PERGAMON

Phytochemistry 51 (1999) 199–210

PHYTOCHEMISTRY

Structural characterization of the *N*-linked oligosaccharides from tomato fruit

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Received in revised form 9 December 1998; accepted 10 December 1998

Abstract

The primary structures of the *N*-linked oligosaccharides from tomato fruit (*Lycopersicon esculentum*) have been elucidated. For the isolation of the protein fraction, two procedures were employed alternatively: a low temperature acetone powder method and ammonium sulfate precipitation of the tomato extract. After peptic digestion, the glycopeptides were purified by cation-exchange chromatography; the oligosaccharides were released by *N*-glycosidase A and fluorescently labelled with 2-aminopyridine. Structural characterization was accomplished by means of two-dimensional HPLC in combination with exoglycosidase digestions and MALDI–TOF mass spectrometry. Two varieties as well as two stages of ripening were investigated. In all the samples, the same sixteen *N*-glycosidic structures were detected; the two most abundant glycans showed identical properties to those of the major *N*-linked oligosaccharides of horseradish peroxidase and pineapple stem bromelain, respectively and accounted for about 65–78% of the total glycan amount; oligomannosidic glycans occurred only in small quantities (3–9%). The majority of the *N*-glycans were β1,2-xylosylated and carried an α1,3-fucose residue linked to the terminal *N*-acetylglucosamine. This structural element contributes to cross-reactions among non-related glycoproteins and has been shown to be an IgE-reactive determinant (Tretter, Altmann, Kubelka, März, & Becker, 1993). The presented study gives a possible structural explanation for reported immunological cross-reactivities between tomato and grass pollen extracts due to carbohydrate IgE epitopes (Petersen, Vieths, Aulepp, Schlaak, & Becker, 1996), thereby demonstrating the importance of the structural characterization of plant *N*-glycans for a more reliable interpretation of immunological data.

Keywords: *Lycopersicon esculentum*; Tomato; *N*-glycans; Fucose; HPLC; MALDI–TOF

1. Introduction

The elucidation of *N*-glycans in plants has raised increasing interest in the past years due to several physiological roles which could be attributed to these structures: correct folding, biological activity and stability of proteins can be affected by the presence of *N*-glycans (Driouich, Gonnet, Makkie, Laine, & Faye, 1989; Sheldon, & Bowles, 1992; Nagai, & Yamaguchi, 1993). Moreover, the involvement of *N*-linked oligosaccharides in plant development was demonstrated (Ettlinger, Schindler, & Lehle, 1986). Besides these physiological roles, *N*-glycans can influence the immunogenicity of glycoproteins, representing antigenic epitopes by themselves:

(i) The injection of horseradish peroxidase (HRP) into rabbits resulted in polyclonal anti-HRP antiserum which also reacted with many other plant glycoproteins carrying glycans similar to those from horseradish peroxidase. β1,2-xylose, present in all the glycoprotein–glycans of the study, but absent in mammalian organisms, was supposed to be responsible for the observed cross-reactivity (Faye, Johnson, Sturm, & Chrispeels, 1993).

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0031-9422/99/$ - see front matter
PII: S0031-9422(98)00764-X

(ii) The crossreactivity of a polyclonal antiserum against HRP with other plant glycoproteins and with bee-venom phospholipase A_2, due to a common carbohydrate epitope could be demonstrated. (Prenner, Mach, Glössl, & März, 1992; Wilson, Harthill, Mullin, Ashford, & Altmann, 1998). Chemical defucosylation as well as ELISA inhibition experiments indicated that α1,3-fucose is sufficient to create an immunogenic epitope.

The assumption that *N*-glycans or parts of these structures can not only represent antigenic, but also allergenic epitopes was confirmed by Tretter, Altmann, Kubelka, März, and Becker (1993); in the case of bee-venom allergic patients, the reactivity of IgE antibodies to glycopeptides of phospholipase A_2 was demonstrated; the proximal α1,3-fucose residue was identified as the IgE-reactive determinant.

