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Structure of Ten Free N-Glycans in Ripening Tomato Fruit[1]

Arabinose Is a Constituent of a Plant N-Glycan

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The concentration-dependent stimulatory and inhibitory effect of N-glycans on tomato (*Lycopersicon esculentum* Mill.) fruit ripening was recently reported (B. Priem and K.C. Gross [1992] Plant Physiol 98: 399-401). We report here the structure of 10 free N-glycans in mature green tomatoes. N-Glycans were purified from fruit pericarp by ethanolic extraction, desalting, concanavalin A-Sepharose chromatography, and amine-bonded silica high performance liquid chromatography. N-Glycan structures were determined using 500 MHz ^{1}H-nuclear magnetic resonance spectroscopy, fast atom bombardment mass spectrometry, and glycosyl linkage methylation analysis by gas chromatography-mass spectrometry. A novel arabinosyl-containing N-glycan, Manα1→6(Ara*f*α1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc, was purified from a retarded concanavalin A fraction. The location of the arabinosyl residue was the same as the xylosyl residue in complex N-glycans. GlcNAc[5']Man$_3$(Xyl)GlcNAc(Fuc)GlcNAc and GlcNAc[5']Man$_2$GlcNAc(Fuc)GlcNAc were also purified from the weakly retained fraction. The oligomannosyl N-glycans Man$_5$GlcNAc, Man$_6$GlcNAc, Man$_7$GlcNAc, and Man$_8$GlcNAc were purified from a strongly retained concanavalin A fraction. The finding of free Man$_5$GlcNAc in situ was important physiologically because previously we had described it as a promoter of tomato ripening when added exogenously. Mature green pericarp tissue contained more than 1 μg of total free N-glycan/g fresh weight. Changes in N-glycan composition were determined during ripening by comparing glycosyl and glycosyl-linkage composition of oligosaccharidic extracts from fruit at different developmental stages. N-Glycans were present in pericarp tissue at all stages of development. However, the amount increased during ripening, as did the relative amount of xylosyl-containing N-glycans.

Plant *N*-glycans, an integral part of *N*-glycoproteins, are classified into two types based on the presence (complex type) or absence (oligomannosidic type) of a β1→2-linked xylosyl in addition to mannosyl and *N*-acetylglucosaminyl residues. Research on *N*-glycoproteins has included structural analyses, biosynthesis, importance in protein conformation and activity, and the role of *N*-glycans in glycoprotein secretion (Chrispeels, 1991). Recently, free plant *N*-glycans were shown to exhibit biological activity. For example, (a) the xylomannoside Man$_3$(Xyl)GlcNAc(Fuc)GlcNAc was described as a growth factor acting at nanomolar range during early development of *Linum usitatissium* (Priem et al., 1990a); (b) the *N*-glycans Man$_3$(Xyl)GlcNAc(Fuc)GlcNAc and Man$_5$GlcNAc were shown to stimulate tomato (*Lycopersicon esculentum*) fruit ripening (Priem and Gross, 1992); and (c) *N*-glycans prepared from a yeast elicitor extract were found to be suppressors of corresponding glycopeptide elicitors of stress responses in tomato cells (Basse and Boller, 1992). Thus, *N*-glycans might best be considered a new class of oligosaccharins, i.e. plant metabolism effector oligosaccharides (Albersheim et al., 1983; Ryan and Farmer, 1991). In humans, free *N*-glycans are associated with enzyme deficiencies, resulting in physiological disorders (Ockerman, 1969; Nordén et al., 1973).

In addition to the biological activity of *N*-glycans in tomatoes, other reports suggest an importance of *N*-glycoconjugates in tomato fruit metabolism. Blocking *N*-glycosylation with tunicamycin delayed fruit ripening, which suggests that *N*-glycoproteins may be important in the ripening process (Handa et al., 1985). In an attempt to determine the potential physiological significance of our previous results concerning the stimulation of ripening by *N*-glycans (Priem and Gross, 1992), we investigated the occurrence of free *N*-glycans in tomato pericarp. Previously, the *N*-glycans Man$_3$(Xyl)GlcNAc-(Fuc)GlcNAc and Man$_5$GlcNAc were found in the extracellular medium of a *Silene alba* cell-suspension culture (Priem

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Abbreviations: α-MeGlc, α-D-methylglucopyranoside; B-GP, bromelain glycopeptide; EIMS, electron impact mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; GlcNAc[5']Man$_2$(Xyl)GlcNAc(Fuc)GlcNAc, GlcNAcβ1→2Manα1→6Manβ1→4GlcNAcβ→4(Fucα1→3)GlcNAc; GlcNAc[5']Man$_3$(Xyl)GlcNAc(Fuc)GlcNAc, GlcNAcβ1→2Manα1→6(Manα1→3)(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc; Man$_5$GlcNAc, Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAc; Man$_2$(Xyl)GlcNAc(Fuc)GlcNAc, Manα1→6(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc; Man$_3$(Xyl)GlcNAc(Fuc)GlcNAc, Manα1→6(Manα1→3)(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc; Sj-GP, *Sophora japonica* glycopeptide; t, terminal.

et al., 1990b); however, no role as metabolic effectors of the cell suspensions was evident.

Although *N*-glycoproteins are found in most plant tissues, including tomato fruit (Hobson, 1974; Moshrefi and Luh, 1983), *N*-glycans have not been reported to occur in a free form in situ, i.e. unconjugated to *N*-glycoproteins. In this paper, we report the presence and structure of 10 free *N*-glycans in mature green tomato fruit and, consequently, the first evidence for a potential role of plant *N*-glycans. In addition, we report a new *N*-glycan structure characterized by the presence of an arabinosyl residue. A simple technique of purification based on Con A affinity chromatography was developed to fractionate different types of *N*-glycans that will be useful in future investigations.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* Mill., cv Rutgers) plants were grown in a greenhouse using standard practices without supplemental lighting. Flowers were tagged at anthesis and hand pollinated. To encourage uniform development, only one or two fruit were allowed to develop per cluster. Stages of ripeness were determined by number of days after pollination, by surface color, and by condition of the locule tissue as previously described (Kim et al., 1991).

Chemicals

Con A-Sepharose type III-A, *Sophora japonica* lectin, pineapple bromelain, and Sephadex G-15 were from Sigma Chemical Co.; Pronase E was from Merck, Inc.; Dowex 1-X8 was from Bio-Rad; $Man_5GlcNAc$ was from BioCarb (Lund, Sweden); TSK-HW40S was from Tosohaas Co. (Philadelphia, PA); $Man_8GlcNAc$ (a mixture of three isomers from human urine) was generously provided by Dr. Gerard Strecker (University of Lille, France); and Man_3(Xyl)GlcNAc(Fuc)GlcNAc was generously provided by Dr. Henri Morvan (University of Limoges, France).

Preparation of Tomato Fruit Oligosaccharidic Extracts

Five hundred grams of fresh pericarp tissue were excised from mature green tomato fruit. The tissue was lyophilized, placed in 1 L of boiling 80% (v/v) ethanol, and stirred for 15 min. The sample was then homogenized using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) and filtered through sintered glass. Ethanol was removed and the sample was concentrated to 15 mL by evaporation in vacuo at 40°C. After filtering the sample through a glass fiber filter, 5 mL was desalted on a column of Sephadex G-15 (65 × 2.5 cm) in distilled water. The desalted G-15 fraction was loaded on a Dowex 1-X8 column (1 × 5 cm) equilibrated in 2 mM pyridinium acetate (pH 5.0), and the column was washed with 10 mL of buffer, thereby purifying the nonretained fraction from acidic oligosaccharides and other compounds that bound to the resin. After lyophilization, this fraction was dissolved in a minimal volume of water and it was subjected to a second desalting using fast protein liquid chromatography on TSK-HW40S (2 × 30 cm Waters glass column, Series AP) equilibrated in 0.1% (v/v) acetic acid at a flow rate of 1 mL/min. Carbohydrate was monitored using a refractometer. A broad peak of oligosaccharides, which eluted between 30 and 52 min, was collected and lyophilized.

