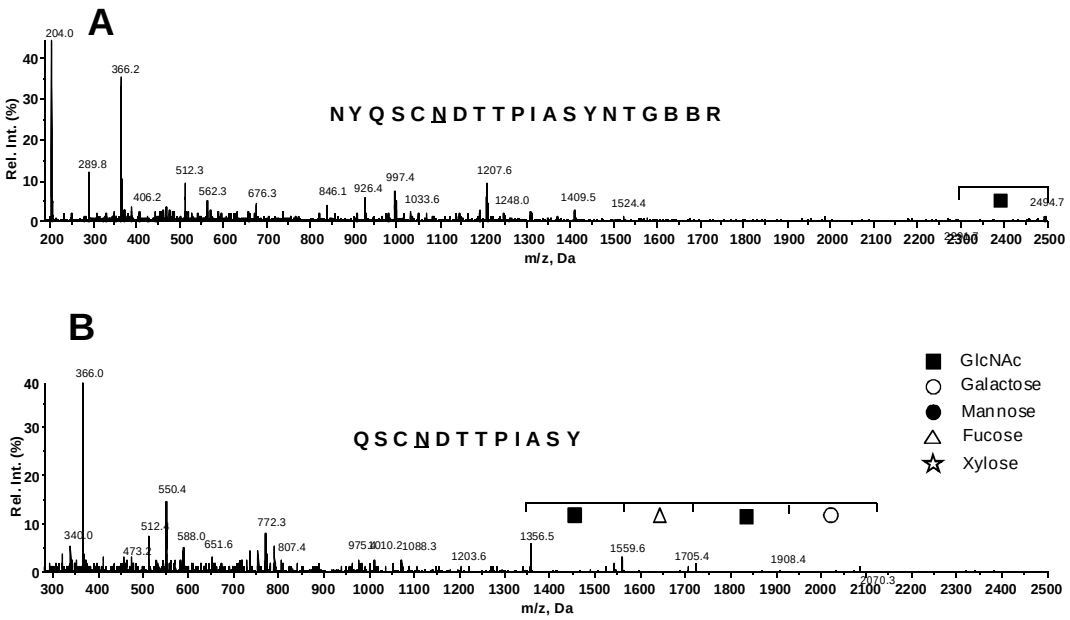

Figure3.3: LC/MS/MS spectra of (A) trypsin- and (B) chymotrypsin-derived N-glycopeptides from lacquer stellacyanin. Observed mass numbers at (A) m/z=2291.7 and (B) m/z=1356.5 were due to oligopeptides, NYQSCNDTTPIASYNTGBBR and QSCNDTTPIASY at Asn60 site, respectively.

Trypsin-digested oligopeptides and oligoglycosylpeptides 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 oligoglycosylpeptides (data are shown in supporting information).The strongestsignalappeared at *m/z*=4487.4 due to the glycopeptides at Asn60 bearingacomplex type N-linked glycan with the molecular weight of 2192.From the MALDI TOF MS analysis, the fucose and xylose-containing complex-type biantennary glycan was linked at the three Asnsites in lacquer stellacyanin.Fucose and xylose should be attached to the GlcNAc next to Asn and at the central mannose residues, respectively.

3.4.3 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.

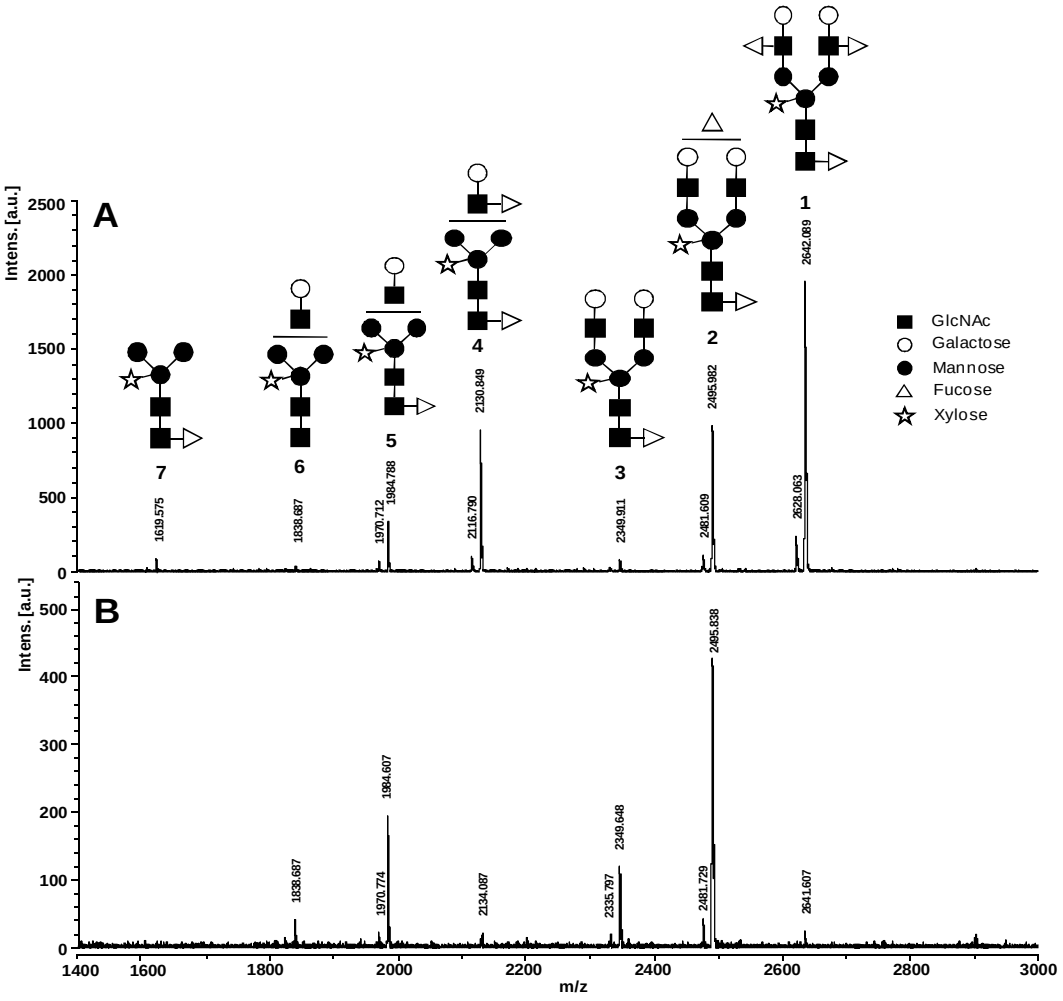

Figure3.4: MALDI TOF MS profiles of aoWR labelled N-linked glycans after (A) PNGase A and (B) PNGase F digestion of lacquer stellacyanin. N-Glycan mass=[observed mass - 447.21 + 17].

The aoWR-labelled N-linked glycans were mixed with the matrix (DHB) and then subjected to MALDITOF MS to provide initial information on the relative quantities of N-linked glycans presented in lacquer stellacyanin. **Figure 3.4** 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 4A and 4B, the intensity was slightly different. These results suggest that the N-linked glycan in the lacquer stellacyanin have fucose at the glucosamine next to Asn because PNGase A releases N-linked glycans with and without α-1, 3-fucosylated glucosamine next to Asn and PNGase F cleave N-linked glycans withα-1, 6-fucosylated glucosamine.

