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Glycan characterisation and antioxidant activity of a novel N-linked glycoprotein from okra

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Abstract

Glycoproteins are present in all living beings, and have many biological functions. The characterisation of glycan structures of plant glycoproteins has become increasingly important in biotechnology and agricultural applications. In the present work, the antioxidant activities of the okra glycoprotein were assessed. The glycan structures of the okra glycoprotein were analysed using lectin microarray combined with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. The okra glycoprotein showed relatively strong 2,2-diphenyl-1-picrylhydrazyl-scavenging ability and reducing power. In addition, the glycan structures of the okra glycoprotein mainly contained N-acetylglucosamine, mannose, and galactose. Furthermore, complex-type N-glycans were the major type of glycan structures from the okra glycoprotein. Most of the complex N-glycans of the okra glycoprotein had terminal GalNAc and Gal N-glycan structures; the glycoprotein showed a high level of fucosylated complex-type glycans. Therefore, the okra glycoprotein is a promising antioxidant. Results of the present work might serve as a reference for a better understanding of the structural information and bioactivity of okra glycoprotein.

Introduction

Okra (*Abelmoschus esculentus* L. Moench) is a widely consumed vegetable, which belongs to the Malvaceae or mallow family, and is also known as lady's finger or gumbo (Ndunguru and Rajabu, 2004). It is widely cultivated in warm regions such as Asia, Southern Europe, and Africa (Adelakun *et al.,* 2009). In Asia, okra is a nutritious and healthy food with various health benefits including anti-diabetic, anti-oxidative, and anti-hypertensive (Wang *et al.,* 2018). Okra has long been used as a traditional medicine for treating kidney enuresis, gastritis, and diabetes (Gao *et al.,* 2018). It has highly bioactive ingredients especially dietary fibres, vitamins, polysaccharides, and glycoproteins. Tomoda *et al.* (1980) extracted okra mucilage F from deseeded immature okra using several purification procedures. The mucilage F was a glycoprotein with molecular weight close to 1,700,000 Da (Hirose *et al.,* 2004). In addition, several studies showed that the glycosylated compounds of okra were effective bioactive components to inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa (Lengsfeld *et al.,* 2004). However, to date, published information on the okra glycoprotein is limited, and hence the potential function and detailed structure of the okra glycoprotein remain unclear.

Glycoproteins account for approximately 50% of total proteins in nature. Glycosylation has long been recognised as an important field of biological and medical research. Most glycoproteins originating from plants have pharmacological actions such as anti-inflammatory, antiviral, and anti-allergy (Song *et al.,* 2011). Furthermore, natural glycoproteins have strong free radical-scavenging and anti-oxidative abilities (Li *et al.,* 2020). At present, many major diseases such as diabetes, Alzheimer's disease, and aging are related to oxidative damage caused by free radicals (Uddin *et al.,* 2019). Therefore, natural antioxidants have been traditionally administered to treat or prevent various diseases. Some studies showed that N-linked glycans extracted from plant glycoproteins containing α 1,3-fucose and β1,2-xylose had strong antigenicity (Li *et al.,* 2016). In addition, a previous study on the physiological significance of N-glycans in rice Golgi apparatus indicated that glycoproteins with N-glycans were critical in the early development of rice (Fanata *et al.,* 2013). These findings suggested that N-glycans

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might play an important role in the biological activity, function, and growth of plant glycoproteins.

Glycans could alter the carbohydrate structure, and consequently regulate the biological activity of glycoproteins. Therefore, the characterisation of glycoprotein structures is critical for analysing the functional activity of glycoproteins. However, identifying glycoprotein structures has historically been difficult to study due to overexpressed glycosylated proteins and their inherent flexibility (Frenz *et al.,* 2019). In the last decade, lectin microarray has been used as a common tool for detecting the carbohydrate structure of glycoconjugates, which can quickly and sensitively analyse the original samples of unreleased glycans (O'Riordan *et al.,* 2014), thus reflecting the real state of the glycan structures in the sample. However, the result of the lectin microarray is generally partial structural information of the glycan. Therefore, lectin microarray needs to be combined with mass spectrometry to obtain an accurate glycan structure. Notably, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has high mass accuracy, resolution, and sensitivity; and is commonly used as a first step to analyse glycan structures. Also, it can be used directly to identify complex samples with a high tolerance of buffers and salts (Bai *et al.,* 2016).

In the present work, the *in vitro* antioxidant activities of the okra glycoprotein were investigated, including 1,1-diphenyl-2-picrylhydrazyl radical-scavenging ability and reducing power. Subsequently, the glycan structures of the okra glycoprotein were analysed by lectin microarray. Finally, the N-glycans of the glycoprotein released by PNGase A and F were identified by MALDI-TOF-MS, aiming to elucidate the preliminary structure of the glycoprotein. These results provided certain theoretical guidance for further elucidation of the structure-function relationships of okra glycoprotein.