Preparation of Glycopeptides

Native *S. japonica* lectin (15 mg) and denaturated pineapple bromelain (60 mg) were digested with 2% pronase E (w/v) in 10 mL of 10 mM Ca-acetate (pH 8.0) at 40°C. Additional pronase E was added at the same concentration to the mixture at 12, 24, and 40 h. After 48 h, the sample was adjusted to pH 5.0 with acetic acid, boiled for 5 min, and centrifuged. The supernatant was concentrated in vacuo at 40°C and desalted on TSK-HW40S.

Affinity Chromatography on Con A-Sepharose

N-Glycans were fractionated on an immobilized Con A-Sepharose column using a method similar to that of Montreuil et al. (1986). The column (1 × 10 cm) was equilibrated in 5 mM sodium acetate (pH 5.2) containing 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0.02% (v/v) sodium azide, at a flow rate of 13.5 mL/h at 22 to 24°C. The desalted oligosaccharidic fraction was dissolved in 1 mL of buffer and applied to the column. The column was then sequentially eluted with 52.5 mL each of 0, 10, and 300 mM α-D-methylglucoside; 7.5-mL fractions were collected. Fractions were concentrated to 2 mL by evaporation in vacuo at 40°C and desalted by fast protein liquid chromatography on TSK-HW40S as described above.

FAB-MS

FAB-MS spectra were obtained by Liquid Matrix Secondary Ion Mass Spectrometry on a VG Instrument ZAB2SE high-mass, high-resolution, double-focusing mass spectrometer with a VG 11-250 data system. Per-*O*-methylated oligoglycosylalditols (1–5 μg) were dissolved in 10 μL of methanol; 2 μL were used for analysis. Metanitrobenzyl alcohol was used as the liquid matrix. Ionization was performed with a cesium ion gun (37 kV, 1–4 μA). Spectra were recorded under the following conditions: mass range, 500 to 2400 D; accelerating voltage, 9 kV; scanning mode, exponential down; scanning speed, 20 s/decade; resolution, 2500:1; mass calibration, cesium iodide.

HPLC

N-Glycans were purified by HPLC on an amine-bonded silica column (5 μm AS-5A; Brownlee Labs, Santa Clara, CA; 25 × 0.4 cm) equilibrated in 65% (v/v) acetonitrile. UV detection at 200 nm was performed during a 1-h isocratic run. Acetonitrile was evaporated with a stream of N_2 at 40°C, and the resulting fractions were lyophilized.

NMR Spectroscopy

Samples (80–150 μg) were exchanged in D_2O three times and dissolved in 300 μL of high-purity D_2O under N_2 and sealed in NMR tubes. Spectra were recorded on a GE GN-

500 (500.11 MHz ^{1}H) and a GE Omega-600 (599.71 MHz ^{1}H) spectrometer at 25 and 60°C. ^{1}H chemical shifts were determined relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate with acetone as standard (2.225 ppm downfield). DQF-COSY experiments were recorded at 600 and 500 MHz with standard pulse sequence (Rance et al., 1983).

Glycosyl and Glycosyl-Linkage Composition by GC-EIMS

Total sugar content was measured colorimetrically using the phenol sulfuric assay (Dubois, 1956). Glycosyl composition was determined by hydrolyzing *N*-glycans in 2 N TFA for 1 h at 120°C. Samples were then reduced and acetylated using a procedure similar to that of Blakeney et al. (1983). Alditol acetates were separated on a 50-m 5% (w/w) phenylmethylsilicone capillary column (0.2-mm i.d.) and detected using flame ionization.

For glycosyl-linkage composition analyses, samples were reduced with $NaBH_4$, desalted on TSK-HW40S, and lyophilized prior to TFA hydrolysis. In both cases, $NaBD_4$ was used as the second reducing agent. Partially per-*O*-methylated, per-*O*-acetylated alditol acetates were prepared as previously described (Tong and Gross, 1990), separated by GC as described above, and quantified by EIMS (Hewlett-Packard 5970A).

The percentage of reducing t-*N*-acetylglucosamine was calculated from the relative abundance ratios of m/e 84:85 and 144:145. Methylation analysis of reduced oligosaccharides was carried out using NaOH as the base agent (Ciucanu and Kerek, 1984). Per-*O*-methylated oligosaccharides were extracted in chloroform. After several washes of the chloroform phase with distilled water, the fraction was taken to dryness with a stream of N_2 at 40°C and hydrolyzed with 4 N TFA for 1 h at 120°C. Individual partially methylated alditol acetates were identified and quantified by GC-EIMS and GC-flame ionization detection as described above.

RESULTS

Tomato Fruit Soluble Carbohydrate Content

Total sugar content of the 80% (v/v) ethanol extract obtained from 500 g fresh weight of mature green fruit was 10.9 g of Glc equivalents. After desalting on Sephadex G-15, the recovered oligosaccharidic extract contained 15.6 mg of carbohydrate, i.e. about 0.1% of the total. *N*-Acetylglycosaminyl and mannosyl residues, typical of *N*-glycans, represented 27% of the total neutral sugar (data not shown), suggesting the presence of *N*-glycans or *N*-glycopeptides in the extract. Anion-exchange chromatography on Dowex 1-X8 resulted in the elimination of rhamnosyl-containing polymers, i.e. pectic polymers. Further desalting on TSK-HW40S resulted in elimination of glucosyl and galactosyl residues. The recovery of mannosyl and *N*-acetylglucosaminyl residues was 75%, compared with a total yield of 54% (data not shown).

Fractionation on Con A-Sepharose

Con A is a plant lectin that exhibits specificity for α-anomers of Man (and Glc), Gal, and L-Fuc, in descending order. However, its greatest affinity is for Manα3(Manα6)-Manβ-containing *N*-glycans. Two complex-type glycopeptides were prepared for column calibration: Man_2(Xyl)GlcNAc(Fuc)GlcNAc and Man_3(Xyl)GlcNAc(Fuc)GlcNAc, from pineapple bromelain (Ishihara et al., 1979) and *S. japonica* lectin (Fournet et al., 1987) pronase E digestions, respectively. The percent recovery after TSK-HW40S was 7.9% for Sj-GP and 1% for B-GP (data not shown). Glycopeptides, as well as Man_5GlcNAc, were applied to the Con A column and eluted with increasing concentrations of α-MeGlc. B-GP eluted first, followed by Sj-GP; neither glycopeptide bound to the column. However, Man_5GlcNAc did bind and was eluted with 300 mM α-MeGlc.

The desalted extract (6.4 mg of carbohydrate) was fractionated on Con A-Sepharose (Fig. 1a; tubes 1–21b). Most oligosaccharides eluted in tubes 3a and 4a (non retained, NR). Nevertheless, the two concentrations of α-MeGlc successively released a weakly retained (WR) fraction (10 mM; tubes 10a and 11a) and a strongly retained (SR) fraction (300 mM; tubes 17a and 18a). After desalting, these fractions had a composition characteristic of complex and oligomannoside type *N*-glycans, respectively, and represented about 10% of the initial sample (data not shown). These results, supported by the high specifity of Con A, demonstrated the presence of *N*-glycans and/or *N*-glycopeptides in the tomato extract.