Table3.2: MALDI TOF MS assignment of the N-linked glycans from lacquer Stellacyanin

Peak	Observed m/z[a]		Theoretical m/z[b]		
No	PNGaseA	PNGaseF	N-glycan	aoWR-glycan	N-linked glycan[c]
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

[a]MALDI TOF MS signal digested with PNGaseA and PNGaseF.

[b]Theoretical molecular weight of N-linked glycan.

[c]HexNAc: N-acetylglucosamine, Hex: mannose or glucose,Fuc: fucose,Xyl: xylose.

Table3. 2 shows the structure of N-linked glycans based on the m/z in MALDI TOF MS spectra in **Figure 3.4**. The most intense peak (No. 1) at the *m/z* of 2642 was assigned to a complex type N-linked glycan consisting of four glucosamines (HexNAc)4, five hexoses (Hex)5, three fucoses (Fuc)3, 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 anα-1,3 linked fucosylated GlcNAc next to Asn and a β-1,2linked xylose should be attached to the central mannose residue. Other signal peaks, Nos. 2-7 in Figure 3.4, 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 3.4. 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-H_2O, and *m/z*=657.1 due to Hex+HexNAc+NeuAc are diagnostic or marker ions originating from sialylatedglycopeptides. 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.

3.5 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 Asnresidues (Asn28, Asn60, and Asn102) in lacquer stellacyanin, indicating that the N-linked glycan had the same structureat the three Asn sites and was a biantennarycomplex type N-linked glycan containing fucose at GlcNAc next to Asnand xyloseat the centralmannose 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 phytocyanin family of plant glycoproteins. Characterization of N-linked glycans in lacquer laccase is also under investigation.

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Chapter 4

Structure elucidation of N-linked glycan of Lacquer Laccase using Mass Spectroscopies

4.1 Abstract

Laccase (EC 1.10.3.2) of lacquer sap, is a multicopper oxidoreductive enzyme of catehols, plays key role for formation of a durable film with a glorious surface. Laccase (100kDa) is heavily glycosylated protein that containing 15 possible N-glycosylation sites. Structure of N-glycans in purified lacquer laccase was elucidated using enzymatic digestions and labeling methods in combination with mass spectrometries. Quantitatively confirmation of N-glycans in laccase, both complex- and hybride-type N-linked glycans having a common plant-specific structural unit of β-xylose, core α-fucose and Lewis epitope, terminal trisaccharides (GlcNAc-Gal-Fuc) were observed. N-glycopeptide analysis by LC/MS/MS, 13 N-glycosylation sites in laccase were occupied with complex type N-glycan, $GlcNAc_4Man_3Gal_2Fuc_3Xyl_1$, and among them Asn5, 233 and 381 were found to be glycosylated with hybride–type N-glycan, mainly $GlcNAc_{2\text{-}3}Man_{4\text{-}5}Fuc_1Xyl_1$. N-glycosylation sites at Asn 364 and 519 were not found to be glycosylated.

4.2 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 [Kumanotani, 1995]. Laccase (EC 1.10.3.2), a blue multicopper oxidoreductive enzyme from the Japanese lacquer tree, *Rhus vernicifera* was first discovered in 1883 by Yoshida [Yoshida, 1883]. Since that, laccase and laccase-like proteins have been described in many plants, fungi (ascomycetes and basidiomycetes), insects and bacteria [Madhavi, 2009 and Dwivedi, 2011].

The physiological functions of laccase are different in the various organisms but they all catalyse polymerization or depolymerization processes. In plants, laccase are involved in cell wall formation and together with peroxidases, in lignifications, wound healing and iron oxidation [Dwivedi, 2011]. Nowadays, a number of industrial applications of laccase have been reported in agricultural, industrial and medical areas are recently getting more attention for prospective applications in the broad industrial fields, because of their diverse roles in nature, substrate usage varieties, termostablity and environmentally friendly catalyst [Dwivedi, 2011].

Despite of origin and functions, laccases are glycosylated protein especially in plant and fungi has heavily glycosylated that could be affective for the protein secretion, stability the protein centre, protecting against proteolysis, termostability and enzyme activity [Dwivedi *et al*, 2011 and Rodgers *et al*., 2009]. N-linked oligosaccharide heterogeneity of plant laccase was studied in sycorome, *Acer pseudoplatanus* L and xylose-containing the biantennary complex type were identified [Tezuka, 1993].

Wan *et al* isolated hydrosoluble components of Chinese lacquer and their structural properties was evaluated including two laccase isoenzymes (L1 and L2) [Wan *et al*., 2006]. Authors also studied the effect of other lacquer components such as polysaccharides, stellacyanin and laccase isoenzymes on enzyme activity of free and immobilised laccase, result releaved that polysaccharides and stellacyanin have a negative effect, but isoenzymes have synergenic effect on laccase activity [Wan *et al*., 2010]. Lu *et al* investigated enzyme activity and stability of free and immobilized lacquer laccase result releaved that immobilized laccase has more stable and it suggest a repeated use of enzymes [Lu *et al*., 2012].

We have previously reported the N-linked glycan structure of the lacquer stellacyanin, another copper containing glycoprotein in lacquer sap, and we found that xylose and fucose containing complex type N-linked glycans to be attached in stellacyanin [Tumurbaatar and Yoshida, 2015].

Lacquer laccase is heavily glycosylated protein, with 15 possible N-glycosylation sites (Asn-X-Thr/Ser), predicted from its amino acid sequence has not accomplished yet [Nitta *et al*., 2002]. In the present study, we elucidate the N-linked glycans in lacquer laccase using enzymatic digestions and chemical labeling in combination with mass spectrometries. Protease digested laccase treated with glycopeptidase (PNGase F and A) and then released N-linked glycans were labeled with oaWR and analysed by MALDI TOF MS. Result revealed that both complex- and hydride-type N-linked glycans to existing in lacquer laccase, quantitatively. For further analysis of N-linked glycan structures at each glycosylation site, trypsin and chymotrypsin digested glycoprotein were subjected to LC/MS/MS analysis and the predictable N-glycopeptides in peptides/glycopeptides mixture were selectively detected by using carbohydrate-specific marker ion (GlcNAc, *m/z*=204).

We identified that 13 N-glycosylation sites in laccase were glycosylated frequently with complex type N-glycan, $GlcNAc_4Man_3Gal_2Fuc_3Xyl_1$, and among them Asn5, 233 and 381 were found to be glycosylated with hybride–type N-glycan, mainly $GlcNAc_2Man_{4\text{-}5}Fuc_1Xyl_1$. In this study, N-linked glycan at Asn 364 and 519 were not confirmed.

4.3 Experimental

4.3.1 Materials

Lacquer acetone powder was prepared by addition of acetone to lacquer sap according to the method reported by Reinhammar [Reinhammar, 1970]. Trypsin from porcine pancreas (≥10,000 units/mg),α-chymotrypsin from bovine pancreas (61.75 units/mg), 1, 4-dithiothreitol (DTT), iodoacetamide (IAA), and trifluoroacetic acid (TFA) were obtained from Sigma Aldrich, Japan. Peptide-N-Glycosidase A (PNGaseA) from almond and peptide-N-Glycosidase F (PNGaseF) 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 Daltonik 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.