The involvement of carbohydrate structures in food allergies is presently discussed quite controversial. However, there is increasing evidence that certain carbohydrates, especially *N*-glycans, might play a role in these adverse food reactions (Aalberse, Koshte, & Clemens, 1981), deduced from the following observations: many proteins which are of allergological importance are glycosylated; many allergic individuals show hypersensitivity to various types of allergens which are not taxonomically related, thus suggesting common, ubiquitous structures as glycans; and finally, many pollen allergic patients are also sensitive to certain food stuffs, which also implies common structural epitopes (Möller et al., 1997).

In immunological investigations, mostly indirect methods are applied which shall prove the involvement of glycans in allergic reactions, such as deglycosylation, lectin binding and compositional analysis (Petersen, Becker, Moll, Blümke, & Schlaak, 1995; Batanero, Villalba, Monsalve, & Rodriguez, 1996; Tsuji et al., 1997); however, structural information is a prerequisite for making correct conclusions about the allergenicity of *N*-glycans or epitopes, respectively.

We report here on the structural elucidation of the *N*-linked oligosaccharides in tomato fruit, which shall help to understand some observed cross-reactivities reported recently by Petersen, Vieths, Aulepp, Schlaak, and Becker (1996).

2. Results

2.1. Preparation of N-glycans from tomato fruit

To isolate the protein fraction from tomato fruit, two procedures were carried out alternatively: on the one hand, the tomatoes were homogenized in an appropriate extraction buffer and the proteins were precipitated by addition of ammonium sulfate. On the other hand, a low-temperature acetone powder method, first described by Clements (1965), was employed; the proteins precipitated in acetone, whereas small hydrophobic compounds (flavonoids, carotinoids) as well as most of the salts were effectively removed; the proteins were extracted from the resulting powder, yielding concentrations of 180–310 μg/ml as determined according to Bradford (1976); these values are in the same order of magnitude with those reported by Vieths, Schöning, Brockmann, and Aulepp (1992), who used this procedure for the preparation of several fruit and vegetable extracts. A cation-exchange chromatography eliminated most of the by-products in the sample. The binding fraction was assayed for the amount of *N*-acetylglucosamine by OPA-aminosugar analysis, yielding 170–260 nmol GlcNAc, dependent on the sample. From these values it could be deduced that about 85–130 nmol of *N*-glycans are present in the glycopeptide fractions (assuming 2 moles of GlcNAc/mol of oligosaccharide). The oligosaccharides were released from the glycopeptides using *N*-glycosidase A, separated from peptides and residual glycopeptides by reverse-phase chromatography and derivatized with the fluorescent tag 2-aminopyridine.

2.2. Separation and structural elucidation of the N-linked oligosaccharides

The pyridylaminated oligosaccharides were first size-fractionated on MicroPak AX-5 (Fig. 1A); 11 fractions were manually collected and rechromatographed if necessary. Individual fractions were subjected to reverse-phase HPLC, yielding a total of sixteen subfractions (additionally, an aliquot of the total glycan pool was applied to the reverse-phase system, see Fig. 1B). The elution position of each isolated oligosaccharides was determined on both columns and compared to those from reference oligosaccharides.

The individual peaks were then subjected to exoglycosidase treatment using α-mannosidase, β-*N*-acetylhexosaminidase and endoglycosidase H. The digests were again chromatographed on reverse-phase and MicroPak to monitor the change in the elution behavior (Tables 1 and 2). Whenever possible, a tentatively identified peak was coinjected with the corresponding reference oligosaccharide to verify the identical elution behavior. In the following, the structural assignment of each peak is described; the proposed structures are depicted in Table 3.

2.3. Complex and truncated complex glycans

The retention data of oligosaccharide 1-A, a minor component present in all the tomato samples, did not fit with any of the available reference compounds.