A second fractionation was performed to assure that a portion of the nonretained fraction was not a result of overloading the binding capacity of the column. Tubes with high amounts of *N*-acetylglucosamine (4a and 5a) were refractionated (Fig. 1b, tubes 1–21b). A small amount of material was

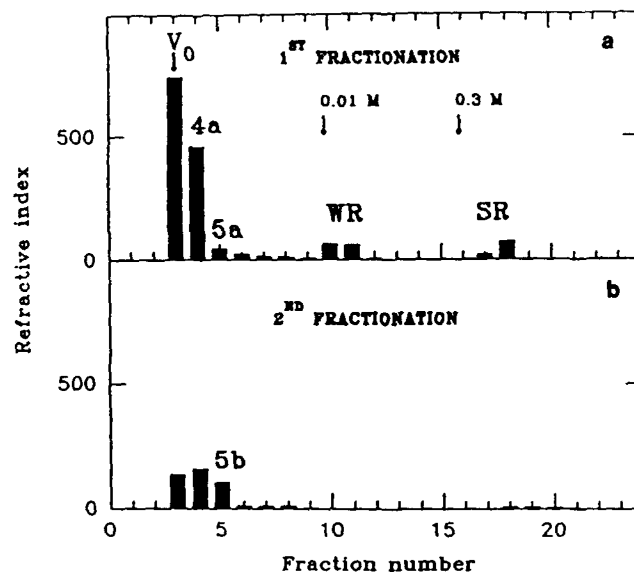


Figure 1. Con A chromatography of the neutral oligosaccharidic extract of mature green tomato fruit pericarp. After desalting on TSK-HW40S, the extract was fractionated on Con A-Sepharose (a). Arrows indicate the void volume (V_o) and the beginning of successive elutions with 0.01 and 0.3 M α-MeGlc, which released fractions "WR" (weakly retained) and "SR" (strongly retained). The nonretained fractions 4a and 5a, rich in *N*-acetylglucosamine, were pooled and subjected to a second fractionation (b).

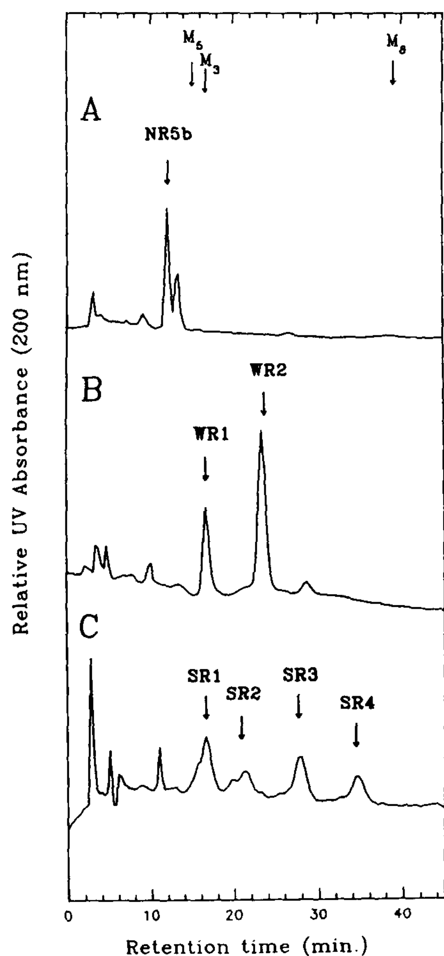


Figure 2. Amine-bonded silica HPLC of *N*-glycan-containing fractions from mature green tomato fruit pericarp. Fractions 5b, WR, and SR, after Con A-Sepharose (Fig. 1), were lyophilized, dissolved in 400 μL of 50% (v/v) acetonitrile, and injected (20–100 μL) on an amine-bonded silica HPLC column. The column was eluted with 65% (v/v) acetonitrile under isocratic conditions. Resulting oligosaccharide-containing fractions were lyophilized and subjected to structural analysis. Arrows in the upper panel indicate labeled-glycan elution positions: M_3, $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$; M_5, $Man_5GlcNAc$; M_8, $Man_8GlcNAc$.

released during α-MeGlc elution. However, nonretained fractions eluted at a later relative volume, suggested by the higher carbohydrate content in tube 5b (data not shown). Tube 3b did not contain *N*-acetylglucosamine but was rich in arabinosyl and xylosyl residues. Tube 4b was 5.4% *N*-acetylglucosamine, but Glc was the major sugar component (69%). Tube 5b contained a relatively high amount of *N*-acetylglucosamine (17%) and residues typical of complex-type *N*-glycans.

Purification by HPLC on Amine-Bonded Silica

The Con A-purified fractions NR5b (tube 5b), WR, and SR were purified using amine-bonded silica HPLC (Fig. 2). NR5b exhibited one main peak (NR5b) that eluted before Man_5-GlcNAc and $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$ standards. A smaller peak eluted after NR5b, but it was composed only of glucosyl residues and was therefore discarded. WR eluted as two peaks, the first and smallest peak (WR1) eluted before $Man_5GlcNAc$; the second and main peak (WR2) eluted substantially after $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$. SR eluted as four peaks between $Man_5GlcNAc$ and $Man_8GlcNAc$, inclusively. The *N*-glycans purified after this step were then subjected to structural characterization.

Structure of Tomato Fruit *N*-Glycans

The glycosyl composition of *N*-glycans purified after amine-bonded silica HPLC was determined (Table I). Subsequently, glycosyl-linkage composition (Table II) and FAB-MS analyses were performed on reduced and permethylated samples, as well as 500 MHz ^{1}H-NMR spectroscopy of the native molecules. Results of the structural analyses are described below under subheadings for each group of *N*-glycans, based on their affinity for Con A, which is related to the general structural features of that group.

Oligosaccharides SR1 TO SR4

Glycosyl composition revealed that mannosyl and *N*-acetylglucosaminyl residues predominated; glucosyl residues were also detected in SR4. Methylation analysis showed that these *N*-glycans contained glycosyl linkages characteristic of oligomannoside type *N*-glycans, i.e. *N*-acetylglucosaminyl residues appeared as reducing, 1,4-linked only; mannosyl

Table I. *Sugar composition of tomato N-glycans purified on amine-bonded silica HPLC column*

Sugars[a]	NR5b	WR1	WR2	SR1	SR2	SR3	SR4
				mol %			
Fuc	0.17	0.12	0.14				
Ara	0.16						
Xyl			0.12				
Man	0.33	0.34	0.36	0.77	0.74	0.84	0.79
Glc	Tr[b]	Tr	Tr		Tr		0.10
GlcNAc	0.34	0.53	0.37	0.23	0.26	0.16	0.11
Total[c]	90	60	120	55	46	110	80

[a] Analyzed as their alditol acetate derivatives. [b] Trace amounts were detected but could not be quantified accurately. [c] Amounts are in μg.

Table II. *Methylation analysis of tomato N-glycans after amine-bonded silica HPLC*
Oligosaccharides were reduced with $NaBH_4$ before methylation.

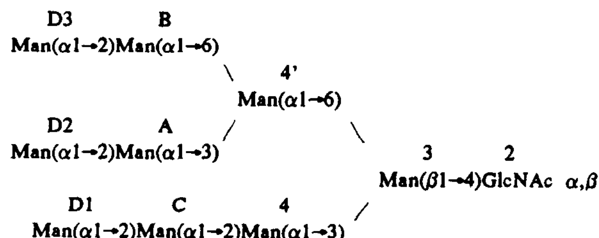
Methylated Sugar[a]	NR5b	WR1	WR2	SR1	SR2	SR3	SR4
				mol %			
2,3,5-Me_3-Ara	0.18						
2,3,4-Me_3-Fuc	0.11	0.05	0.05				
2,3,4-Me_3-Xyl			0.14				
2,3,4,6-Me_4-Man	0.27	0.33	0.24	0.65	0.54	0.42	0.28
2,3,4,6-Me_4-Glc							0.07
3,4,6-Me_3-Man			0.21	Tr[b]	0.17	0.33	0.36
2,3,4-Me_3-Man		0.33		Tr			
1,3,5,6-Me_4-GlcNMeAc-*ol*	0.03	0.04	0.04	0.09	0.08	0.06	0.07
3,4-Me_2-Man	0.33						
2,4-Me_2-Man				0.17	0.21	0.19	0.22
1,5,6-Me_3-GlcNMeAc-*ol*	0.03	0.08	0.02				
4-Me-Man			0.19				
3,6-Me_2-GlcNMeAc	0.04	0.06	0.04				
3,4,6-Me_3-GlcNMeAc		0.11	0.06				

[a] Separated as partially methylated alditol acetates and characterized by retention times and fragmentation patterns by GC-EIMS. [b] Trace amounts were detected but could not be quantified accurately.

residues were nonreducing t-, 1,2-, and 1,3,6-linked. Nonreducing t-glucosyl residues were detected in SR4. Consequently, SR1 to SR4 may belong to endoglucosaminidase-released oligomannosides, with the structure $Man_xGlcNAc$. These results agree with the high affinity of these *N*-glycans for Con A, since they were strongly retained on Con A under our experimental conditions.