4.3.2 Measurements

The MALDI TOF MS spectrum was recorded on an Ultraflex II instrument (Bruker Daltonics) in reflection mode with an acceleration voltage of 25kV in the positive ion mode. The MS spectrum was automatically provided by using Flex Control software 3.0 version. External calibration was carried out with singly charged monoisotopic peaks of peptide standards (1046.5Da angiotensin

II, 1296.6Da angiotensin I, 1347.7Da 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

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 electro spray ionization (ESI). An ODS column (TSK-Gel ODS-80Ts (5μm) with ID 2.5mmx15cm anda TSK guard cartridge (5μm) with ID 3.2mmx1.5cm) were used at 30ºC. The eluents used were (A) 0.1% TFA in 90% acetonitrile and (B) 0.1% TFA in 10% acetonitrile with a flow rate of 0.2ml/minin a gradient program for 120min. and MS/MS spectra were acquired in the positive ionization mode. MS data were processed with DataAnalyst 4.0 by using a default setting for glycopetide analysis. Peptide sequences in glycopeptides obtained by proteases were annotated by BioAnalyst (peptide sequence), and MS/MS analyses of attached glycans were interpreted manually.

4.3.3 Protein digestion and N-glycan labeling

Lacquer laccase (1mg) was suspended in 0.1M ammonium bicarbonate (NH_4HCO_3) solution, DTT (5μl, 120mM) was added, and then the mixture was incubated for 30 min at 60°C. To the mixture was added 10μl of IAA (123mM) and incubated in the dark for 1h at room temperature, then trypsin (1mg) or chymotrypsin (1mg) solution (10μl) was added to the above carboxyamidemethylated solution, and the mixture was incubated overnight at 37°C and 25°C, respectively, then heated at 95°C to digest enzymes.

Glycopeptidase treatment and N-glycan labeling

The trypsin-digested stellacyanin was deglycosylated by adding 0.25U of PNGase A and PNGase F, respectively, at pH 5.0 (adjusted by 0.1%HCOOH)

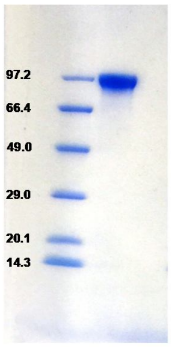
overnightat 37°C. The digested N-glycans were labeled withN^{α}-((aminooxy)acetyl)tryptophanylargininemethyl ester (aoWR) by using a commercial BlotGlyco™glycan purification and labeling kit according to the manufacturer's protocol [BlotGlycoR protocol]. The chymotrypsin-digested stellacyanin was also treated with 5 U PNGaseA and PNGase F, respectively, by the same procedure as above at pH 8.5 to give aoWR-labelled glycans.

4.3.4 Peptide identification by Database Search

Peak lists were generated from the MS/MS spectra using Bruker Flex Analyst were processed by the Mascot algorithm (Matrix Science Ltd.) to assign peptides generated protein digests. The database searched for tryptic, chymotryptic and glycoprotein digested both of trypsin and chymotrypsin were used in once with up to one miscleavage. Carboxymethylation of cystein residues in proteins were allowed.

4.4 Results and Discussion

The lacquer laccase 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 4.1** shows the SDS PAGE profile (A) and amino acid sequence (B) of lacquer laccase. The purified lacquer laccase was confirmed by SDS PAGE as one major band around 100kDa. **Figure 4.1B** exhibits the amino acid sequence of the lacquer laccase according to the literature [Nitta *et al.*, 2002].



(A)

VDVHNYTFVL QERNFTKWCS TKSMLVVNGS FPGPTITARK GDTIFVNVIN QGKYGLTIHW HGVKQPRNPW 70
SDGPEYTQC PIKPGINFIY EVILSTEEGT LWWHAHSDWT RATVHGALVI LPANGTTYPF PPPYQEQTIV 140
LASWFKGDVM EVITSSEETG VFPAAADGFT INGELGDLYN CSKETTYRLS VQPNKTYLLR IVNAVLNEEK 210
FFGIAKHTLT VVAQDASYIK PINTSYIMIT PGQTMDVLFT TDQTPSHYYM VASPFHDAID TFANFSTNAI 280
IQYNGSYKAP KSPFVKPLPV YNDIKAADKF TGKLRSLANE KFPVNVPKVN VRRIFMAVSL NIVKCANKSC 350
NNNIGHSTSA SLNNISFALP QTDVLQAYYR NISGVFGRDF PTVQKKANFS LNTAQGTQVL MIEYGEAVEI 420
VYQGTNLGAA TSHPMHLHGF NFYLVGTGAG TFNNVTDPPK YNLVDPPELN TINLPRIGWA AIRFVADNPG 490
VWFLHCHFER HTTEGMATVV IVKDGGTINT SMLPSPAYMP PCS 533

(B)

Figure 4.1: Laccase isolated from lacquer sap: SDS-PAGE profile (A) and amino acid sequence (B) of lacquer laccse. N-glycosylation sites are underlined and copper binding sites in blue, respectively.

The lacquer laccase had 533 amino acid residues and the molecular weight of the amino acid residues was 59054Da, thus the molecular weight of the carbohydrate portions of the lacquer laccase is around 40% of glycoprotein. Protein information is available in UniProt Protein Database with ID Q94IDO and entry name Q94IDO-TOXVR (http://www.uniprot.org/uniprot/Q94IDO).

4.4.1 MALDI TOF MS analysis of N-linked glycans in laccase

N-linked glycans in lacquer laccase were released with PNGaseA and PNGase F, respectively, and 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 the relative quantities of N-linked glycans presented in lacquer laccase. The observed mass numbers increased 447.21 due to the aoWR labelling reagent compared to the underived N-linked glycans. **Figure4.2** 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 laccase.