Materials and methods

Chemicals and materials

Okra was purchased from a local market in Jinzhou, Liaoning Province, China. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Pepsin and bovine serum albumin fraction V were purchased from Solarbio Life Sciences (Beijing, China). N-glycosidase F (PNGase F) and N-glycoamidase A (PNGase A) were purchased from Roche CustomBiotech (Penzberg, Germany). All the lectins were purchased from Calbiochem (Merck Biosciences GmbH, Germany). 2,5-Dihydroxy-benzoic acid (DHB) was purchased from Bruker Daltonik GmbH (Germany). All other chemicals and reagents used were of analytical grade.

Extraction of okra glycoprotein

The okra was cut into pieces of 3 - 5 mm thickness, and dried for 6 h at 65°C in a hot air oven. The dried material was pulverised into powder, and 2 g of which was dissolved in 80 mL of buffer (pH 6.4, 0.1 mol/L NaCl solution with 0.2 mol/L potassium phosphate), and then extracted at 40°C for 4 h. The supernatants were collected using centrifugation at 9,000 *g* for 20 min (TG16KR, Sichuan, China), and concentrated. The concentrated solution was further precipitated with 80% ammonium sulphate, and maintained overnight at 4°C. The obtained precipitate was dissolved and dialysed against distilled water at 4°C for 48 h. The dialysed solution was collected and lyophilised to obtain an okra glycoprotein with a molecular weight of more than 3000 U.

In vitro antioxidant activities of okra glycoprotein
DPPH radical-scavenging assay

The DPPH radical-scavenging ability of the okra glycoprotein was analysed by DPPH method with minor modifications (Shu *et al.,* 2019). In brief, the prepared 1.5 mL of DPPH ethanol solution (2×10^{-4} mol/L) was added to 1.5-mL samples of different concentrations (0.25 - 1.25 mg/mL). After that, the mixture was shaken and stored in the dark at room temperature, and the absorbance of the mixture at 517 nm was detected after 30 min. Vitamin C was used as a positive control. Eq. 1 was used to evaluate the DPPH scavenging capacity:

$$\text{Scavenging effect (\%)} = [1 - (A_1 - A_2)/A_0] \times 100\% \quad \text{(Eq. 1)}$$

where, A_0, A_1, and A_2 = absorbance of the DPPH, absorbance of the sample with DPPH, and absorbance of the sample with ethanol, respectively.

Reducing power assay

The reducing ability of the okra glycoprotein was determined by the previous method with slight modifications (Liu *et al.,* 2010). Further, 1.0 mL of different mass concentrations (0.5 - 2.5 mg/mL) of samples were dissolved in 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 0.1% potassium ferricyanide, and stored for 20 min at

50°C. The reaction solution was added to 2.5 mL of 10% TCA to terminate the reaction. Afterwards, it was centrifuged at 5,000 *g* and 4°C for 10 min to obtain the supernatant. Then, 2.5 mL of the supernatant was added to 0.5 mL of 0.1% ferric chloride and 2.5 mL of deionised water, then stored at room temperature. Subsequently, the absorbance at 700 nm of the reaction solution was measured after 10 min. Vitamin C was used as the positive control.

Lectin microarray analysis

The structure of the glycoprotein was detected using lectin microarray by the method previously described by Zhao *et al.* (2018b). The okra glycoprotein was labelled with the fluorescent dye Cy3, and the excess fluorescent reagent was removed from the labelled samples using the Sephadex-G25 column. After that, 5 μg of labelled glycoprotein dissolved in 0.5 mL of incubation buffer (0.5M glycine, 2% BSA, and 0.1% Tween-20 in sodium phosphate buffer (PBST)) was used to block the lectin microarray. The slide was stored for 3 h at 37°C, and rinsed with 1× PBST and phosphate-buffered saline, each for 5 min. The lectin microarray date was determined using an FV 1000 laser scanning confocal microscope at laser power settings of 100%, and a photomultiplier tube of 70%. The obtained images were analysed using Genepix 3.0 software at 532 nm.

Release of N-glycans

N-glycans with PNGase A were released from the okra glycoprotein using the previously described method by Triguero *et al.* (2010). The okra glycoprotein sample (0.5 g) was digested with 0.64 g of pepsin in 150 mL of HCl (pH 2.0) at 37°C for 16 h, and the pH was adjusted to 2.0 every half hour. The solution was boiled for 10 min to deactivate the enzyme, and the reaction mixture was further lyophilised. Following lyophilisation, the sample was deglycosylated with PNGaseA in 0.1 M citric acid buffer (pH 5.0) at 37°C for 48 h. Then, the mixture was heated for 5 min at 100°C to deactivate the enzyme, and then freeze-dried.