Analysis of SR1 by FAB-MS revealed a spectrum that exhibited molecular ions $[M + H]^+$ and $[M + Na]^+$ of 1328.6 and 1350.6, respectively (data not shown). These data correspond to a mol wt of 1327.6, which is in agreement with permethylated $Man_5GlcNAc$-*ol*. Similarly, $[M + H]^+$ and $[M + Na]^+$ of *N*-glycans SR2, SR3, and SR4 were found, corresponding to mol wts of 1531.7, 1735.7, and 1939.9, respectively (data not shown), in agreement with reduced and permethylated $Man_6GlcNAc$-*ol*, $Man_7GlcNAc$-*ol*, and $Man_8GlcNAc$-*ol*. They differed from one another by a value of 204, corresponding to one per-*O*-methylated mannosyl residue.

The 500 MHz ^{1}H-NMR spectra of SR1 to SR4 *N*-glycans are given in Figure 3 and their ^{1}H-NMR spectral parameters are listed in Table III. Coding of the glycosyl residues of the oligomannoside type *N*-glycans was as follows:



The ^{1}H-NMR spectrum of SR1 was identical to that of the reference compound $Man_5GlcNAc$ (**1**; Fig. 3, Table III) run under analogous conditions. In addition, the assignments of the anomeric reporter groups of SR1 agreed with the values for corresponding resonances of $Man_5GlcNAc$ previously

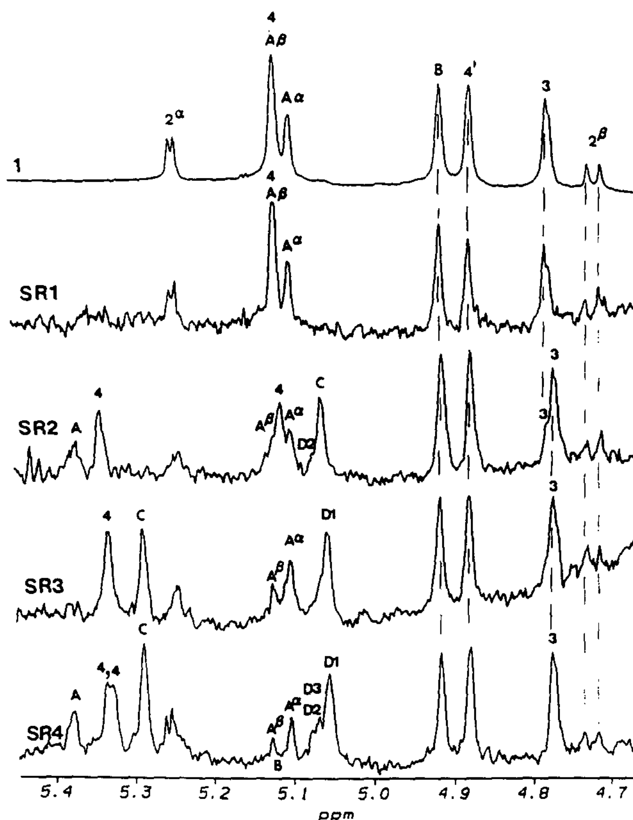


Figure 3. Anomeric reporter group regions of 500 MHz ^{1}H-NMR spectra of oligosaccharides SR1, SR2, SR3, and SR4 at 60°C. **1** is a spectrum of the reference $Man_5GlcNAc$. The numbers and letters in the spectra refer to the corresponding residues in the structure (see text and Fig. 4).

Table III. *Anomeric reportor group chemical shifts of oligosaccharides SR1, SR2, SR3, and SR4*[a]

See Figure 4 and text for complete structure and coding of monosaccharide residues. n.d., Not determined. Superscript α indicates α anomer.

	Compound and Schematic Structure										
	B \ 4′ / A \ 3-2 / 4	B \ 4′ / A \ 3-2 / 4	B \ 4′ / A \ 3-2 / C-4	B \ 4′ / A \ 3-2 / C-4	B \ 4′ / D2-A \ 3-2 / 4	B \ 4′ / D2-A \ 3-2 / 4	B \ 4′ / A \ 3-2 / D1-C-4	B \ 4′ / D2-A \ 3-2 / D1-C-4	B \ 4′ / D2-A \ 3-2 / D1-C-4	D3-B \ 4′ / A \ 3-2 / D1-C-4	D3-B \ 4′ / A \ 3-2 / D1-C-4
Residue T°C	SR1	1[a]	SR2′	2[b]	SR2″	3[b]	SR3	SR4′	4[b]	SR4″	5[b]
GN-2											
α 60	5.246	5.246	5.244		5.244		5.242	5.252			5.244
25	5.246	5.246	n.d.	5.245	n.d.	5.230	5.245	5.228	5.228	5.250	5.249
β 60	4.723	4.724	4.724		4.724		4.724	4.726		4.726	4.727
25	n.d.	4.708	n.d.	n.d.	n.d.	4.708	n.d.	n.d.	4.714		n.d.
Man-3											
60	4.785	4.784	4.776		4.785		4.774	4.755		4.775	4.774
25	n.d.	n.d.	n.d.	4.774[α]	n.d.	4.783[α]	n.d.	n.d.	4.775	n.d.	
Man-4											
60	5.120	5.119	5.343		5.12[c]		5.330	5.325		5.334	5.331
25	5.107	5.100	5.350	5.348	5.09[c]	5.094	5.344	5.337	5.334	5.346	5.346
Man-4′											
60	4.879	4.879	4.878		4.878		4.879	4.878		4.878	4.884
25	4.878	4.873	n.d.	4.872[α]	n.d.	4.872	4.872	n.d.	4.871	n.d.	4.871
Man-A											
α 60	5.101	5.099	5.109		5.372		5.101	5.375		5.101	5.124
β	5.120	5.119	5.12[c]		5.372		5.124			5.124	5.098
α 25	5.084	5.078	5.080	5.080	5.403	5.395	5.080	5.408	5.399	5.102	5.106
β	5.107	5.100	5.09[c]	5.108	5.403	5.404	5.105		5.407		5.071
Man-B											
60	4.916	4.915	4.915		4.915		4.916	4.915		—[c]	5.124
25	4.914	4.908	4.907	4.904	4.907	4.904	4.909	4.907	4.905		5.153
Man-C											
60			5.063				5.287	5.287		5.287	5.288
25			5.053	5.053			5.305	5.306	5.305	5.306	5.312
Man-D1											
60							5.053	5.052		5.054	5.059
25							5.042	5.040	5.046	5.040	5.043
Man-D2											
60					—[c]			—[c]			
α 25						5.053			5.055		
β						5.060			5.060		
Man-D3											
60										—[c]	5.059
25											5.043

[a] The ^{1}H-NMR spectrum of reference compound **1** was identical to that reported by Priem et al. (1990b). [b] Chemical shifts of reference compounds **2**, **3**, and **4** are those of corresponding structures described by Michalski et al. (1990). The values of compound **5** are those for $Man_8GlcNAc$ from yeast invertase reported by Byrd et al. (1982). [c] Values could not be detected more accurately due to close overlap.

reported by Cohen et al. (1980), Priem et al. (1990b), and Michalski et al. (1990). Thus, SR1 was clearly $Man_5GlcNAc$ (Fig. 4).

SR2 was identified as a mixture of two isomers, SR2′ and SR2″ (Fig. 4), on the basis of chemical shift analogy with $Man_6GlcNAc$ as described by Michalski et al. (1990) (**2** and **3**; Table III). Analogous to compounds **2** and **3**, the characteristic doubling of the H-1 signals of Man-4 and Man-A isomers suggested that both occur in both t- and α1,2-linked positions. The Man-C residue was characterized by its reporter anomeric resonance at 5.063 ppm (60°C). The chemical shift of the H-1 signal of Man-D2 overlaps the resonance of H-1 of Man-C and could not be detected. However, two resonances were observed in the anomeric reporter group region of the β-Man-3 residue. One resonance at 4.785 ppm supported the presence of the SR2″ isomer based on chemical shift analogy to the value of the corresponding protons of SR1 also possessing a β-Man-3 residue substituted by Man-4. The second resonance of H-1 of Man-3 was shifted upfield at 4.776 ppm, which was previously described as typical for the introduction of α1,2-linked Man-C (Vliegenthart et al., 1983). The ratio SR2′:SR2″ in the mixture was estimated to be approximately 2:1.