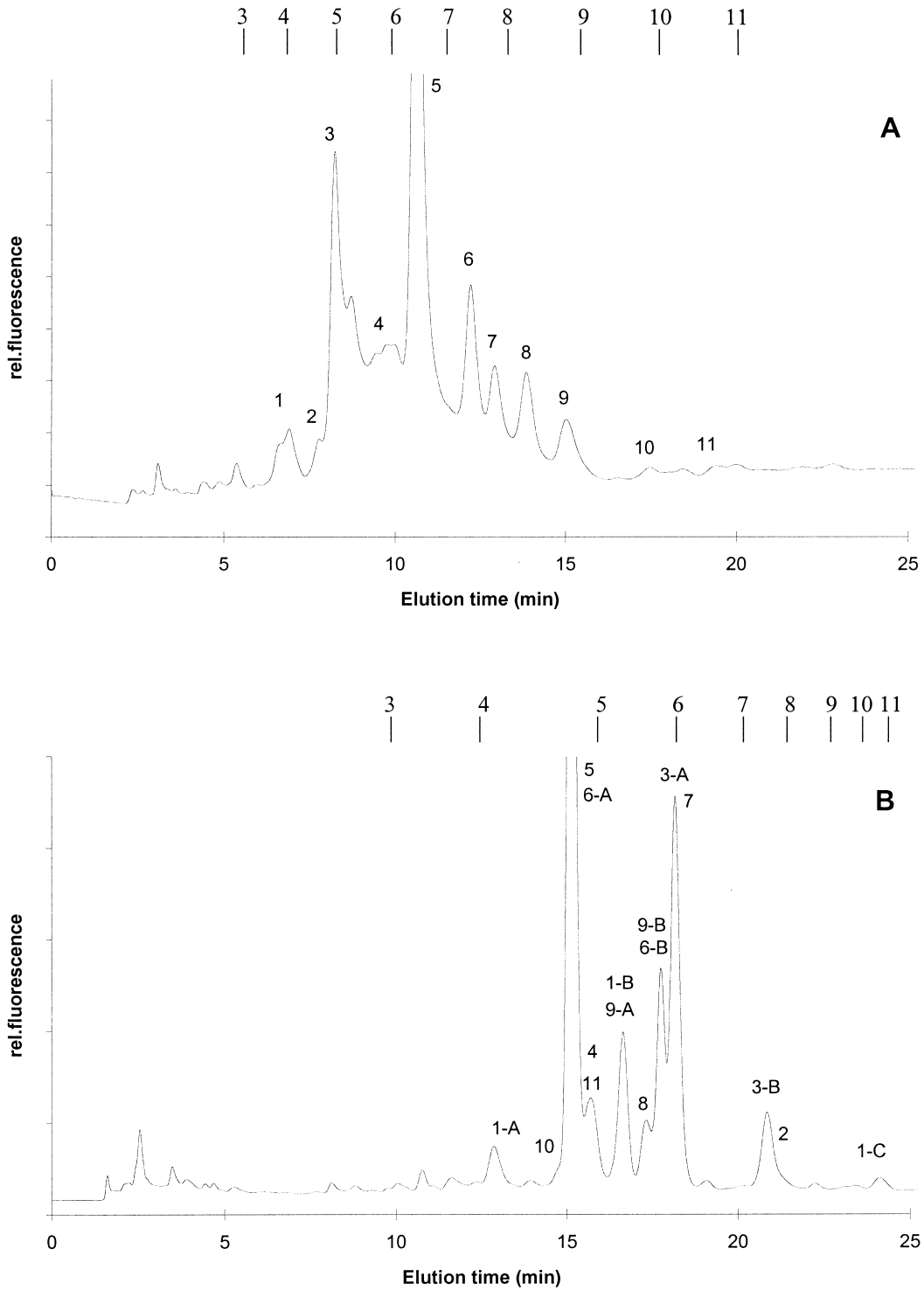


Fig. 1. HPLC profiles of pyridylaminated oligosaccharides from tomato fruit (sample NotAP2). The oligosaccharides released with *N*-glucosidase A were derivatized with 2-aminopyridine and fractionated on MicroPak AX-5 (A) and on Hypersil ODS (B). The corresponding elution times of the individual fractions (indicated by numbers in the charts) on both columns are given in Table 3. Numbers at the top indicate the elution positions of isomaltooligosaccharides with the respective degree of polymerization.