The method of releasing N-glycans by PNGase F from the okra glycoprotein has been previously described by Zhao *et al.* (2018a). Briefly, 1.5 mg of the okra glycoprotein was added to 8 M urea and 10 mM dithiothreitol to denature the glycoprotein. After that, the reaction solution was washed with 40 mM NH_4HCO_3, and further digested with 2 μL of PNGase F at 37°C for 12 h. The N-glycans of the okra glycoprotein were collected with Supelclean ENVI-Carb SPE tubes (Sigma-Aldrich, Inc., Bellefonte, PA), and lyophilised. The reaction mixture released from PNGase A was mixed with the reaction mixture released from PNGase F, and detected by MALDI-TOF-MS.

MALDI-TOF-MS analysis

MALDI-TOF-MS analysis (Bruker Daltonics, Bremen, Germany) was performed to determine the detailed N-glycan structures (Zhao *et al.,* 2018a). The lyophilised N-glycans of the okra glycoprotein were added to 5 μL of methanol:water (MW; 1:1, v/v). Then, 1 μL of the sample mixture was deposited on the MTP target plate, and air-dried. The same volume of 20 mg/mL DHB in MW was spotted to recrystallise the glycans. The instrument was externally calibrated using peptide calibration standards. The spectrum was obtained in the positive ion mode with the maximum accelerating voltage of 25 kV. The *m/z* data and the structure of the N-glycans were checked using GlycoWorkbench software mainly designed to assist the annotation of MS/MS data by allowing the user to quickly define a candidate structure and test its fragmentation against a peak list (Tissot *et al.,* 2008).

Results and discussion

In vitro antioxidant activities of okra glycoprotein

DPPH radical-scavenging assay of okra glycoprotein

DPPH is a free radical that produces a violet solution in ethanol. When the DPPH solution encounters a proton-free radical scavenger, the purple colour of the DPPH solution rapidly disappears (Yamaguchi *et al.,* 1998). This property has been commonly used to identify the scavenging ability of antioxidants. Therefore, the antioxidant activity of the okra glycoprotein was analysed using DPPH radical-scavenging ability. This activity and vitamin C changed in a concentration-dependent manner (Figure 1); as the concentration of the okra glycoprotein increased from 0.25 to 1.25 mg/mL, the DPPH-scavenging ability of the okra glycoprotein increased from 25.87 to 71.57%. In addition, the IC_{50} value of the okra glycoprotein was 0.65 mg/mL. The okra glycoprotein showed significant DPPH radical-scavenging ability. It had relatively high antioxidant activity and could be used as a free radical scavenger.

Reducing power assay of okra glycoprotein

Studies showed that reducing power was related to the presence of reductones, which could exert an antioxidant action because they stabilised

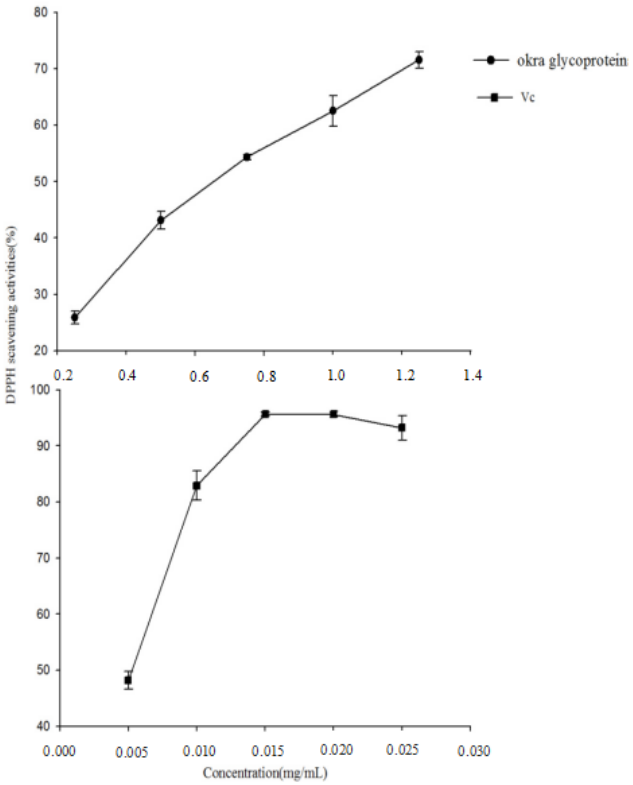


Figure 1. DPPH radical scavenging ability of okra glycoprotein and vitamin C.

and blocked free radical chain reactions by reacting with free radicals (Govindan *et al.,* 2016). Therefore, the reducing power of a compound could be used as an important indicator for evaluating its antioxidant activity. The reducing power of the okra glycoprotein is shown in Figure 2. The absorbance was 0.153, 0.217, 0.277, 0.355, and 0.378 at the concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL, respectively. The result of the reducing power of the okra glycoprotein showed that it had good reducing ability and potential antioxidant activity.