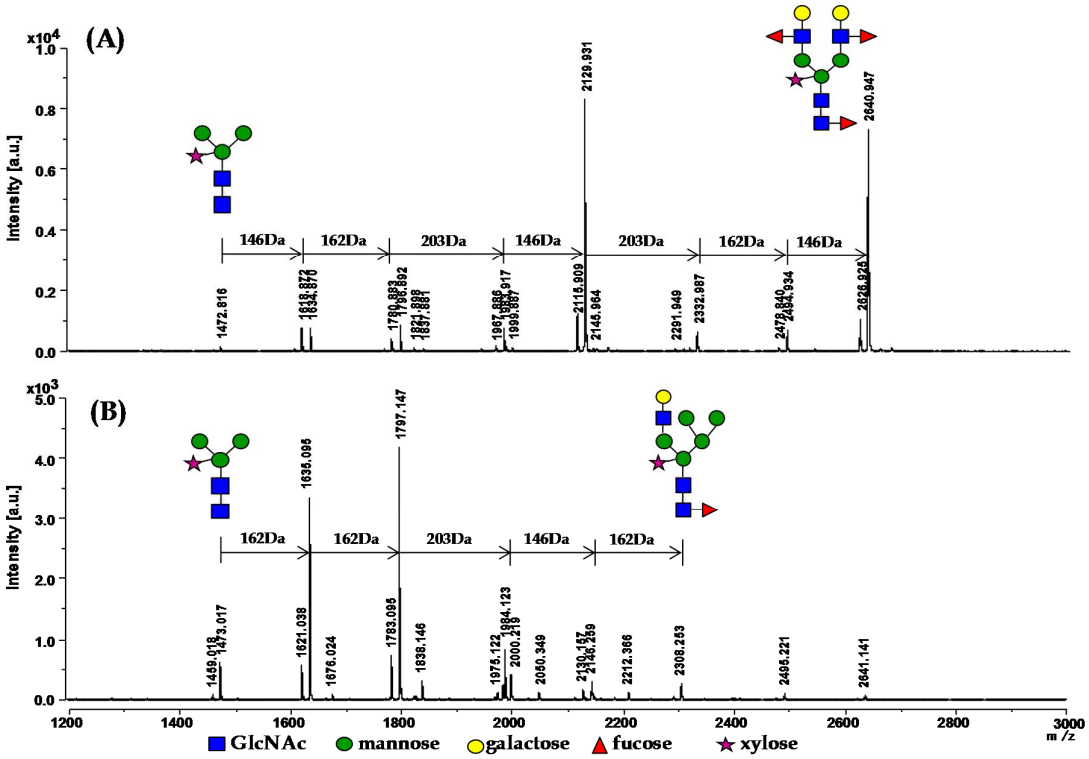


Figure 4.2: MALDI TOF MS profiles of aoWR labelled N-linked glycans after (A) PNGase A and (B) PNGase F digestion of lacquer laccase. N-Glycan mass=[observed mass - 447.21 + 17].

MALDI TOF MS profiles of aoWR labelled N-linked glycans shows that glycan mass signals were similar, but intensity were quite different after PNGase A and PNGase F digestion of lacquer laccase. The observed mass numbers increased 447.21 due to the aoWR labelling reagent compared to the underived N-linked glycans. Glycomod search revealed that the observed m/z value in PNGase A treated samples found to be identical with to plant specific complex type N-glycan structure. Peaks have the most intensity at m/z 2640 [numbered as 1] assigned as the plant specific complex type N-linked glycan consisting of four glucosamines (HexNAc)4, five hexoses (Hex)5, three fucoses (Fuc)3, and one xylose (Xyl), that is, (HexNAc)4(Hex)5(Fuc)3Xyl. *m/z* 2129 [13] assigned as (HexNAc)3(Hex)4(Fuc)1Xyl that decreased mass of 512, indicating HecNAc-Hex-Fuc were cleaved from the complex type N-linked glycan (HexNAc)4(Hex)5(Fuc)3Xyl as illustrated in **Figure 4.2**.

In contrast, peaks at m/z 1635 and 1797 were observed with high intensity PNGase F treated laccase, that observed low intensity comparing PNGase F. These m/z values found to hybride type N-glycan structure, which are assigned as (HexNAc)2(Hex)4Xyl and (HexNAc)2(Hex)5Xyl with core xylose at central mannose. As illustrated **Figure 4.2B**, peaks were observed at *m/z* 2000, 2146 are 2308 with low intensity assigned as (HexNAc)3(Hex)5Xyl [12], (HexNAc)3(Hex)5FucXyl [14] and (HexNAc)3(Hex)6FucXyl [16], respectively. All the observed signals [1-20] and their proposed structure were summerized in **Table4.1**.

These results of the quantitation analyses by MALDI TOF MS suggest that complex and hybride type N-linked glycan were attached to lacquer laccase.

Table4.1: Observed labeled N-glycans released from lacquer laccase in MALDI TOF MS

Peak	Theoretical m/z		Observed m/z		
№	N-glycan	Labeled-glycan	PNGaseA	PNGaseF	N-glycan composition
1	1024.36	1472.57	1473.61	1473.40	(HexNAc)2 (Hex)3 Xyl
2	1170.41	1618.62	1619.57		(HexNAc)2 (Hex)3 (Fuc) Xyl
3	1186.41	1634.62	1635.76	1635.53	(HexNAc)2 (Hex)4 Xyl
4	1227.43	1675.64		1677.56	(HexNAc)3 (Hex)3 Xyl
5	1332.47	1780.68	1781.76		(HexNAc)2 (Hex)4 (Fuc) Xyl
6	1348.46	1796.67	1797.82	1797.59	(HexNAc)2 (Hex)5 Xyl
7	1373.49	1831.70	1822.81		(HexNAc)3 (Hex)3 (Fuc) Xyl
8	1389.49	1837.70	1838.85	1838.63	(HexNAc)3 (Hex)4 Xyl
9	1494.52	1942.73	1942.89		(HexNAc)2 (Hex)5 (Fuc) Xyl
10	1519.55	1967.76	1967.88		(HexNAc)3 (Hex)3 (Fuc)2 Xyl
11	1535.55	1983.76	1984.58	1984.58	(HexNAc)3 (Hex)4 (Fuc) Xyl
12	1551.54	1999.75	1999.88	2000.21	(HexNAc)3 (Hex)5 Xyl
13	1681.60	2129.81	2129.93	2130.15	(HexNAc)3 (Hex)4 (Fuc)2 Xyl
14	1697.60	2145.81		2146.62	(HexNAc)3 (Hex)5 (Fuc) Xyl
15	1843.66	2291.87	2291.94		(HexNAc)3 (Hex)5 (Fuc)2 Xyl
16	1859.65	2307.86		2308.25	(HexNAc)3 (Hex)6 (Fuc) Xyl
17	1884.68	2332.89	2332.98		(HexNAc)4 (Hex)4 (Fuc)2 Xyl
18	2030.74	2478.95	2478.84		(HexNAc)4 (Hex)4 (Fuc)3 Xyl
19	2046.74	2494.95	2494.93	2495.22	(HexNAc)4 (Hex)5 (Fuc)2 Xyl
20	2192.27	2640.48	2640.94	2641.14	(HexNAc)4 (Hex)5 (Fuc)3 Xyl

HexNAc, acetylglucosamine; Hex, mannose or glucose; Fuc, fucose; Xyl, xylose.
Observed mass = [Glycan+447.21]+H^+, matrix DHB in ACN:water (7:3)

4.4.2 LC/MS/MS analysis of glycopeptides

For analyse of the N-glycosylation sites, lacquer laccase were digested both trypsin and chymotrypsin which contain peptides and glycopeptides were directly analysed by LC/MS/MS without any separation and purification. Glycopeptides with potential N-glycosylation sites in laccase obtained in different length of peptides from trypsin and chymotrypsin cleavages. For identification of glycopeptides, glycopeptides contained in the mixture sample was selectively detected by extracted ion chromatography (XIC) to reveal that it contains a glycan marker ionof *m/z* 204.1 due to glucosamine, $GlcNAc^+$.