However, the glycan exhibited resistance to α-mannosidase treatment. The identity could be presumed from complete digestion of MMF^3 from phospholipase A_2 with α-mannosidase, resulting in a peak with the same retention behavior as oligosaccharide 1-A on both columns. 1-A is therefore presumed to be $00F^3$.

Oligosaccharide *1-B* also was resistant to α-mannosidase. Together with its size and having the same reten-

Table 1
Influence of α-mannosidase treatment on HPLC retention behavior of pyridylaminated oligosaccharides. Aliquots of about 50–100 pmol of collected HPLC fractions were subjected to α-mannosidase for 20 h and rechromatographed. The lower enzyme dose was applied for the selective removal of α1,3-mannose residues, the higher dose for a complete removal of α-mannose residues. Conversion to products was not necessarily quantitative

Fraction	Enzyme dose	Elution positions on		Product coeluting with
		MicroPak AX-5	Hypersil ODS	
1-C	50 mU	–	8.5	
2	5 mU	3.7	8.7	
	50 mU	2.9	7.2	
3-A	50 mU	4.1	5.2	1-B
3-B	5 mU	4.0	11.0	1-C
	50 mU	–	8.5	
4	5 mU	4.7	5.1	
	50 mU	3.7	4.4	1-A
5	5 mU	5.0	6.0	MOXF[a], 3-A
	50 mU	4.1	5.2	1-B
6-B	5 mU	6.0	7.8	
7	50 mU	3.7	8.7	
		2.9	7.2	
9	50 mU	3.8	8.7	
		2.9	7.1	
10	50 mU	3.7	8.7	
		–	7.2	
11	50 mU	3.7	8.7	
		–	7.2	

[a] The structures of the reference oligosaccharides are given in Section 4.

tion time on reverse-phase HPLC as the product of α-mannosidase digestion of the major structure of horseradish peroxidase (MMXF^3), 1-B was deduced to be *00XF*^{3}.

The third peak present in the first MicroPak fraction, *1-C*, showed a remarkably high retention time on reverse-phase (11.0 glucose units) and sensitivity to α-mannosidase, resulting in a reduction of the retention time to 8.5 glucose units. This corresponds to the retention time of completely digested MMX from zucchini ascorbate–oxidase (Altmann, 1998). Coelution of the limited digestion product from the reference oligosaccharide with 1-C yields a single, homogenous peak as calculated from addition of the peak areas; 1-C was therefore found to be *M0X*.

Peak 2 from MicroPak gave a single peak on reverse-phase, which was sensitive to α-mannosidase; two peaks, lacking one and two mannose residues as deduced from the retention behavior on MicroPak HPLC, were obtained. The second peak on the size fractionation column exhibited the same retention time as fraction 1 of phospholipase A_2. Therefore, the structure of peak 2 was assumed to be *MM*. This was confirmed by coinjection of MM (prepared from bovine

Table 2
Influence of β-*N*-acetylhexosaminidase treatment on HPLC retention behavior of pyridylaminated oligosaccharides. Aliquots of about 50–100 pmol of collected HPLC fractions were digested with β-*N*-acetylhexosaminidase for 20 h and rechromatographed. The lower enzyme dose was applied for partial removal of β-*N*-acetylglucosamine residues, the higher enzyme dose for a complete removal of β-*N*-acetylglucosamines. Conversion to products was not necessarily quantitative

Fraction	Enzyme dose	Elution positions on		Product coeluting with
		MicroPak AX-5	Hypersil ODS	
6-A	10 mU	6.5	4.8	MMXF[a], 5
6-B	10 mU	6.5	4.8	MMXF[a], 5
8	2 mU	7.3	5.8	6-B
		7.3	4.8	6-A
	10 mU	6.5	4.7	MMXF[a], 5

[a] The structures of the reference oligosaccharides are given in Section 4.

fibrin), resulting in a single peak. Furthermore, the α-mannosidase digest of fibrin MM behaved exactly the same way as the digest of peak 2 on both HPLC columns.