Identification of glycan structures of okra glycoprotein by lectin microarray

Lectins are a family of carbohydrate-recognising proteins which are frequently used as an analytical tool to identify the carbohydrate moieties of intact glycoproteins. In the present work, all fluorescently labelled samples of the okra glycoprotein were profiled on a microarray consisting of 37 lectins.

As shown in Table 1, 11 lectins (Jacalin, ECA, PHA-E, SJA, PNA, AAL, GSL-1, Con A, PTL-II, GNA, and SNA) showed that the positive signals exceeded 100 in the okra glycoprotein. Among these, the fluorescent intensity of PHA-E, Con A, and SNA was 268.80, 201.12, and 210.36, respectively. This suggested that these three types of lectins had significant binding to the okra

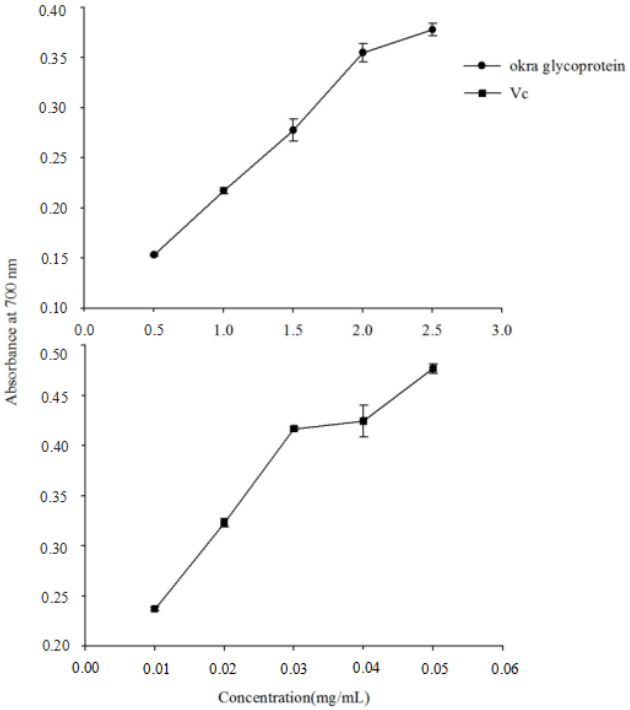


Figure 2. Reducing power of okra glycoprotein and vitamin C.

glycoprotein. PHA-E and SNA bind to GlcNAc, and Con A binds to mannose. This indicated that the glycan structures of the okra glycoprotein contained GlcNAc and mannose. In addition, galactose-identified lectins such as Jacalin, ECA, PNA, and PTL-II had significant fluorescence signals, thus suggesting that the glycan structures of the okra glycoprotein contained a certain amount of galactosylated glycan structures.

Characterisation of N-glycan structure by MALDI-TOF-MS

MALDI-TOF-MS is an advanced analytical tool for high-resolution and rapid characterisation of post-translational modifications of proteins. In general, PNGase A and F are available to release N-glycans. However, PNGase F cannot release N-glycans containing α1,3-linked Fuc from proteins (Karg *et al.,* 2009), but PNGase A can cleave α 1,3-linked Fuc. Further treatment with PNGase A and F could enzymatically release the N-glycans of okra glycoprotein. The N-glycans of the okra glycoprotein were further characterised by MALDI-TOF-MS. The structures of the oligosaccharides in the sample were annotated by the GlycoMod tool based on the *m/z* values of the major peaks in each MS spectrum.

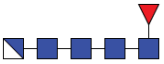
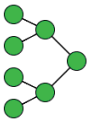
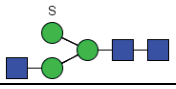
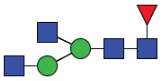
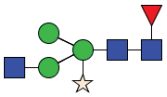
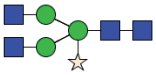
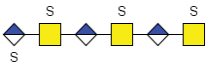
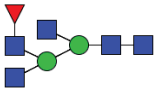
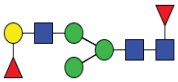
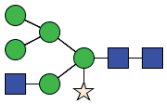
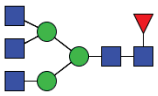
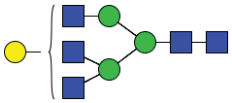
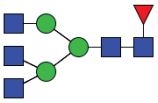
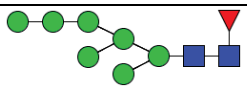
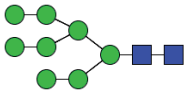
As shown in Table 2 and Figure 3, the N-glycans of the okra glycoprotein showed a high diversity when compared with other plants. A total