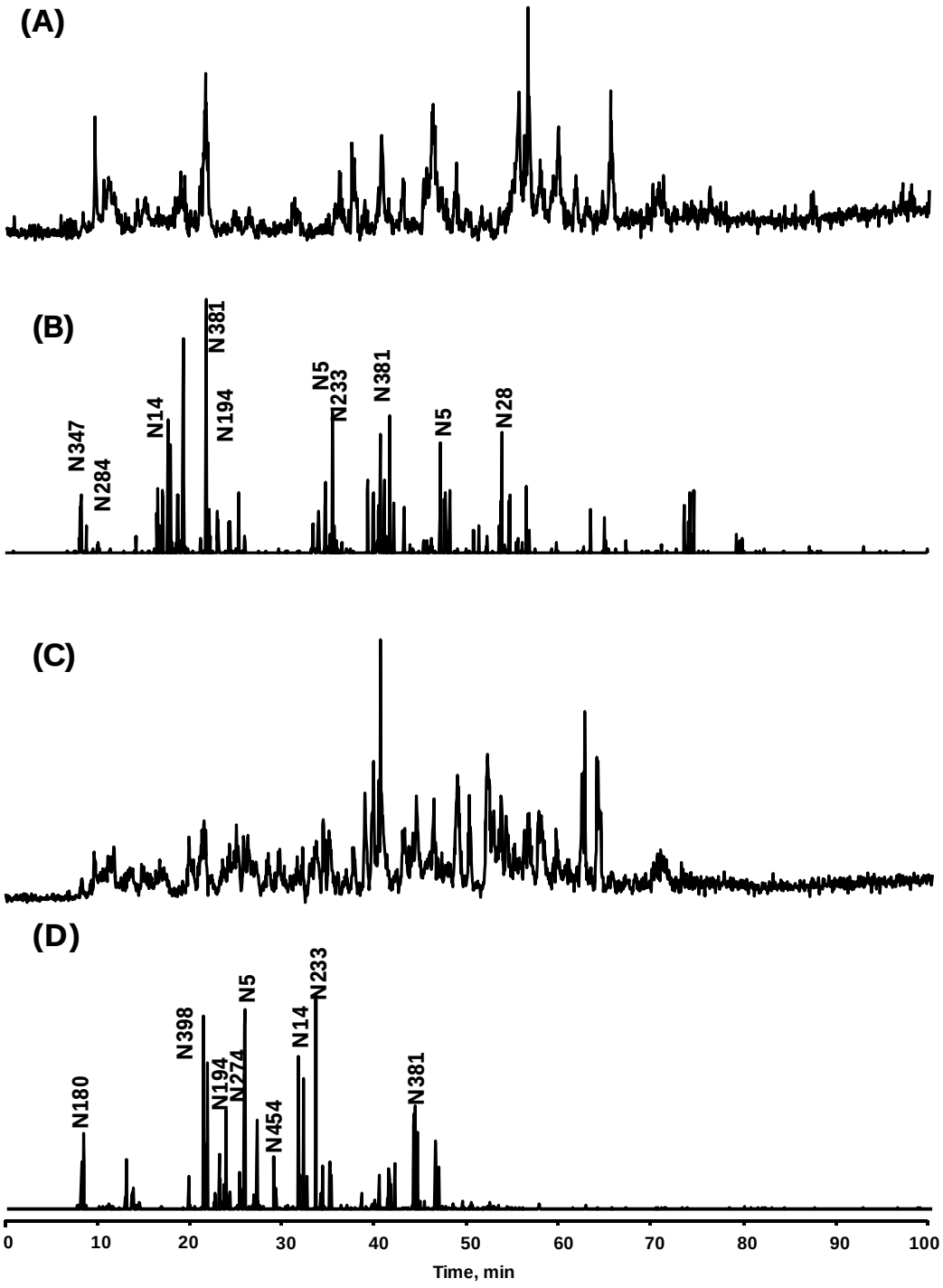


Figure4.3: Total Ion Chromatography [TIC] and Extracted Ion Chromatography [XIC] of laccase digest in LC/MS/MS. Obtained TIC are in (A) and (C), XIC containing marker ion of m/z=204 (HexNAc) in (B), (D) from trypsin and chymotrypsin digested lacquer laccase, respectively.

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. Each glycopeptides detected in XIC were analysed using their individual MS/MS spectrum. There are three kinds of fragmentation ions (*m/z* 204, 366 and 512 etc), glycan related fragment ion, peptide related fragment ion (y and b series fragmentation) and glycopeptides fragment ions generated of glycopeptides in the MS/MS of N-glycopeptides.

TIC and XIC of trypsin and chymotrypsin showed in **Figure 4.3**, where detected peak were analysed based on their peptide mass and fragmentation, then structure of attached glycan was determined. By elucidating corresponding MS/MS in XICs, we confirmed 9 tryptic glycopeptides, VDVHNYTFVLQEK, K.NFTK, K.SMLVVNGSFPGPTITAR, Y.NCSK, R.LSVQPNK, Y.NGSYK, K.CANK, R.NISGVFGR and K.KANF, which are contain potential N-glycosylation sites at Asn5, 14, 28, 180, 194, 284, 347, 381 and 398 respectively. MS/MS spectrum of chymotrypsin digest of laccase, we could identified 9 chymotryptic glycopeptides, including 4 new glycopeptides matches are following L.VILPANTTY, Y.IKPINTSY, F.ANF and F.NNVDPPKY that contain potential N-glycosylation sites at Asn124, 233, 174 and 454, respectively. However, form the MS/MS spectra in XIC peaks, two glycopeptides have possible N-glycosylation sites at Asn 364 and 519 were not confirmed. We also perform trypsin digestion and subsequent chymotrypsin digestion of laccase to obtain a shorter peptides and glycopeptides as possible as. We could not obtain the glycopeptides to correspond Asn 364 and 519, this result suggest these two glycosylation sites were not glycosylated.

LC/MS/MS results of obtained glycopeptides were summarized in **Table 4.2**. MS/MS spectrum of tryptic and chmotryptic glycopeptides were assigned by manually, attached N-glycan mass was calculated from observed mass and

charge, where N-glycan structure was presented by numbers with in MALDI TOF MS result. As the result, all obtained glycopeptides in lacquer laccase were glycosylated with complex type N-glycan. Most frequently observed glycan structures were [20] and [13] that consisted of (HexNAc)4 (Hex)5 (Fuc)3 Xyl and (HexNAc)3 (Hex)4 (Fuc)2 Xyl as shown in **Table 4.1**. Also paucimannose type N-linked glycan of [2] have molecular weight of 1170.4 was observed to be attach at Asn14, 194, 233, 381 and 454, respectively.

Hybride type N-linked glycan assigned by oaWR labeled glycan by MALDI TOF MS represented in [3], [5] and [9] were found to be attached some tryptic and chymotryptic peptides. Tryptic derived peptide, K.SMLVVNGSFPGPTITAR was glycosylated only with (HexNAc)2 (Hex)4 Xyl of 1186.4 [3]. The most common hybride type N-linked glycan structure was (HexNAc)2 (Hex)4 (Fuc) Xyl, *m/z* 1332.4 and it also attached to Asn 5, 233 and 381 as shown in result. The largest structure of hybride type N-linked glycan detected in LC/MS/MS analysis of glycopeptides was (HexNAc)2 (Hex)5 (Fuc) Xyl [9] and found only at Asn 381, tryptic derived glycopeptides, R.NISGVFGR as 1172.4 (+2). However, some structure of N-linked glycans are following: *m/z* 2000, 2146 are 2308 were assigned as (HexNAc)3(Hex)5Xyl [12], (HexNAc)3(Hex)5FucXyl [14] and (HexNAc)3(Hex)6FucXyl [16], respectively, were not identified to be attaching any of these characterized peptides in LC/MS/MS. It might be caused their low abundance in the digest mixture of peptide and glycopeptides, these peaks also have very low intensity in MALDI TOF MS.