SR3 was a single structural isomer of $Man_7GlcNAc$. The ^{1}H-NMR evidence in support of the above interpretation was the downfield shift at 5.287 ppm (60°C) of the H-1 resonance of Man-C in conjunction with the additional reporter resonance at 5.053 (60°C), characteristic for H-1 of Man-D1. These values were in agreement with those deduced previously for $Man_8GlcNAc$ isomers that also contain α1,2-linked Man-C (compare SR3 and **4**; Table III). As compared with SR2′, the chemical shifts of all other structural reporter groups of SR3 remained essentially unaltered. Thus, the structure of SR3 was established as a single $Man_7GlcNAc$ isomer (Fig. 4).

The ^{1}H-NMR spectrum of SR4 resembled that of the thyroglobulin $Man_8GlcNAc$, described as a mixture of three isomers (Byrd et al., 1982). The two resonances for H-1 of Man-A at $5.101^{\alpha}/5.124^{\beta}$ and at 5.375 ppm, together with the characteristic downfield shift of H-1 of α1,2-linked Man-C at 5.287 ppm, suggested the presence of both SR4′ and SR4″ isomers. Also, the spectrum at 25°C showed two anomeric proton signals for Man-4 at 5.346 and 5.337 ppm, which are characteristic reporter groups of Man-4 in the presence of t- and α1,2-linked Man-A, respectively. The doubling of the signal of H-1 of the Man-4 confirmed the presence of both SR4′ and SR4″ isomers. The relative decrease in the signal intensity of the anomeric proton of the t-Man-B at 4.915 ppm suggested a small contribution (about 15%) of α1,2-linked Man-B Man_8-isomer, i.e. SR4‴, to the $Man_8GlcNAc$ mixture. However, this assumption was hard to confirm due to close overlap of both H-1 resonances of Man-D1, D2, and D3 at 5.04 to 5.06 ppm and α1,2-linked Man-B with t-linked Man-A at 5.124 ppm. All other structural reporter resonances of SR4 agreed with those deduced for corresponding isolated Man_8-isomers **4** and **5** (Table III). In conclusion, at least two isomers assigned to SR4′ and SR4″ (Fig. 4) were present in the $Man_8GlcNAc$ mixture, and the presence of the third, SR4‴, cannot be excluded by ^{1}H-NMR data. Structures of the $Man_8GlcNAc$ isomers are presented in Figure 4.

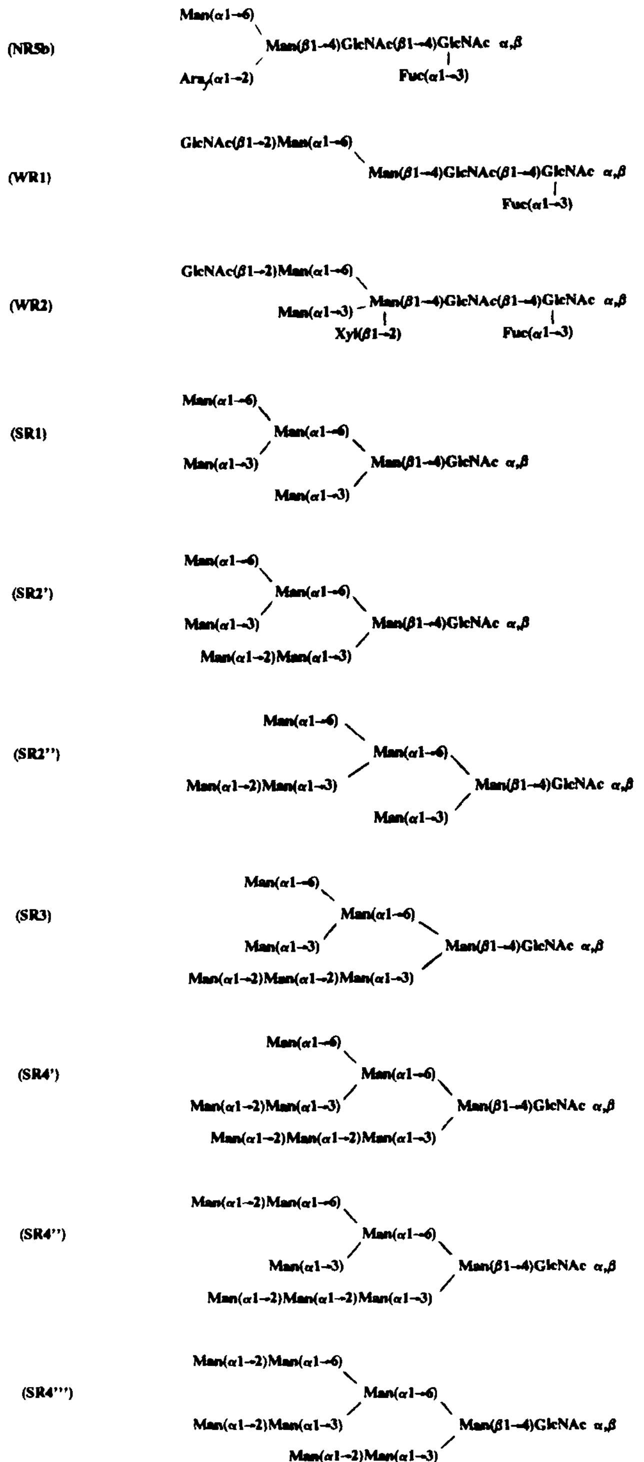


Figure 4. Structures of the free *N*-glycans in mature green tomato.

Oligosaccharide WR2

Glycosyl composition showed fucosyl, xylosyl, mannosyl, and *N*-acetylglucosaminyl residues in a ratio of 1:1:3:3. Methylation analysis revealed the presence of 1,2,3,6-linked mannosyl residues, found in *N*-glycans as part of the core Manα1→6(Manα1→3)(Xylβ1→2)Man. The presence of nonreducing t-linked xylosyl and t-linked mannosyl residues also support this contention. However, the presence of nonreducing t-linked *N*-acetylglucosaminyl and 1,2-linked mannosyl residues suggested the presence of one GlcNAcβ1→2Man. Fucosyl residues were found as nonreducing t-linked. Presence of 1,4-linked and reducing 1,3,4-*N*-acetylglucosaminyl suggested a chitobiose core →4GlcNAcβ1→4(Fucα1→3)GlcNAc as a part of WR2. The low amount of t-linked fucosyl residues and the presence of reducing 4-linked *N*-acetylglucosaminyl residues may be explained by partial loss of the labile fucosyl residues during heating at 60°C in the NMR tube prior to methylation analysis (Sharon, 1975).

The FAB-MS spectrum of WR2 (data not shown) exhibited molecular ions $[M + H]^+$ and $[M + Na]^+$ of 1745.8 and 1767.8, suggesting a mol wt of 1744.8, in agreement with permethylated GlcNAcMan(Xyl)Man$_2$GlcNAc(Fuc)GlcNAc-*ol*. An A1-type fragment of 1276.8 was present, resulting from fission of the chitobiose core.