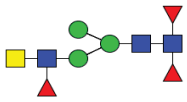
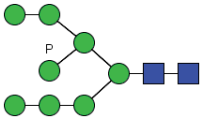
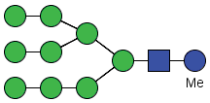
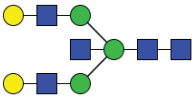
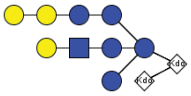
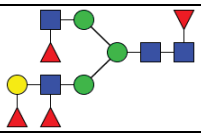
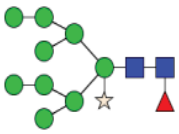
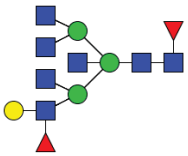
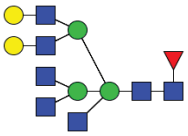
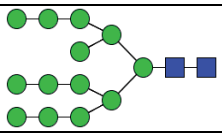
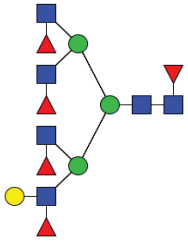
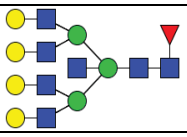
Table 1. The identified glycan structures of okra glycoprotein by lectin microarray.

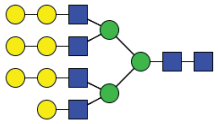
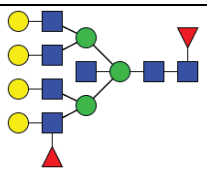
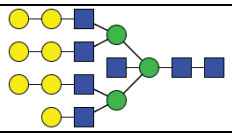
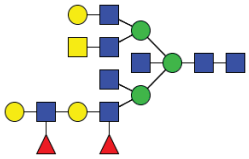
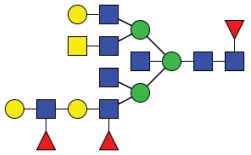
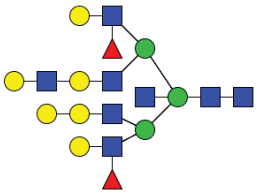
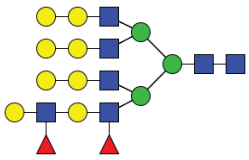
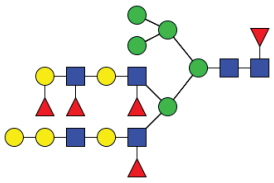
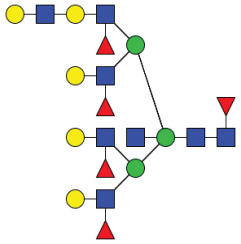
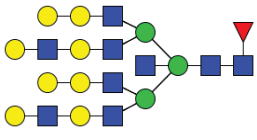
Lectin	Specificity	Fluorescence intensity
Jacalin	Galβ1-3GalNAcα-Ser/Thr(T), GalNAcα-Ser/Thr(Tn), GlcNAcβ1-3-GalNAcα-Ser/Thr(Core3), sialyl-T(ST) not bind to Core2, Core6, and sialyl-Tn (STn)	110.22
Erythrina cristagalli Lectin (ECA)	Galβ-1,4GlcNAc (type II), Galβ1-3GlcNAc (type I)	128.10
Hippeastrum Hybrid Lectin (HHL)	High-Mannose, Manα1-3Man, Manα1-6Man, Man5-GlcNAc2-Asn	/
Wisteria floribunda Lectin (WFA)	Terminating in GalNAcα/β1-3/6Gal	/
Griffonia simplicifolia Lectin II (GSL-II)	GlcNAc and α-or β-linked N-acetylglucosamine residues on the non-reducing terminal of oligosaccharides, agalacto-type, tri- or tetra-antennary N-glycans	/
Maackia amurensis Lectin II (MAL-II)	Siaα2-3Galβ1-4Glc(NAc)/Glc, Siaα2-3Gal, Siaα2-3, Siaα2-3GalNAc	6.11
Phaseolus vulgaris Erythroagglutinin (PHA-E)	Bisecting GlcNAc, biantennary complex-type N-glycan with outer Gal	268.80
Psophocarpus tetragonolobus Lectin I (PTL-I)	GalNAc, GalNAcα-1,3Gal, GalNAcα-1,3Galβ-1,3/4 Glc	/
Sophora japonica Agglutinin (SJA)	Terminal in GalNAc and Gal, anti-A and anti-B human blood group	102.89
Peanut Agglutinin (PNA)	Galβ1-3GalNAcα-Ser/Thr(T)	167.15
Euonymus europaea Lectin (EEL)	Galα1-3(Fucα1-2)Gal (blood group B antigen)	32.02
Aleuria aurantia Lectin (AAL)	Fucα1-6 GlcNAc(core fucose), Fucα1-3(Galβ1-4) GlcNAc	135.24
Lotus tetragonolobus Lectin (LTL)	Fucα1-3Galβ1-4GlcNAc, Fucα1-anti-H blood group specificity	24.01
Maclura pomifera Lectin (MPL)	Galβ1-3GalNAc, GalNAc	/
Lycopersicon esculentum Lectin (LEL)	LacNAc and poly LacNAc	/
Griffonia simplicifolia Lectin I (GSL-I)	αGalNAc, αGal, anti-A and B	131.56
Dolichos biflorus Agglutinin (DBA)	αGalNAc, Tn antigen, GalNAcα1-3((Fucα1-2))Gal (blood group A antigen)	3.61

