Structural Study of Asparagine-linked Oligosaccharide Moiety of Taste-modifying Protein, Miraculin*

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The structures of the N-linked oligosaccharides of miraculin, which is a taste modifying glycoprotein isolated from miracle fruits, berries of Richadella dulcifica, are reported. Asparagine-linked oligosaccharides were released from the protein by glycopeptidase (almond) digestion. The reducing ends of the oligosaccharide chains thus obtained were aminated with a fluorescent reagent, 2-aminopyridine, and the mixture of pyridylamino derivatives of the oligosaccharides was separated by high performance liquid chromatography (HPLC) on an ODS-silica column. More than five kinds of oligosaccharide fractions were separated by the one chromatographic run. The structure of each oligosaccharide thus isolated was analyzed by a combination of sequential exoglycosidase digestion and another kind of HPLC with an amide-silica column. Furthermore, high resolution proton nuclear magnetic resonance (¹H NMR) measurements were carried out. It was found that 1) five oligosaccharides obtained are a series of compounds with xylose-containing common structural core, Xylα1→2(Manα1→6)Manβ1→4-GlcNAcβ1→4(Fucα1→3)GlcNAc, 2) a variety of oligosaccharide structures are significant for two glycosylation sites, Asn-42 and Asn-186, and 3) two new oligosaccharides, B and D, with unusual structures containing monoantennary complex-type were characterized.

Red berries of Richadella dulcifica, which is a native shrub of tropical West Africa, have a property in modifying sour taste into sweet taste; e.g. lemons elicit a sweet taste after chewing pulps of the berries. Because of this unusual property, the berry has been called “miracle fruit.” Recently the active principle of miracle fruit, which is called miraculin, was completely purified (1), and its amino acid sequence was determined (2). Miraculin was a glycoprotein with 191 amino acid residues, and its molecular weight calculated on the basis of the amino acid sequence and the carbohydrate content (13.9%) was 24,600. The carbohydrate chains were elucidated to link to Asn-42 and Asn-186.

In the present paper, we report the detailed structures of N-linked oligosaccharides of miraculin and demonstrate the distribution of carbohydrate chains into two glycosylation sites. The processing pathway of asparagine-linked oligosaccharides present in plant glycoproteins will be discussed briefly.

EXPERIMENTAL PROCEDURES

Enzymes and Reference Compounds—N-Oligosaccharide glycopeptidase from almond (obtainable as glycopeptidase A), β-galactosidase and β-N-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo Co. α-L-Fucosidase from bovine kidney was purchased from Boehringer Mannheim. Pepsin was purchased from Sigma, lysyl endopeptidase (Achromobacter lyticus protease I) from

[Diagram of oligosaccharide structures]

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The structures of oligosaccharides A–E were determined and are summarized in Table II.

Wako Pure Chemical Industries. A series of reference compounds of xylose-containing biantennary-complex types that share a common structural unit, Xyl\_\_1→3\_\_2\_\_Man\_\_1→6\_\_2\_\_Man\_\_1→4\_\_2\_\_GlcNAc\_\_1→4\_\_2\_\_Fuc\_\_1→3\_\_2\_\_GlcNAc\_\_1→3\_\_2\_\_Glucose units (obtainable as PA-glucose oligomers, Takara Shuzo Co.).

Preparation of Miraculin Protein—Miraculin was purified from miracle fruits (R. dulcifica) as described previously (1).

The abbreviations used are: Xyl, d-xylose; PA, pyridylamino; Fuc, L-fucose; HPLC, high performance liquid chromatography; LEP, lysyl endopeptidase.

Preparation of Oligosaccharides from Miraculin and Two Glycopeptides—Oligosaccharides were released from 10 mg of miraculin protein by sequential digestion with pepsin and N-oligosaccharide glycopeptidase (almond) (5). Lysyl endopeptidase-digested glycopeptides were directly treated with the glycopeptidase. After the oligosaccharide fraction was collected by gel filtration on a Bio-Gel P-4 column, it was reductively aminated with 2-aminopyridine by the use of sodium cyanoborohydride (4). The pyridylamino derivatives of oligosaccharides were purified by gel filtration on a Sephadex G-15 column and analyzed on the following HPLC procedure.