Oligosaccharide *3-A* exhibited the same elution behavior in both HPLC systems as the reference oligosaccharide from pineapple stem bromelain, *M0XF³*. α-mannosidase digestion of 3-A resulted in a single peak, which coeluted with compound 1-B (5.2 glucose units on reverse-phase HPLC).

Peak *3-B* was identified based on its size and retention times before and after α-mannosidase digestions in comparison with the standard MMX from fraction 1 of ascorbate oxidase. Partial digestion with α-mannosidase resulted in a peak which coeluted with compound 1-C. The final proof of peak identity was again provided by coinjection of the *MMX* reference compound.

Peak 4, which appeared to be non-homogenous on MicroPak eluted as one major peak (>85%) on the reverse-phase column. The minor components (at least five peaks) were present in minute quantities and could not be analyzed further. The major peak was subjected to α-mannosidase digestion, yielding two signals on reverse-phase HPLC with retention times of 5.1 and 4.4 glucose units, thus indicating the presence of two mannose residues in the structure.

The first peak on reverse-phase coeluted with compound 1-A, thus indicating peak 4 to represent *MMF³*. This assumption could be proved by (i) coinjection of phospholipase A_2-compound 5A (MMF^3), resulting in a single peak and (ii) by digesting the reference compound with α-mannosidase, yielding the same two degradation products as for peak 4.

The major oligosaccharide (5) in all the samples, representing 47–59% of the total *N*-glycan amount, was shown to be susceptible to α-mannosidase digestion, resulting in two peaks: the first eluted at retention times identical to those from oligosaccharide 3-A and the $M0XF^3$ reference compound, the second coeluted with oligosaccharide 1-B, already being elucidated as $00XF^3$. Furthermore, additional evidence for the peak being *MMXF³* was obtained from coinjection of the reference oligosaccharide from horseradish peroxidase, yielding a single peak.

Fraction 6 from MicroPak gave two peaks on the reverse-phase column with relative retention times of 4.7 and 5.8 glucose units, respectively. These peaks were digestible by β-*N*-acetyl-hexosaminidase to a common new compound, exhibiting the same elution behavior as compound 5. Furthermore, hornbeam $GnGnXF^3$ digestion with the same enzyme resulted in formation of the two peaks mentioned above. Again, the final proof, that the two peaks represent *MGnXF³* and *GnMXF³* was accomplished by coinjection of the corresponding oligosaccharides obtained from limited β-*N*-acetylglucosaminidase digestion of hornbeam pollen $GnGnXF^3$.

Oligosaccharide 8 also gave one major peak on reverse-phase HPLC. An initial hexosaminidase digestion resulted in the presence of the peaks with 4.8 and 5.8 glucose units on reverse-phase, thus presumably representing the above mentioned components $MGnXF^3$ and $GnMXF^3$. Complete digestion with β-*N*-acetyl-hexosaminidase yielded a single peak, coeluting with compound 5 and the reference compound $MMXF^3$. Hence, oligosaccharide 8 can be designated to be *GnGnXF³*.

2.4. Oligomannosidic glycans

From the relative retention times on MicroPak of 7.7, 8.7, 9.7 and 10.6 glucose units, oligomannosidic glycans were predicted.