The ^{1}H-NMR spectrum of WR2 is presented in Figure 5 and its spectral parameters are reported in Table IV. Coding of the glycosyl residues of complex type *N*-glycans was as follows:

```
   5'            4'
GlcNAc(β1→2)Man(α1→6)
                     \   3            2            1
                      Man(β1→4)GlcNAc(β1→4)GlcNAc  α,β
         4           /    |                    |
     Man(α1→3)            |                    |
                          X                    F
                      Xyl(β1→2)            Fuc(α1→3)
                          or
                      Araf(α1→2)
```

The structure of WR2 was deduced by comparison of its ^{1}H-NMR spectrum with those of two xylomannoside oligosaccharides previously described by Lhernould et al. (1992), i.e. (GlcNAc$^{5'}$)Man$_3$(Xyl)GlcNAc(Fuc)GlcNAc (the reference chemical shifts are given in parentheses in Table IV) and an oligosaccharide lacking the nonreducing t-GlcNAc-5,Man$_3$(Xyl)GlcNAc(Fuc)GlcNAc (**6**, Table IV). The characteristic upfield shift of H-1, the downfield shift of H-2 of Man-4′ of WR2 (in comparison with **6**), and the additional doublet at 4.546 ppm confirmed the presence of nonreducing t-linked GlcNAc-5′, analogous to the oligosaccharides mentioned above (Lhernould et al., 1992), and its pyridylamino derivative, (GlcNAc$^{5'}$)Man$_3$(Xyl)GlcNAc(Fuc)-GlcNAc-PA (Takayama et al., 1989). The H-2, H-3, and H-4 resonances of GlcNAc-5′ at 3.706, 3.546, and 3.454 ppm, respectively, were assigned from DQF-COSY cross peaks (data not shown). Due to the small amount of sample, complete assignment of the resonances by DQF-COSY experiments was not possible. However, cross peaks that could be assigned (the values are marked with an asterisk in Table IV) were in agreement with values reported for compound **6**. The structure of WR2 is given in Figure 4.

Oligosaccharide WR1

Fucosyl, mannosyl, and *N*-acetylglucosaminyl residues were detected in the ratio 0.7:2:3. Methylation analysis showed that 1,2- and 1,6-linked mannosyl residues were found in equimolar amounts. The absence of nonreducing t-linked mannosyl residues and the presence of nonreducing t-linked *N*-acetylglucosaminyl residues suggested that a structure of GlcNAcβ1→2Manα1→6Manβ1→ was contained in WR1. The same glucosaminyl and fucosyl structural features were found in WR1 as in WR2, again suggesting the presence of →4GlcNAcβ1→4(Fucα1→3)GlcNAc in this *N*-glycan.

FAB-MS of WR1 showed molecular ions $[M + H]^+$ and $[M + Na]^+$ of 1380.7 and 1402.7, respectively, suggesting a mol wt of 1379.7 (data not shown). This agreed with permethylated GlcNAcMan$_2$GlcNAc(Fuc)GlcNAc-*ol*. An A1-type fragment of 913.5 was seen, resulting from fission of the chitobiose core.

^{1}H-NMR data for WR1 were in accord with the proposed structure for this oligosaccharide based on methylation analysis. Due to the small amount of sample, assignments were limited to the anomeric reporter groups. The ^{1}H-NMR spectrum showed the characteristic H-1 resonance of GlcNAC-5′

Figure 5. 500 MHz ^{1}H-NMR spectrum of the WR2 oligosaccharide at 25°C. The numbers and letters refer to the corresponding residues in the structure. For example, 1^F designates H-1 of Fuc and 2^3 designates H-2 of Man-3 (see text and Fig. 4).

Table IV. *Structural reporter group chemical shifts of oligosaccharides WR2, WR1, and NR5b*

See Figure 4 and text for complete structure and coding of monosaccharide residues. n.d., Not determined.

Residue	Reporter Group	5′-4′ \ 3-2-1 / | | 4 X F WR2 25°C	4′ \ 3-2-1 / | | 4 X F 6[a] 25°C	5′-4′ \ 3-2-1 | F WR1 25°C	 WR1 60°C	4′ \ 3-2-1 | | Ara$_f$ F NR5b 25°C	 NR5b 60°C
GlcNAc-1	H-1α	5.079	5.084	5.086	5.107	5.081[c]	5.105
	H-1β	4.669[c]	4.689	n.d.	n.d.	4.687[c]	4.667
	H-2α	4.131	4.139			4.139[c]	
	H-2β	3.751[c]	3.872			3.868[c]	
	H-3α		3.996			3.992[c]	
	CH3	2.028	2.031α 2.025β	2.027	2.033	2.027	2.035
GlcNAc-2	H-1	4.555[c]	4.561	—[d]	—[d]	4.561[c]	4.566
	H-2	3.764[c]	3.770α			3.782[c]	
	CH3	2.048	2.048α 2.051β	2.048	2.048	2.058	2.058
Man-3	H-1	4.853	4.853		4.730	n.d.	4.811
Man-4	H-1	5.123[c]	5.122				
	H-2	4.043[c]	4.040				
	H-3	3.831[c]	3.833				
Man-4′	H-1	4.899[c] (4.898)	4.910	4.908	4.902	4.916[c]	4.919
	H-2	4.107[c] (4.104)	3.975			3.967[c]	
	H-3	3.867[c]	3.847				
GlcNAc-5′	H-1	4.548[c]		4.545	4.561		
	H-2	3.706[c]					
	H-3	3.546[c]					
	H-4	3.454[c]					
	CH3	2.048		2.048	2.048		
Xyl	H-1	4.456[c]	4.465				
	H-2	3.380[c]	3.374				
	H-3	3.447[c]	3.452				
	H-5ax	3.253[c]	3.256				
	H-5eq	4.017[c]	4.018				
Fuc	H-1	5.123[c]	5.122α	5.121	5.129	5.123[c]	5.126
	H-2	3.710[c]	3.706			3.709[c]	
	H-5	4.718[c]	4.714			4.732[c]	
	H-6	1.272[c]	1.272	1.271	1.271	1.272[c]	1.265
Ara$_f$	H-1					5.302[c]	5.292
	H-2					4.202[c]	
	H-3					3.968[b]	
	H-4					4.286[c]	
	H-5S					3.808[b]	
	H-5R					3.695[c]	

[a] Reference compound previously described by Lhernould et al. (1992). Values in parentheses in column 1 were taken from the same study for an oligosaccharide having the same structure as WR2. [b] Low intensity DQF-COSY cross peaks. [d] Values determined by DQF-COSY experiment. [d] Values could not be detected more accurately due to close overlap.

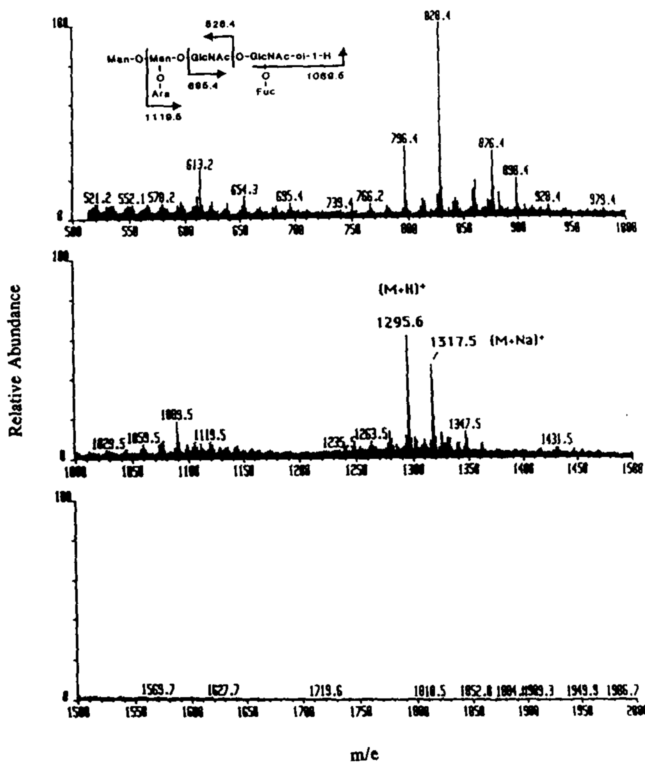


Figure 6. FAB-MS spectrum of the arabinosyl-containing tomato *N*-glycan NR5b.

at 4.545. The anomeric resonance of Man-4′ of WR1 at 4.908 ppm was compared with the corresponding resonances of reference linear glycopeptides lacking the GlcNAc-5′ residue, Man(α1→6)Man(β1→4)GlcNAc(β1→4)GlcNAc-Asn and Man(α1→6)Man(β1→4)GlcNAc(β1→4)(Fucα1→6)-GlcNAcAsn, for which the anomeric protons of Man-4′ resonate at 4.915 and 4.916 ppm, respectively (see Van Kuik et al., 1986). The characteristic downfield shift ($\Delta\delta = 0.008$ ppm) confirmed the presence of GlcNAc-5′ β-1,2-linked to Man-4′ of WR1. All other resonances fit the corresponding signals of WR2. Based on the above, WR1 was assigned to the structure given in Figure 4.