Isolation and Identification of PA-Oligosaccharides by HPLC (Two-dimensional Sugar Mapping Technique)—First, sample oligosaccharide derivative was separated by HPLC on a reverse-phase, Shim-pack CLC-ODS column (5 mm) (Shimadzu). Elution was performed at a flow rate of 1.0 ml/min at 55 °C using two solvents, A and B. Solvent A was 10 mM sodium phosphate buffer, pH 3.8, and solvent B was 10 mM sodium phosphate buffer, pH 3.8, containing 0.5% 1-butanol. After injection of a sample, the ratio of solvent B to A was increased with a linear gradient from 20:80 to 50:50 in 60 min. Each oligosaccharide fraction that separated as a peak was collected and evaporated to dryness. Second, the residue was dissolved in a solvent C and injected on the second column. The second was size fractionation HPLC with a TSK-GEL Amide-80 (4.6 × 250 mm, TOSOH). Elution was performed at a flow rate of 1.0 ml/min at 40 °C using two solvents, C and D. Solvent C was composed of 3% acetic acid in water with triethylamine, pH 7.3, and acetonitrile, 35:65 by volume. Solvent D was composed of 3% acetic acid in water with triethylamine, pH 7.3, and acetonitrile, 50:50 by volume. After injection of a sample, the ratio of solvent D to C was increased with a linear gradient from 0 to 100% in 50 min. In this HPLC systems, PA-oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively. Third, the elution position of the sample obtained by above two columns was plotted as coordinates (expressed by glucose unit numbers) on the "sugar map" whose data base is prepared by the use of 113 kinds of standard oligosaccharides (6).

\(^{1}\) H NMR Measurements—Prior to NMR measurements, PA-oligosaccharide (10–100 µg each as neutral sugar) isolated by HPLC was desalted by gel filtration on a Sephadex G-15 column. Samples were dissolved in 99.8% D\(_2\)O, lyophilized, and dissolved again in 99.8% D\(_2\)O at concentration of 30–300 µM. NMR measurements were made...
on a Bruker AM-400 spectrometer operating at 400 MHz in the Fourier transform mode. Measurements were made at 30 °C. Typically, 2000 transients were accumulated for each measurement. Chemical shifts are expressed in parts/million (ppm) from internal acetone. The chemical shift of acetone was determined to be 2.216 ppm in D$_2$O at 30 °C. Chemical shifts are expressed in parts/million (ppm) from internal DSS but were actually measured with internal acetone (δ = 2.216 ppm in D$_2$O at 30 °C). The symbols used are ●, GlcNAc; ■, Gal; •, Man; □, Fuc; □, Xyl.

RESULTS AND DISCUSSION

Carbohydrate Composition—The complete amino-acid sequence analysis of miraculin has shown that all of the carbohydrate of miraculin is associated with Asn-42 and Asn-186 and these sites are fully glycosylated (2). The carbohydrate composition of miraculin (500 µg) was determined after hydrolysis with trifluoroacetic acid. The moles/mole protein of 1 xylose residue/3 mannose residues. This result suggests that the major parts of the carbohydrate chains of the miraculin are xylose-containing N-acetyllactosamine-type structure which are specific to plant protein. Moreover, no N-acetylgalactosamine residue was detected, indicating that usual O-linked oligosaccharides are not present in the protein.

Isolation of Two Glycopeptides Containing Asn-42 and Asn-186—We prepared two kinds of miraculin glycopeptides, each of which contained glycosylation site Asn-42 or Asn-186, by digestion with lysyl endopeptidase as described in the previous paper (2). It was confirmed that each glycopeptide is highly pure and does not contain other glycopeptides (2).
Table II

Proposed structures of N-linked oligosaccharides obtained from miraculin

|    | A          | B          | C          | D          | E          |
|----|------------|------------|------------|------------|------------|
|    | Manα1       | Manβ1      | Manα1       | Fucα1      | Fucα1      |
|    | →Manβ1 →GlcNAcβ1 →GlcNAc | →Manβ1 →GlcNAcβ1 →GlcNAc | →Manβ1 →GlcNAcβ1 →GlcNAc | →Fucα1      | →Fucα1      |
|    |           |            | Xylβ1       |            |            |
| %  | 58.0       | 11.4       | 18.8        | 4.1        | 7.2        |
| %  | 37.6       | 4.9        | 33.3        | 7.2        | 1.5        |
| %  | 80.5       | 6.7        | 6.7         | 1.5        | 1.5        |

Hydrates of the original protein remained undigested by this procedure. The oligosaccharide fraction of each of two kinds of glycopeptides containing Asn-42 and Asn-186, respectively, was also separately released by the glycopeptidase digestion.