Lens culinaris Agglutinin (LCA)	α-D-Man, Fucα-1,6GlcNAc, α-D-Glc	/
Ricinus communis Agglutinin I (RCA120)	β-Gal, Galβ-1,4GlcNAc (type II), Galβ1-3GlcNAc (type I)	66.02
Solanum tuberosum Lectin (STL)	Trimers and tetramers of GlcNAc, core (GlcNAc) of N-glycan, oligosaccharide containing GlcNAc and MurNAc	/
Bandeiraea simplicifolia-1 (BS-I)	α-Gal, α-GalNAc, Galα-1,3Gal, Galα-1,6Glc	44.19
Concanavalin A (ConA)	High-Mannose, Manα1-6(Manα1-3)Man, terminal GlcNAc	201.12
Psophocarpus tetragonolobus Lectin II (PTL-II)	Gal, blood group H, T-antigen	111.70
Datura stramonium Agglutinin (DSA)	β-D-GlcNAc, (GlcNAcβ1-4)n, Galβ1-4GlcNAc	4.51
Soybean Agglutinin (SBA)	α- or β-linked terminal GalNAc, (GalNAc)n, GalNAcα1-3Gal, blood-group A	49.90
Vicia villosa Agglutinin (VVA)	Terminal GalNAc, GalNAcα-Ser/Thr(Tn), GalNA cα1-3Gal	/
Narcissus pseudonarcissus Agglutinin (NPA)	High-Mannose, Manα1-6Man	/
Pisum sativum Agglutinin (PSA)	Fucα-1,6GlcNAc, α-D-Man, α-D-Glc	/
Amaranthus caudatus Agglutinin (ACA)	Galβ1-3GalNAcα-Ser/Thr (T antigen), sialyl-T(ST) tissue staining patterns are markedly different than those obtained with either PNA or Jacalin	38.84
Wheat Germ Agglutinin (WGA)	Multivalent Sia and (GlcNAc)n	/
Ulex europaeus Agglutinin I (UEA-I)	Fucα1-2Galβ1-4Glc(NAc)	/
Pokeweed Mitogen (PWM)	Oligomers of β(1,4)-linked N-acetyl-D-glucosamine, N-acetyllactosamine	/
Maackia amurensis Lectin I (MAL-I)	Galβ-1,4GlcNAc	25.42
Galanthus nivalis Agglutinin (GNA)	High-Mannose, Manα1-3Man	157.34
Bauhinia purpurea Lectin (BPL)	Galβ1-3GalNAc, Terminal GalNAc	20.21
Phaseolus vulgaris Agglutinin (PHA-E+L)	Bisecting GlcNAc, bi-antennary N-glycans, tri-and tetra-antennary complex-type N-glycan	79.19
Sambucus nigra Lectin (SNA)	Sia2-6Gal/GalNAc	210.36

Table 2. Summary of the information of the N-glycans released from okra glycoprotein as analysed by MALDI-TOF-MS.

m/z	Proposed structure	Monosaccharide composition	Type of glycan	Ion type
1133.444		$(GlcNAc)_3(GlcA)_3$	Truncated	$[M+Na]^+$
1160.444		$(GlcNAc)_4(GlcA)_3(Fuc)_1$	Truncated	$[M+Na]^+$
1175.370		$(Man)_7$	Truncated	$[M+Na]^+$
1194.371		$(Man)_3(GlcNAc)_3$	Complex	$[M+H]^+$
1301.499		$(Man)_2(GlcNAc)_4(Fuc)_1$	Truncated	$[M+H]^+$
1392.515		$(Man)_3(GlcNAc)_3(Xyl)_1(Fuc)_1$	Complex	$[M+H]^+$
1449.536		$(Man)_3(GlcNAc)_4(Xyl)_1$	Complex	$[M+H]^+$
1476.179		$(GalNAc)_3(GlcA)_3$	Truncated	$[M+H]^+$
1526.560		$(Man)_2(GlcNAc)_5(Fuc)_1$	Truncated	$[M+Na]^+$
1590.565		$(Man)_3(GlcNAc)_3(Fuc)_2(Gal)_1$	Complex	$[M+Na]^+$
1592.544		$(Man)_5(GlcNAc)_3(Xyl)_1$	Hybrid	$[M+Na]^+$
1666.631		$(Man)_3(GlcNAc)_5(Fuc)_1$	Complex	$[M+H]^+$
1682.626		$(Man)_3(GlcNAc)_5(Gal)_1$	Complex	$[M+H]^+$
1688.613		$(Man)_3(GlcNAc)_5(Fuc)_1$	Complex	$[M+Na]^+$
1705.604		$(Man)_7(GlcNAc)_2(Fuc)_1$	Hybrid	$[M+H]^+$
1721.599		$(Man)_8(GlcNAc)_2$	High-mannose	$[M+H]^+$