Table 4.2: Observed N-glycopeptides from lacquer laccase analysed by LC/MS/MS

Glycosylation sites	Trypsin derived glycopeptides		Chymotrypsin derived glycopeptides		Proposed structure of N-linked glycan
	Peptide sequence	Observed mass, *m/z*	Petide sequence	Observed mass, *m/z*	
Asn 5	VDVHNYTFVLQEK.	1263 (+3) 1092. (+3) 1043. (+3)	VDVHNY.	1214.4 (+2) 1470.6 (+2) 1141.4 (+2) 1039.8 (+2)	2192.2 [20] 1681.6 [13] 1535.5 [11] 1332.4 [5]
Asn 14	K.NFTK.	1351.5 (+2) 1095.8 (+2) 840.1 (+2)	F.VLQEKNF.	1024.6 (+3) 1280.5 (+2)	2192.2 [20] 1681.6 [13] 1170.4 [2]
Asn 28	K.SMLVVNGSFPGPTITAR.	978.6 (+3)	-		1186.4 [3]
Asn 124	-		L.VILPANTTY.	1365.8 (+2)	1681.6 [13]
Asn 180	Y.NCSK.	1351.1 (+2) 1095.4 (+2)	-		2192.2 [20] 1681.6 [13]
Asn 194	R.LSVQPNK.	1489.9 (+2) 994.1 (+3) 1234.3 (+2) 978.3 (+2) 1059.3 (+2)	L.SVQPNKTY.	1044.1 (+3) 1309.5 (+2) 1053.7 (+2)	2192.2 [20] 1681.6 [13] 1332.4 [5] 1170.4 [2]
Asn 233	-	-	Y.IKPINTSY.	1309.6 (+2) 1155.1 (+2) 1053.7 (+2) 1044.0 (+3) 1134. (+2)	2192.2 [20] 1681.6 [13] 1373.4 [7] 1332.4 [5] 1170.4 [2]
Asn 274	-	-	F.ANF.	1273.5 (+2) 1118.3 (+2) 1016.8 (+2)	2192.2 [20] 1884.6 [17] 1681.6 [13]

Asn 284	Y.NGSYK.	1381. (+2) 1125. (+2)	-	-	2192.2 [20] 1681.6 [13]
Asn 347	K.CANK.	1344.6. (+2) 1087.3 (+2)	-	-	2192.2 [20] 1681.6 [13]
Asn 364			ND		
Asn 381	R.NISGVFGR.	1266.5 (+2) 1010.4 (+2) 1172.4 (+2) 1091.3 (+2) 1193.1 (+2) 1015.1 (+3)	Y.RNISGVF.	1493.3 (+2) 1237.8 (+2)	2192.2 [20] 1681.6 [13] 1535.5 [11] 1494.5 [9] 1332.4 [5] 1170.4 [2]
Asn 398	K.KANF.	1080.8 (+2)	Q.KKANF.	933.9 (+3) 1400.6 (+2)	2192.2 [20] 1681.6 [13]
Asn 454	-	-	F.NNVDPPKY.	1365. (+2) 1109. (+2)	1681.6 [13] 1170.4 [2]
Asn 519			ND		

ND; not determined, (-) ; peptide or *m/z* are not observed

Figure 4.4 shows the MS/MS spectrum acquired *m/z* 994.4, (3+) at 23 min and *m/z* 785 was calculated to be peptide mass with R.LSVQPNK that contain possible N-glycosylation sites at Asn 194. The molecular mass of attached glycan was calculated 2210.8, which suggest the plant specific complex type N-glycan consisted of HexNAc4, Hex5, Fuc3 and Xyl1.

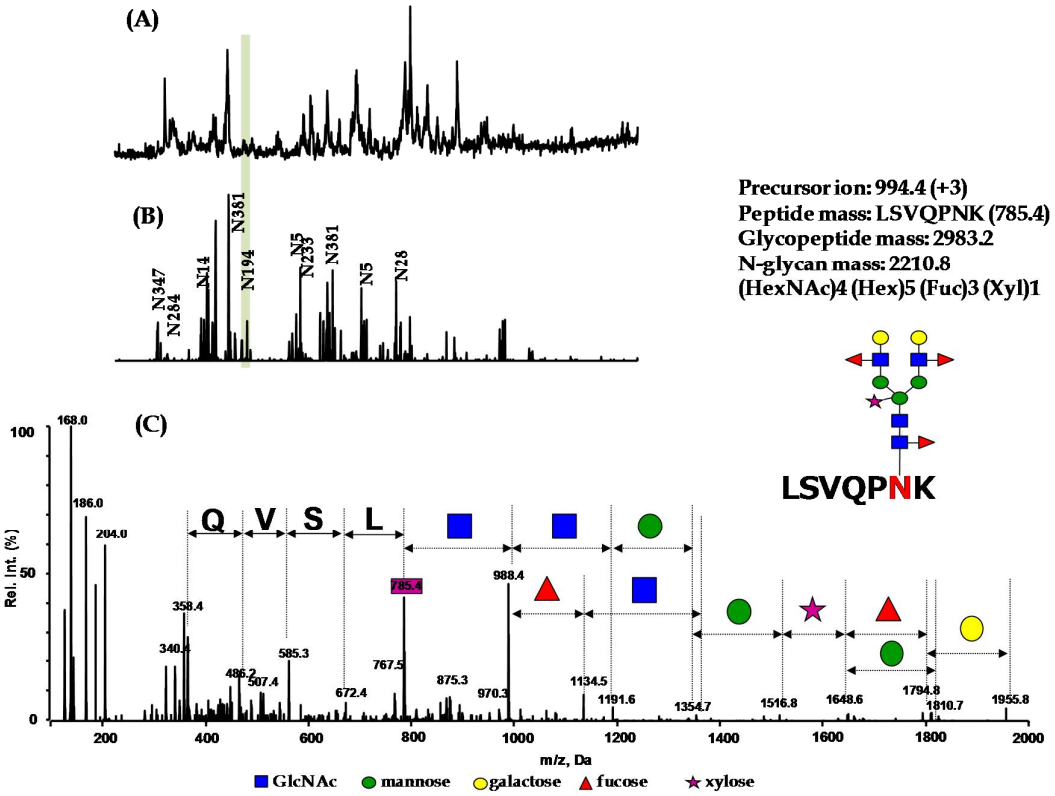


Figure 4.4: LC/MS/MS analysis of N-glycopeptide R.LSVQPNK. derived trypsin digestion of lacquer laccase. Total Ion Chromatography (A); Extracted Ion Chromatography (B); MS/MS spectrum (C) of trypsin digested glycopeptide R.LSVQPNK. carring complex type glycan 994.4, 3+ at Asn 194.

LC/MS/MS spectrum of hybride type N-linked glycan attached chymotryptic glycopeptides, VDVHNY that containing potential N-glycosylation sites at Asn 5 shown in **Figure 4.5**.