Fractionation of Oligosaccharides and Comparison of Distribution of Each Oligosaccharide between Two Glycosylation Sites—On reverse-phase HPLC (Fig. 1), isocratic elution was performed in this case with 10 mM sodium phosphate buffer, pH 3.8, instead of gradient elution. Pyridylamino derivative of each oligosaccharide prepared from whole protein and two glycopeptides containing Asn-42 and Asn-186, respectively, was separated into more than five peaks. Oligosaccharides A–E shown on Fig. 1 were further examined for the homogeneity by use of an amide absorption Amide-80 column (Fig. 2). The elution positions of oligosaccharides on the Amide-80 column reflect primarily the molecular sizes of oligosaccharides. It was revealed that oligosaccharides A–E were all homogeneous not only on an ODS-silica column but also in terms of molecular size. Fig. 1, moreover, clearly shows that a significant degree of variety exist between two glycosylation sites, Asn-42 and Asn-186. Although several minor peaks are present in addition to the lettered components on Fig. 1, they were obtained in an amount too small for further structural analyses.

1H NMR Analyses of Oligosaccharides A–E—Chemical shift values for the anomeric protons and the methyl protons of oligosaccharides A–E along with four reference compounds are compiled in Table I. Compounds a, c, and f from lactase, and that from bromelain are xylose-containing N-acetyllactosamine-type oligosaccharides of plant origin glycoprotein (3). 1H NMR spectral data for the H-1 and methyl groups clearly indicate that the chemical shifts of oligosaccharides C, A, and E are all in good agreement with those obtained from bromelain, lactase a, and lactase f, respectively. The structures of oligosaccharides B and D, however, have not yet been proved to exist. Comparisons of the chemical shift data...
N-Linked Oligosaccharides of Miraculin

for B and D with those of reference compounds, laccase c and laccase f clearly indicate that the substitutions of outer N-acetylgalactosamine residue by galactose residue through β1→4 linkage and fucose residue through α1→6 linkage cause the anomeric proton signals to shift the downfield, from 4.511 (laccase c) to 4.533 ppm (laccase f) for GlcNAc-5, from 4.546 (laccase c) to 4.576 ppm (laccase f) for GlcNAc-5'. Oligosaccharides B and D each have 1 outer GlcNAc residue linking to galactose and Fuc-2 residues. The H-1 resonance observed at 4.535 in oligosaccharide B is characteristic for GlcNAc-5'. The chemical shift of an anomeric proton at 4.576 ppm in oligosaccharide D is characteristic for GlcNAc-5'. These results indicate that in oligosaccharides B and D, the outer GlcNAc residue links to Man-4 and Man-4', respectively.

The chemical shift of the H-1 proton of Man-4 of oligosaccharide D is identical with that for oligosaccharide A. By contrast, chemical shifts observed for Man-4 of oligosaccharides B and E are identical but are significantly different from those observed for oligosaccharides A and D. These results indicate that 1) in oligosaccharides A and D, Man-4 is at a non-reducing terminal and 2) an N-acetylgalactosamine residue is bonded to Man-4 in oligosaccharides B and E. This situation is quite similar for the chemical shift of H-1 proton of xylose residue, that is, in oligosaccharides A and D, the chemical shifts of H-1 proton of xylose residue are 4.439–4.437 ppm, and in B and E, are 4.429–4.426 ppm. The chemical shift of the H-1 proton of Man-4' of oligosaccharide B is essentially the same as that of oligosaccharide A. This means that oligosaccharides A and B possess Man-4' at a non-reducing terminal. Man-4' observed for oligosaccharide D and E give identical chemical shifts, which are identical but significantly different from those for A and B. This indicates that an N-acetylgalactosamine residue is bonded to Man-4' in oligosaccharides D and E. All of these results are quite consistent with the structure of oligosaccharides A–E given in Table II.

Characterization of Oligosaccharides A–E Using Two-dimensional Mapping Technique—Each oligosaccharide which was clearly separated on ODS-silica column (Fig. 1) was collected separately. They were subjected to a size fractionation on an amide-silica column. The values of elution positions (expressed as glucose unit numbers) of oligosaccharides on ODS- and amide-silica columns were plotted on the two-dimensional sugar map prepared using 116 different oligosaccharide standards (6) (Fig. 3). The elution positions of oligosaccharides A, C, and E derived from miraculin coincide within the experimental error with those of reference compounds laccase a, bromelain, and laccase f, respectively.