1735.567		$(Ara)_{12}$	Truncated	$[M+H]^+$
1777.649		$(Man)_3(GlcNAc)_3(GalNAc)_1(Fuc)_3$	Complex	$[M+Na]^+$
1823.547		$(Man)_8(GlcNAc)_2$	High-mannose	$[M+Na]^+$
1856.641		$(Man)_9$ $(GlcNAc)_1$ (Methylated $Glc)_1$	Truncated	$[M+H]^+$
1866.661		$(Man)_3(GlcNAc)_5(Gal)_2$	Complex	$[M+Na]^+$
1980.618		$(GlcNAc)_1(Glc)_5(Gal)_3(KDo)_2$	Truncated	$[M+Na]^+$
2085.760		$(Man)_3(GlcNAc)_4(Fuc)_4(Gal)_1$	Complex	$[M+Na]^+$
2183.734		$(Man)_9(GlcNAc)_2(Xyl)_1(Fuc)_1$	Hybrid	$[M+Na]^+$
2380.901		$(Man)_3(GlcNAc)_7(Fuc)_2(Gal)_1$	Complex	$[M+H]^+$
2396.895		$(Man)_3(GlcNAc)_7(Fuc)_1(Gal)_2$	Complex	$[M+H]^+$
2531.863		$(Man)_{13}(GlcNAc)_2$	High-mannose	$[M+H]^+$
2615.995		$(Man)_3(GlcNAc)_6(Fuc)_5(Gal)_1$	Complex	$[M+H]^+$
2742.983		$(Man)_3(GlcNAc)_7(Fuc)_1(Gal)_4$	Complex	$[M+Na]^+$

2880.004		$(Man)_3(GlcNAc)_6(Gal)_7$	Complex	$[M+Na]^+$
2889.041		$(Man)_3(GlcNAc)_7(Fuc)_2(Gal)_4$	Complex	$[M+Na]^+$
3083.084		$(Man)_3(GlcNAc)_7(Gal)_7$	Complex	$[M+Na]^+$
3133.147		$(Man)_3(GlcNAc)_8(Fuc)_2$ $(Gal)_3(GalNAc)_1$	Complex	$[M+Na]^+$
3279.205		$(Man)_3(GlcNAc)_8(Fuc)_3$ $(Gal)_3(GalNAc)_1$	Complex	$[M+Na]^+$
3394.244		$(Man)_3(GlcNAc)_8(Fuc)_2(Gal)_6$	Complex	$[M+H]^+$
3515.270		$(Man)_3(GlcNAc)_7(Fuc)_2(Gal)_8$	Complex	$[M+H]^+$
3610.294		$(Man)_5(GlcNAc)_6(Fuc)_5(Gal)_5$	Hybrid	$[M+Na]^+$
3696.269		$(Man)_3(GlcNAc)_8(Fuc)_5(Gal)_5$	Complex	$[M+Na]^+$
3775.371		$(Man)_3(GlcNAc)_9(Fuc)_1(Gal)_8$	Complex	$[M+H]^+$