Oligosaccharide NR5b

NR5b was composed of arabinosyl, fucosyl, mannosyl, and *N*-acetylglucosaminyl residues in a 1:1:2:2 ratio. Like WR1 and WR2, glycosyl linkages typical of the core -4GlcNAcβ1-4(Fucα1-3)GlcNAc were found. Methylation analysis revealed that arabinosyl residues were nonreducing, t-linked, and in the furanose isomeric form. Mannosyl residues were found in equimolar amounts of nonreducing t- and 1,2,6-linked. No xylosyl residues were detected. These data correspond to structures Man1-6(Ara1-2)Man→ and/or Ara1-6(Man1-2)Man1→.

The FAB-MS spectrum of NR5b exhibited molecular ions $[M + H]^+$ and $[M + Na]^+$ of 1295.6 and 1317.5, respectively, suggesting a mol wt of 1294.6, in agreement with permethylated Man(Ara)ManGlcNAc(Fuc)GlcNAc-*ol* (Fig. 6). An A1-type fragment of 828.4 resulting from fission of the chitobiose core was found.

The ^{1}H-NMR spectra of NR5b at 60 and 25°C are given in Figure 7, a and b, respectively. The assignments of the reporter resonances were based on 1D and DQF-COSY NMR spectroscopy (data not shown). The reporter groups of Fuc, GlcNAc-2, and H-1 of GlcNAc-1 agree with those reported for WR2 (Table IV). The spectrum lacked the characteristic structural reporter groups for both xylosyl and Man-4 residues at 3.250 to 3.374 ppm and 5.122 ppm, respectively. Comparison of structural reporter groups of NR5b with the reference glycopeptides Man(α1→6)Man(β1→4)GlcNAc(β1→4)GlcNAc-Asn and Man(α1→6)Man(β1→4)-GlcNAc(β1→4)(Fucα1→6)GlcNAc-Asn, as described by Van Kuik et al. (1986), conveyed the following information. The anomeric resonances of the Man-4′ were essentially identical, 4.916 ppm, for NR5b and the two reference glycopeptides. This fact agreed with the presence of a nonreducing, t-linked Man-4′ residue. Furthermore, the downfield shift of H-1 of Man-3 of NR5b at 4.811 ppm relative to the value 4.77 ppm

Figure 7. 500 MHz ^{1}H-NMR spectrum of NR5b at 60°C (a) and 25°C (b). The numbers and letters refer to the corresponding residues in the structure (see text and Fig. 4).

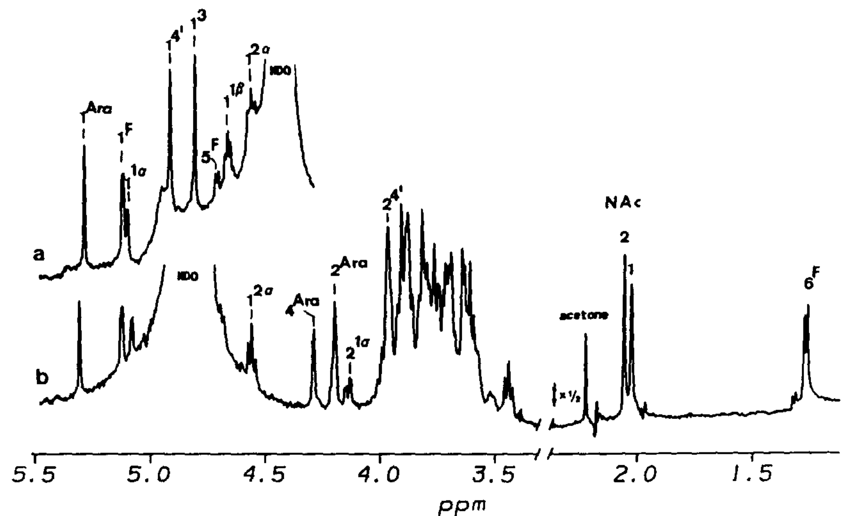


Table V. *Methylation analysis of the HW40 fraction from immature (IM), mature green (MG), and red ripe (RR) tomato fruit and the glycopeptide prepared from S. japonica lectin (Sj-GP)*

Methylated Sugar[a]	Developmental Stage			Sj-GP
	IM	MG	RR	
	mol %[b]			
2,3,4-Me_3-Fuc	1.00	1.00	1.00	1.00
2,3,4-Me_3-Xyl	1.91	1.15	1.08	0.88
2,3-Me_2-Xyl_p and 3,4-Me_2-Xyl_p[c]	3.67	2.44	2.24	
2,3,5-Me_3-Ara	0.85	1.07	1.05	
2,3-Me_2-Ara_f	3.08	2.38	2.29	
2,3,4,6-Me_4-Man	2.67	3.00	2.99	3.47
3,4,6-Me_3-Man	1.63	1.73	1.94	
2,3,6-Me_3-Man	0.94	1.15	0.73	
3,4-Me_2-Man	0.72	0.95	1.28	
2,4-Me_2-Man	1.11	1.18	1.25	
2,3-Me_2-Man	0.30	0.28	0.38	
4-Me-Man	1.19	1.22	1.38	2.30
2,3,4,6-Me_4-Gal	1.74	1.55	0.88	
2,3,4-Me_3-Gal	2.06	2.82	1.53	
2,3,4,6-Me_4-Glc	4.72	1.67	0.77	
2,3,6-Me_3-Glc	7.20	3.97	1.43	
2,3-Me_2-Glc	0.48	0.67	0.61	
3,4,6-Me_3-GlcNMeAc	0.29	0.29	0.30	
3,6-Me_2-GlcNMeAc	0.35	0.47	0.43	0.43
6-Me-GlcNMeAc	0.15	0.15	0.15	0.67
1,3,5,6-Me_4-GlcNMeAc-*ol*	0.57	0.48	0.35	
1,5,6-Me_3-GlcNMeAc-*ol*	0.54	0.61	0.67	
2,3,4-Me_3-Glc or -Man[c]	1.59	0.97	0.73	

[a] Separated as partially methylated alditol acetates and characterized by retention times and mass spectra. [b] Calculated relative to 2,3,4-Me_3-Fuc. [c] Not separated.

for the linear glycopeptides was consistent with glycosylation of Man-3 at C2 by Ara_f. The H-1 and H-2 reporter resonances of Ara_f were assigned from DQF-COSY cross peaks (data not shown). Assignment of the H-3 of Ara_f was confounded by the presence of two unassigned peaks, at the same chemical shift as H-2 of Ara_f. Under the plausible assumption that the cross peak at 3.968 ppm was that of the H-3 resonance of Ara_f, the cross peaks corresponding to the H3 through H5 resonances agreed with chemical shifts reported for α-L-arabinofuranosides by Hoffmann et al. (1992) (see Table IV). Both methylation glycosyl-linkage analysis and our interpretation of the ^{1}H-NMR data were consistent with the structure for NR5b given in Figure 4.

Changes in Glycosyl Composition of the Oligosaccharidic Extract during Development

Extracts of fruit at different stages of development were compared by glycosyl-linkage analysis of partially per-*O*-methylated, per-*O*-acetylated alditol acetates. The most prominent change was the decrease in glucosyl and partially methylated glucosyl derivatives during maturation, which was likely due to starch hydrolysis. A decrease in galactosyl content was also observed. Partially methylated galactosyl derivatives also decreased, and this is most likely related to the net loss of galactosyl-containing cell wall polymers during ripening (Gross and Wallner, 1979).