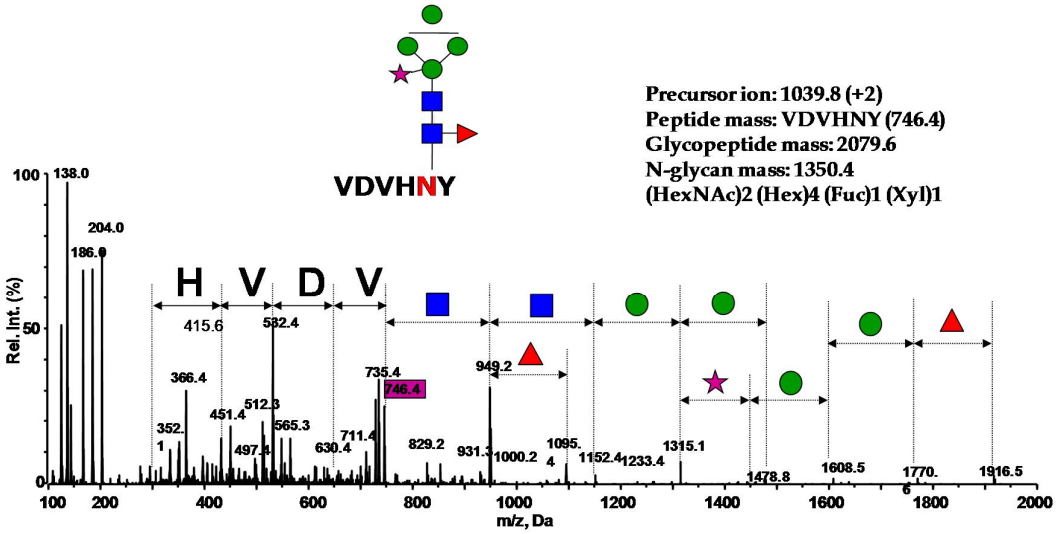


Figure 4.5: MS/MS spectrum of chymotrypsin derived glycopeptide VDVHNY. carring hybride type glycan 1039.8, 2+ at Asn 5.

4.4.3 Peptide mapping by Mascot search

For peptide identification of lacquer laccase, MS/MS spectra obtained trypsin and chymotrypsin digest were submitted for a Mascot database search with one missed cleavage, peptide tolerance of 1.2 and variable modification of cystein (carboxymethylation). We expect that all peptides were generated trypsin and chymotrypsin digest except glycopeptides, if there are not modified to be identified.

The Mascot search results were showed in **Table 4.3** and protein recovery of individually search was 23%, 25% and 30%, respectively. When these individual searching results were put in together, protein recovery score was 54%. **Figure 4.6** shows protein sequence analysis including peptides matches result provided by Mascot search and peptides sequences were also confirmed

by glycopeptides analysis. These results indicate that 64% of the glycoprotein was identified in the study. We expect that detection of unoccupied peptides containing N-glycosylation sites at Asn364 and 519. However peptides were not registered in online peptide search in their free and this situation suggest that these parts of protein might be modified other protein modification, especially O-glycosylation.

```
  1 VDVHNYTFVL QEKNFTKWCS TKSMLVVNGS FPGPTITARK GDTIFVNVIN
    QGKYGLTIHW HGVKQPRNPW SDGPEYITQC PIKPGTNFIY EVILSTEEGT
101 LWWHAHSDWT RATVHGALVI LPANGTTYPF PPPYQEQTIV LASWFKGDVM
    EVITSSEETG VFPAAADGFT INGELGDLYN CSKETTYRLS VQPNKTYLLR
201 IVNAVLNEEK FFGIAKHTLT VVAQDASYIK PINTSYIMIT PGQTMDVLFT
    TDQTPSHYYM VASPFHDALD TFANFSTNAI IQYNGSYKAP KSPFVKPLPV
301 YNDIKAADKF TGKLRSLANE KFPVNVPKVN VRRIFMAVSL NIVKCANKSC
    NNNIGHSTSA SLNNISFALP QTDVLQAYYR NISGVFGRDF PTVQKKANFS
401 LNTAQGTQVL MIEYGEAVEI VYQGTNLGAA TSHPMHLHGF NFYLVGTGAG
    TFNNVTDPPK YNLVDPPELN TINLPRIGWA AIRFVADNPG VWFLHCHFER
501 HTTEGMATVV IVKDGGTTNT SMLPSPAYMP PCS
```

Figure 4.6: Identified peptides and glycopeptides of lacquer laccase in the study. Matched peptide sequences identified by Mascot search represented in red, which are obtained from independent search results of laccase digested samples. Glycopeptides are in green characterized by LC/MS/MS analysis of lacquer laccase. Unidentified peptide sequences by both Mascot search and glycopeptide analysis are in black, and N-glycosylation sites are underlined, respectively.

Table4.3: Matched peptide list from Mascot Database (NCBInr) search

Matched peptide		Observed mass, *m/z*	Expected mass, *m*
Residue number	**Sequence**		
(1)Trypsin digestion			
40-53	R.KGDTIFVNVINQGK.	766.8 (+2)	1531.8
41-53	K.GDTIFVNVINQGK.	702.9 (+2)	1403.7
54-64	K.YGLTIHWHGVK.	655.8 (+2), 437.7 (+3)	1309.6
54-67	K.YGLTIHWHGVKQPR.	846.3 (+2), 564.6 (+2)	1690.9
201-210	R.IVNAVLNEEK.	564.7 (+2), 1128.5 (+1)	1127.6
201-216	R.IVNAVLNEEKFFGIAK.	896.4 (+2)	1790.9
316-328	R.SLANEKFPVNVPK.	721.8 (+2)	1441.7
322-328	K.FPVNVPK.	800.3 (+1), 400.8 (+2)	799.4
333-344	R.RIFMAVSLNIVK.	695.8 (+2)	1389.8
334-344	R.IFMAVSLNIVK.	617.8 (+2)	1233.7
389-395	R.DFPTVQK.	834.0 (+1)	833.4
461-476	K.YNLVDPPELNTINLPR.	934.3 (+2)	1866.9
477-500	R.IGWAAIRFVADNPGVWFLHCHFER.	966.6 (+3)	2897.4
484-500	R.FVADNPGVWFLHCHFER.	1066.2 (+2), 711.3 (+3)	2129.9
501-513	R.HTTEGMATVVIVK.	693.4 (+2)	1384.7
(2) Chymorypsin digestion			
46-54	F.VNVINQGKY.	1034.4 (+1), 517.9 (+2)	1033.5
61-70	W.HGVKQPRNPW.	609.8 (+2)	1217.6