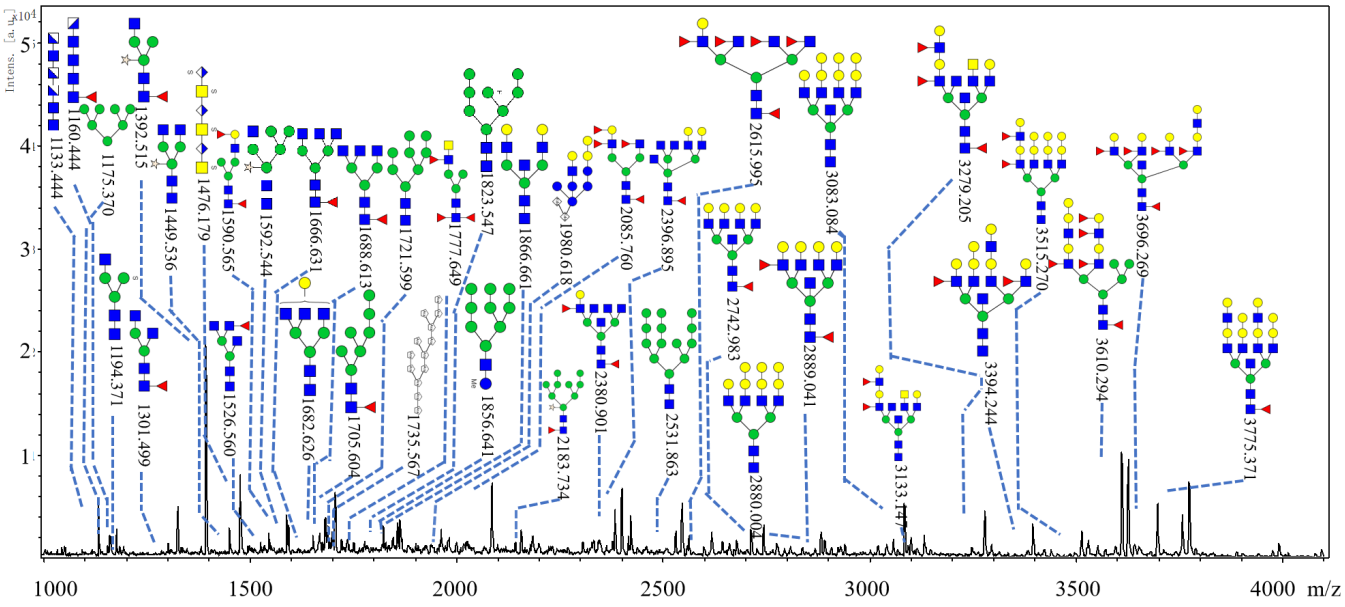


Figure 3. MALDI-TOF-MS spectrum of N-linked glycan from okra glycoprotein.

of 39 N-glycan structures were confirmed from the okra glycoprotein. All MALDI-TOF-MS data were acquired in the mass spectrometer with a positive ion mode analyser. The released N-glycans were predominantly detected as $[M + H]^+$ and $[M + Na]^+$. Furthermore, nine truncated structures were released from the okra glycoprotein, which were the structures at the *m/z* of 1133.444, 1160.444, 1175.370, 1301.499, 1476.179, 1526.560, 1735.567, 1856.641, and 1980.618. The other glycans contained the core structure of N-glycan. A total of 23 complex-type glycans were identified in the okra glycoprotein, which were the structures at the *m/z* of 1194.371, 1392.515, 1777.649, and so on. The complex-type glycan was the major type of N-glycans in the okra glycoprotein. Furthermore, most of the complex N-glycans of the okra glycoprotein had terminal GalNAc and Gal N-glycan structures, which was consistent with the results of the lectin microarray. The most abundant N-glycans in the okra glycoprotein was the complex-type glycan, and it was observed at the *m/z* of 1392.515 ($(Man)_3(GlcNAc)_3(Fuc)1(Xyl)_1$), as shown in Figure 3. This structure was also found in seeds of *Ginkgo biloba* (Wang *et al.,* 2017). Previous studies suggested that N-linked glycans from plants containing α1,3-fucose and β1,2-xylose were associated with food allergies.

Except for the major types of N-glycans, three high-mannose types were identified in the okra glycoprotein with *m/z* of 1721.599, 1823.547, and 2531.863, respectively. Previous studies reported that the $(Man)_8(GlcNAc)_2$ was the most abundant N-glycan in high-mannose-type soybean (Li *et al.,* 2016). Furthermore, four hybrid types were identified in the okra glycoprotein, including the glycan structures at the *m/z* of 1592.544, 1705.604, 2183.734, and 3610.294. The glycan structures were $(Man)_5(GlcNAc)_3(Xyl)_1$, $(Man)_7(GlcNAc)_2(Fuc)_1$, $(Man)_9(GlcNAc)_2$ $(Xyl)_1(Fuc)_1$, and $(Man)_5(GlcNAc)_6$ $(Fuc)_5$ $(Gal)_5$, respectively. More remarkably, the okra glycoprotein had high expression of fucosylated N-glycan structures. PNGase A was effective in releasing the core α1,3-fucose, which could result in the high expression of fucosylated N-glycans in the structures of okra glycoprotein. α 1,3-fucose of rice grain was the major immunogenic resource from plant-specific N-glycans. However, the relationship between okra glycoprotein activity and fucosylated N-glycan structures needs further research and analysis.

Conclusion

The present work analysed the antioxidant activities and glycan structures of okra glycoprotein. The okra glycoprotein showed strong antioxidant activities, including the free radical-scavenging and reducing power, which indicated that the okra glycoprotein should be a potential natural antioxidant. The glycan structures of the okra glycoprotein were analysed by lectin microarray, revealing that GlcNAc, mannose, and galactose were contained in the glycan structures of the okra glycoprotein. According to the MALDI-TOF-MS analysis, the complex N-glycans were the major type of glycan structures from the okra glycoprotein. Furthermore, most of the complex N-glycans of the okra glycoprotein had terminal GalNAc and

Gal N-glycan structures, and the okra glycoprotein had a higher level of fucosylated N-glycan structures. However, the relationship between okra glycoprotein activity and fucosylated N-glycan structures needs further confirmation. The present work revealed the detailed glycan structures and antioxidant activities of the okra glycoprotein which provided new insights for further research on the okra glycoprotein, thus helping to explore its potential applications in food and agriculture.

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