Of the glycosyl residues typical of *N*-glycans, a 25% increase in fucosyl and *N*-acetylglucosaminyl residues was observed from the immature to red-ripe stage of ripeness (data not shown). In contrast, mannosyl content remained constant during ripening. Glycosyl-linkage composition was characteristic of *N*-glycans (Table V). In particular, reducing *N*-acetylglucosaminyl residues were found, and their proportions changed during ripening. The ratio of 1,5-*O*-methyl-*N*-acetylglucosaminitol to 1,3,5-*O*-methyl-*N*-acetylglucosaminitol increased from 0.9 in immature fruit, to 1.3 in mature green, and 1.9 in ripe fruit. This indicated that the relative amount of complex-type *N*-glycans increased during ripening.

The percentage of free, i.e. susceptible to reduction in vitro, glucosamine calculated by differential reduction with $NaBH_4$ versus $NaBD_4$, before versus after hydrolysis, increased significantly during ripening, from 62% in immature, to 64% in mature green, and 90.5% in red-ripe fruit. This suggests that free *N*-glycan content increased during ripening. The glycosyl residue, 6-*O*-methyl-*N*-acetylglucosaminyl, which is characteristic of *N*-glycopeptides, was found in fruit at all stages of development, suggesting the presence of Asn-linked complex-type *N*-glycopeptides.

DISCUSSION

Con A-Sepharose Fractionation

Immobilized Con A has been used extensively for separating animal *N*-glycoconjugates, and the structure-affinity re-

lationship is well documented (Kornfeld and Ferris, 1975; Krusius et al., 1976; Baenziger and Fiete, 1979). It was reported that the common backbone of Manα1→6(Manα1→3)Manβ1→4GlcNAc was necessary for binding to Con A (Ogata et al., 1975). Also, Goldstein et al. (1965) determined that at least two 1,3,4- and/or 1,6-linked mannosyl residues were necessary for binding. Typically, the presence of a β-D-N-acetylglucosaminyl residue linked to C-4 of the β-D-mannosyl residue diminishes their affinity for Con A (Baenziger and Fiete, 1979). However, a β-D-N-acetylglucosaminyl residue linked at C-2 of the 4- and/or 4′-α-D-mannosyl increases affinity for Con A (Kornfeld and Ferris, 1975; Narasimhan et al., 1979).

Contrary to the situation in animals, most literature on plant glycoconjugate-Con A interactions concerns glycoproteins. A major characteristic of many plant N-glycans is the presence of a β-D-xylosyl residue linked at the C-2 position of β-D-mannosyl residues. Using electrophoresis and immunochemical methods, Kitagaki-Ogawa et al. (1986) suggested that the xylosyl residue did not affect binding to Con A. However, other studies using immobilized Con A for purifying xylosyl-containing N-glycoproteins have been reported. For example, germin, a wheat glycoprotein that contains xylosyl-containing N-glycans, was purified using adsorption to Con A (Jaikaran et al., 1990).

Results concerning glycoprotein-Con A interactions must, however, be interpreted with caution for glycopeptide and N-glycan purification purposes. Sturm et al. (1987) distinguished and separated complex-type glycopeptides from oligomannoside-type N-glycans according to their inability to bind to Con A. Indeed, we found that the Sj-GP Man_3-(Xyl)GlcNAc(Fuc)GlcNAc-containing glycopeptide did not bind to Con A under our experimental conditions. However, one complex-type N-glycan fraction from tomato bound to immobilized Con A-Sepharose and was eluted using 10 mM α-MeGlc. The corresponding N-glycans WR1 and WR2 were characterized by the presence of one N-acetylglucosaminyl residue linked to the C-2 position of the 4′-α-D-mannosyl residue. Additional β1,2-N-acetylglucosaminyl residues are known to increase affinity of N-glycans for Con A (Kornfeld and Ferris, 1975; Narasimhan et al., 1979). To explain this apparent contradiction with the report of Sturm et al. (1987), we suggest that the apparent binding was due to the use of pH 5.2 in our system rather than pH 7.0, resulting in different affinities of the N-glycans for Con A.

If the nonreducing N-acetylglucosaminyl residue explains binding of WR2 to Con A, then WR1 contains only two mannosyl residues and, consequently, would not be expected to show considerable affinity for Con A. Regardless of the specific structural features involved in the differential affinity of N-glycans for Con A, we have demonstrated that Con A is an excellent tool for fractionation of plant N-glycans.

Structural Features of Tomato Fruit N-Glycans

Tomato fruit N-glycan structures were determined using glycosyl and glycosyl-linkage composition analysis, 500 MHz ^{1}H-NMR spectroscopy, and FAB-MS. Fraction NR5b was a novel N-glycan containing an arabinosyl residue linked to C-2 of the β-D-mannosyl residue. Previously, N-glycans that were of the complex type all contained a xylosyl residue branched at the same C-2 of the β-D-mannosyl residue. The location of the arabinosyl residue on this N-glycan raises questions concerning the biosynthetic pathway of this group of N-glycans. Moreover, it is intriguing that no N-glycoconjugate containing an N-glycan with this structure has yet been reported. An arabinosyl- or rhamnosyl-containing N-glycan in *Daucus carota* was described hypothetically by Sturm (1991), although the suggested location of the putative arabinosyl or rhamnosyl residue was at the 4′- or 4-α-D-mannosyl residue.

The weakly bound Con A fraction, WR2, was an N-glycan previously described as "compound a" of laccase in *Acer pseudoplatanus* L. (Takahashi et al., 1986). More recently, it was identified as a free N-glycan in *Silene alba* cell-suspension cultures (Lhernould et al., 1992). Fraction WR1 exhibited the same structural features as WR2, except for the absence of β-D-xylosyl and β-D-mannosyl residues. This suggests that it may have been a degradation product of mannosidase and xylosidase activity on WR2. Terminal N-acetylglucosaminyl-containing N-glycans have been described only in secreted plant glycoproteins (Sturm, 1991), suggesting that some free tomato N-glycans may result from degradation of secreted N-glycoproteins.

The oligomannosyl glycans we have characterized in tomato pericarp have the same core structure and differ only by the successive addition of α-D-mannosyl residues to Man_5GlcNAc at C, D_1, and D_2. Free Man_5GlcNAc had been detected previously in a cell-suspension culture of *S. alba* (Priem et al., 1990b). It is possible that tomato oligomannosyl glycans occur as degradative products of Man_8GlcNAc or Man_9GlcNAc, similar to the situation in rat liver, where they result from action of lysosomal mannosidases on Man_9GlcNAc (Michalski et al., 1990).

Physiological Significance of Free N-Glycans on Tomato Fruit Ripening

Free N-glycans constitute a significant fraction of the soluble oligosaccharide pool in tomato fruit pericarp. On the basis of relative amounts of reducing N-acetylglucosaminyl residues, we estimate their content at 5 to 6 μg/gram fresh weight in mature green fruit. In a previous report, we described biological activities of the N-glycans Man_5GlcNAc and Man_3(Xyl)GlcNAc(Fuc)GlcNAc at a concentration of 1 ng/gram fresh weight. Our finding of Man_5GlcNAc in tomato pericarp supports the hypothesis that free N-glycans have a crucial role in regulation of tomato fruit senescence. Although tomato glycans exhibit distinctive structures that may not all contain biological activity, their compartmentation would be a critical factor. Indeed, the amount of the individual N-glycan Man_5GlcNAc in tomato pericarp was >0.1 μg/gram fresh weight, 100-fold higher than that required for activity in our bioassays.

The increase in free N-glycan content during ripening could be due to a corresponding increase in their synthesis and/or N-glycoconjugate degradation by endoglycanase(s). Lernhould et al. (1992) discovered a peptide N-glycanase in *S. alba* cell-suspension cultures potentially responsible for the occurrence of free xylosyl-containing N-glycans found in

those cultures (Priem et al., 1990b). Such an enzyme, as well as endo-β-*N*-acetylglucosaminidase (Ogata-Arakawa et al., 1977), could explain the occurrence of oligomannoside type *N*-glycans in tomato fruit.

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