Oligosaccharides B and D, however, do not correspond to any oligosaccharide on the present map, indicating that they are new or unusual oligosaccharides. Oligosaccharides A–E and reference compounds prepared from sycamore cell laccase and stem bromelain were always processed at the same time and in the same manner and their HPLC profiles were compared. Jack bean β-galactosidase cannot release galactose residue from fucose under usual conditions (5 milliunits of enzyme/500 pmol of substrate) because of the existence of fucose α1→6 residue attached to the neighbor GlcNAc residue. Furthermore, any commercial α-L-fucosidase such as from bovine kidney, Fusarium oxysporum, Corynebacterium, and from Charonia lampas cannot release the fucose α1→3 residue attached to the reducing end GlcNAc residue in oligosaccharides of plant origin (under usual conditions (20 milliunits of enzyme/500 pmol of substrate). It was observed that on incubation with α-L-fucosidase (bovine kidney), each 1 mol of fucose residue is released from oligosaccharides B and D, and 2 mol are released from oligosaccharide E. After sequential digestion of α-L-fucosidase (bovine kidney) and β-galactosidase (jack bean), the elution positions of oligosaccharides B, D, and E were shifted on the map to the positions identical with those of the reference compounds laccase b-2, laccase b-1, and laccase C, respectively (Fig. 3). These results suggest that each 1 mol of fucose and galactose residue is released from both oligosaccharides B and D, and 2 mol of both fucose and galactose residues are released from oligosaccharide E.

As shown in Fig. 3, after following β-hexosaminidase (jack bean) digestion, oligosaccharides B, D, and E were all converted finally to the common structure, which is the same as laccase a, Fuc-3Galβ1→4GlcNAcβ1→4GlcNAcβ1→3Man
c

The structures suggested on the basis of the mapping technique for oligosaccharides A–E are consistent with the results obtained by 1H NMR analyses.

Five kinds of proposed structures of oligosaccharides obtained from miraculin are summarized in Table II. Table II also demonstrates the distribution of carbohydrate chains between different N-glycosylation sites, Asn-42 and Asn-186. The percentage of each component oligosaccharide (A–E) was calculated by its peak area on an HPLC profile since the amounts of oligosaccharides A and C are comparable. In the case of Asn 186-linked sugar chains, oligosaccharide A is predominant, while at position Asn 42 between different N-glycosylation sites, Asn-42 and Asn-186.

About 10 kinds of glycoproteins with xylose-containing oligosaccharides have previously been reported, i.e. bromelain (8), laccase from sycamore cells (3), protease inhibitor from barbados pride seeds (9), etc. α-Hemocyanine from helix pomatia (10) is the only protein of animal origin which contains xylose-containing oligosaccharides. In laccase as xylose containing biantennary oligosaccharides a Galβ1→
4GlcNAc structure was found for the first time (3). Since the xylose residue is specific in plant glycoprotein, the mechanism of biosyntheses of Xyl@1→2 and Fuc@1→3 residues are of great interest. Johnson and Chrispeels (11) and Kimura et al. (12) proposed independently different biosynthetic pathways for plant oligosaccharides. However, oligosaccharides B–E characterized in the present study cannot consistently be placed on either of the pathways. Unless more detailed information about substrate specificities of glycosyl transferases is available, it is virtually impossible to align these oligosaccharides in the order of biosynthesis.

The function of the carbohydrate moiety of miraculin has not yet been elucidated. The functional role of the oligosaccharide moiety of the glycoprotein should be studied by using deglycosylated miraculin molecule. The taste modifying function of miraculin is completely damaged even in the mild condition such as a 7-h incubation at 37 °C in a phosphate buffer, pH 6.8 (data not shown). The release of oligosaccharide moiety by enzyme digestion, therefore, is difficult without loss of the miraculin activity. Sasaki et al. (13) reported that in erythropoietin, the peptide structures surrounding the glycosylation sites are important factors influencing the kind of carbohydrate chains formed. In miraculin molecule, threonine and proline residues are predominant and no charged groups are in the vicinity of Asn-42. By contrast, a number of phenylalanine residues and also amino acid residues with acidic and basic charged groups surround the Asn-186 position. It is difficult to draw any definite conclusion from the observed data about the relationship between the peptide structure and carbohydrate biosyntheses.

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