This surmise was verified by performing α-mannosidase digestions of these compounds: each fraction was accessible to α-mannosidase, resulting in the same two peaks (exhibiting relative retention times of 3.7/8.7 and 2.9/7.2 glucose units on MicroPak and Hypersil ODS, respectively; Table 1) for all four fractions. The assumption of these peaks being Manα1–6Manβ1–4GlcNAcβ1–4GlcNAc–pyridylamine and Manβ1–4GlcNAcβ1–4GlcNAc–pyridylamine was confirmed by a limited α-mannosidase digest of MM, which yielded the same two peaks.

Furthermore, endoglycosidase H-digests of the peaks 7 and 9–11 resulted in the formation of pyridylamino–GlcNAc (3.5 glucose units on reverse-phase column), also proving the presence of oligomannosidic structures. Additionally, the authentic oligosaccharides from soybean 7*S*-glycoprotein were used to confirm the identities of the four above-mentioned peaks.

Coelution of peaks 7, 9 and 10 was observed with the corresponding reference compounds $Man_6GlcNAc_2$–$Man_8GlcNAc_2$. Peak 9 consisted of two structural isomers (*M7.1* and *M7.2*), occurring in relative amounts of 45 and 55% (for sample NotAP2 (Table 4)), respectively.

$Man_8GlcNAc_2$ is a homogenous peak on reverse-phase (4.7 GcU), which can be therefore assigned to be the *M8.1*-isomer. The elution behavior of peak 11 corresponds to published data (Tomiya et al., 1991; Kubelka, Altmann, Kornfeld, & März, 1994) and can therefore assigned to be the Man_9-isomer *M9.1*.

Additional confirmation of the results was obtained by MALDI–TOF analysis of individual HPLC fractions. Fig. 2 shows the mass spectrum of fraction 5, identified as $MMXF^3$; Table 5 summarizes the results, which corroborate the assignments based upon two-dimensional mapping.

Table 3
Structures of the *N*-glycans from tomato fruit. The elution data on size-fractionation and reverse-phase column is given in glucose units, together with the structures of the oligosaccharides

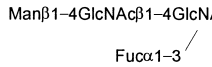
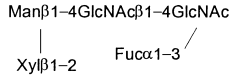
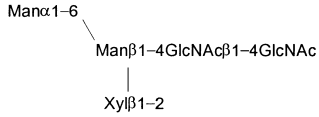
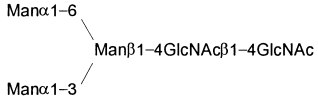
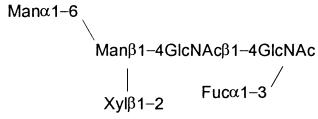
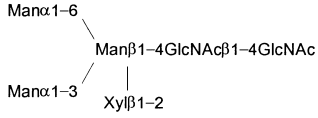
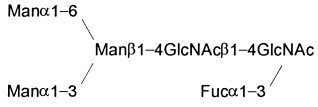
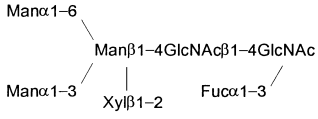
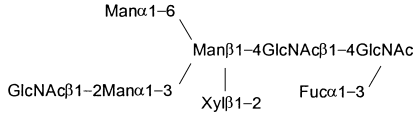
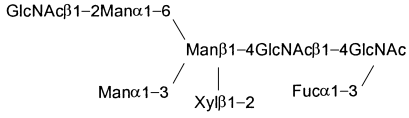
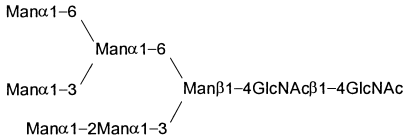
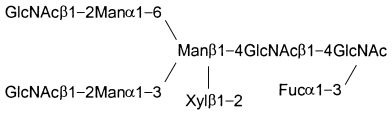
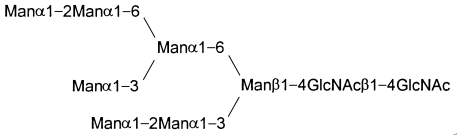
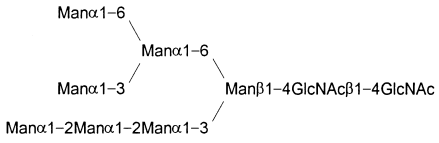
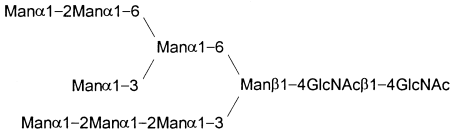
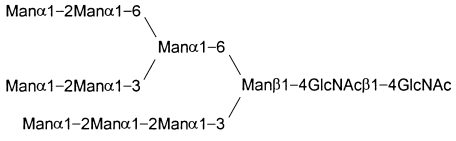
Elution values		Fraction	Structure	Designation
MP	ODS			
3.7	4.4	1-A		$00F^3$
4.0	5.2	1-B		$00XF^3$
4.0	11.0	1-C		M0X
4.7	7.9	2		MM
5.0	6.0	3-A		$M0XF^3$
5.5	7.7	3-B		MMX
5.8	5.0	4		MMF^3
6.5	4.8	5		$MMXF^3$
7.3	4.7	6-A		$MGnXF^3$
7.3	5.8	6-B		$GnMXF^3$