71-88	W.SDGPEYITQCPIKPGTNF.	1012.4 (+2)	2022.9
77-88	Y.ITQCPIKPGTNF.	688.2 (+2)	1374.6
95-102	L.STEEGTLW.	461.2 (+2)	921.4
103-109	W.WHAHSDW.	938.2 (+1)	937.3
146-169	F.KGDVMEVITSSEETGVFPAAADGF.	1229.4 (+2)	2456.1
199-206	L.LRIVNAVL.	449.2 (+2)	896.5
200-211	L.RIVNAVLNEEKF.	716.3 (+2)	1430.7
213-228	F.GIAKHTLTVVAQDASY.	837.3 (+2)	1672.8
220-228	L.TVVAQDASY.	477.2 (+2)	952.4
250-258	F.TTDQTPSHY.	1049.1 (+1)	1048.4
250-259	F.TTDQTPSHYY.	606.7 (+2)	1211.5
266-272	F.HDALDTF.	817.5 (+1)	817.3
288-294	Y.KAPKSPF.	774.2 (+1)	773.4
400-410	F.SLNTAQGTQVL.	566.3 (+2)	1130.5
411-422	L.MIEYGEAVEIVY.	708.4 (+2)	1414.6
415-422	Y.GEAVEIVY.	878.4 (+1)	878.4
(3) Trypsin and chymorypsin digestion			
46-53	F.VNVINQGK.	871.3 (+1), 436.3 (+2)	870.4
54-60	K.YGITIHW.	445.0 (+2)	888.1
68-88	R.NPWSDGPEYITQCPIKGTNF.	1211.1 (+2)	2420.1
103-111	W.WHAHSDWTR.	598.2 (+2)	1194.5
402-422	L.NTAQGTQVLMIEYGEAVEIVY.	582.7 (+4)	2326.7
484-492	R.FVADNPGVW.	502.5 (+2)	1003.4

4.5 Conclusion

Lacquer sap is a water-in-oil emulsion composed mainly of urushiols, polysaccharides, glycoproteins, peroxidase, laccase and stellacyanin. Laccase is a heavily glycosylated protein that containing 15 possible N-glycosylation sites, however, structural studies on the carbohydrate moieties have not been accomplished yet. Protein glycosylation is a common post-translational modification and plays a role in protein biology such as protein folding, interaction, stability and mobility. In this study, we isolated and purified laccase from lacquer acetone powder by continuous Sephadex (C-50 and DEAE A-50) column chromatographies and then the structure of N-glycans by using enzymatic digestions and mass spectrometries was revealed. For quantitative confirmation of N-glycans in lacquer glycoproteins, the released N-glycans with glycopeptidase (PNGase A and F) were labeled with aoWR that is a stable derivatization reagent, and followed by MALDI TOF MS analysis. Both complex- and hybride-type N-linked glycans having common plant-specific structural units of β-xylose, core α-fucose and Lewis epitope, terminal trisaccharides (GlcNAc-Gal-Fuc) were observed in laccase and stellacyanin. For further analysis of N-glycans at each glycosylation site, trypsin and chymotrypsin digested glycoproteins were subjected to LC/MS/MS analysis and the predictable glycopeptides in peptides/glycopeptides mixture were selectively detected by using carbohydrate-specific marker ion (GlcNAc, *m/z*=204).These results suggest that both lacquer stellacyanin and laccase were frequently glycosylated with a biantennary complex type N-linked glycan containing fucose at GlcNAc next to Asn and xylose at the central mannose residues.We also found that 13 N-glycosylation sites in lacquer laccase were glycosylated with a complex type N-glycan, $GlcNAc_4Man_3Gal_2Fuc_3Xyl_1$, in

which 3 sites, Asn5, 233 and 381, were found to be glycosylated with the hybride–type N-glycan, $GlcNAc_{2-3}Man_{4-5}Fuc_1Xyl_1$. The complex type N-linked glycan found in lacquer glycoproteins is the most common N-linked glycan in plants.

N-glycosylation sites at Asn 364 and 519 were not confirmed in glycopeptides analysis,

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Chapter 5

Summary and Conclusion

The aim of this work was to study N-glycosylation of copper-containing glycoproteins, stellacyanin and laccase in lacquer sap from *Rhus vernicifera*. Glycoproteins separated by ion exchange chromatography, such a conventional method according to Reinhammar. To evaluate protein N-glycosylation, enzymatic digestion and chemical labeling of glycans were used prior to MS analysis.

We isolated and purified blue lacquer stellacyanin and laccase from lacquer acetone powder by continuous Sephadex column chromatographies, had a molecular weight of 26kDa and 100kDa, respectively.

By enzymatic digestions and a combination of MALDI TOF MS and LC/MS/MS analyses, we revealed that lacquer stellacyanin is glycosylated with biantennary complex type N-linked glycan at all three Asn residues (Asn28, Asn60, and Asn102). 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 phytocyanin family of plant glycoproteins.

We also elucidated N-glycosylation of lacquer laccase using same characterization strategy with lacquer stellacyanin. Laccase is 100kDa and a heavily glycosylated protein that containing 15 possible N-glycosylation sites. Results releaved that both complex- and hybride-type N-linked glycans having common plant-specific structural units of β-xylose, core α-fucose and Lewis epitope, terminal trisaccharides (GlcNAc-Gal-Fuc) were observed in laccase. We

also found that 13 N-glycosylation sites in lacquer laccase were glycosylated with a complex type N-glycan, $GlcNAc_4Man_3Gal_2Fuc_3Xyl_1$, in which 3 sites, Asn5, 233 and 381, were found to be glycosylated with the hybride–type N-glycan, $GlcNAc_{2-3}Man_{4-5}Fuc_1Xyl_1$.

Occurrence of complex N-linked glycans having plant-specific structural units of β-xylose, core α-fucose and Lewis epitope, terminal trisaccharides (GlcNAc-Gal-Fuc) were also observed in laccase from *Acer pseudoplatanus* L as mentioned in previous chapters. However, functionality of N-glycan on laccase and its enzymatic activity have not been investigated in plant laccase.

As our observation, N-glycosylation sites at Asn 364 and 519 were not confirmed in both glycopeptides analysis of MS/MS analysis and peptide identification by Mascot search, respectively. There is also detailed separation techniques of glycopeptides needed to clearly characterize above N-glycosylation sites. Furthermore, totally 64% of the protein was identified in the study and unidentified peptides, potential O-linked carbohydrate chains are also contemplated.

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Abbreviations Used

ABTS	2, 2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate)
aoWR	N^{α}-((aminooxy)acetyl)tryptophanylarginine methyl ester
API	Armospheric pressure ionization
Asn (N)	Asparagine
CHCA	α-Cyano-4-hydroxycinnamic acid
DHB	2, 5-dihydroxybenzoic acid
DMP	2,6 dimethoxyphenol
DTT	1, 4-dithiothreitol
EMS	Enhanced MS
EPI	Enhanced Product Ion
ER	Endoplasmic Reticulum
ER (in mass analysis)	Enhanced Resolution
ESI	Electrospray Ionization
GalNAc	N-acetyl galactosamine
GluNAc	N-acetyl glucosamine
IAA	Iodoacetamide
IDA	Information Dependent Acquisition
Hex	Hexose (mannose or glucose)
HexNAc	Hexose N-acetylamine (GluNAc or GalNAc)
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography Mass Spectrometry
MALDI	Matrix Assisted Laser Desorption Ionization
MCOs	Multicopper oxydase
M_n	Molecule mass
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PNGaseA	Peptide-N-Glycosidase A

PNGaseF	Peptide-N-Glycosidase F
PTM	Post-translational modification
Ser	Serine
TFA	Trifluoroacetic acid
Thr	Threonine
TIC	Total Ion Chromatography
TOF	Time of Flight
XIC	Extracted Ion Chromatography