Table 3 (*continued*)

Elution values		Fraction	Structure	Designation
MP	ODS			
7.7	6.1	7		[M6.1][a]
8.3	5.5	8		GnGnXF[3]
8.7	5.1	9-A		[M7.2][a]
8.7	5.7	9-B		[M7.1][a]
9.7	4.6	10		[M8.1][a]
10.6	4.9	11		[M9.1][a]

[a] Codes for oligomannosidic structures according to Tomiya et al. (1991).

2.5. Analysis of tomatoes from two varieties. Analysis of red and green tomatoes

Red Notoro-type tomatoes were subjected to the two different isolation procedures in order to monitor the impact of sample preparation on the glycan pattern in terms of quality and quantity. Furthermore, red and green San Marzano-type fruits were analyzed to distinguish differences in the glycan pattern due to the variety and the stage of ripening, respectively. Table 4 shows the condensed results in terms of relative abundances of individual fractions on the total *N*-glycan amounts.

It could be demonstrated that irrespective of the preparation method very similar results were obtained; San Marzano-type tomatoes contain the same sixteen structures and also the relative quantities were rather similar in the examined varieties. Interestingly, also the state of ripening does not seem to have a significant impact on the appearance of the glycan pattern in terms of quality and quantity although it has to be stated that more samples have to be analyzed for a statistical interpretation.

3. Discussion

The *N*-glycans of tomato fruit were structurally elucidated, thereby comparing two varieties as well as two stages of ripening. The low protein content of

Table 4
Relative abundances of the *N*-linked oligosaccharides in tomato fruit. Abbreviations used: Not, variety Notoro; SM, variety San Marzano; AP, acetone power procedure; AS, ammonium sulfate procedure; r, red; gr, green

Fraction, designation[a]		NotAP1	NotAP2	NotAS	SMr1	SMr2	SMgr	*mean value*
1-A	00F	0.8	1.1	1	1.7	1.5	2.8	1.5
1-B	00XF	0.7	0.4	1.9	2	2	2.3	1.6
1-C	M0X	1.2	2.9	0.9	0.8	1.5	1.2	1.4
2	MM	0.9	0.5	1	2.4	1	1.4	1.2
3-A	M0XF	18.4	18.3	17.3	19.6	18.9	22.1	19.1
3-B	MMX	4.5	3.7	3.3	3.4	2.3	2.3	3.2
4	MMF	2	2.6	5.2	1.9	2.2	1.3	2.5
5	MMXF	51.7	49.4	47.7	58.7	53.4	54	52.5
6-B	GnMXF	7.3	6.8	6.4	5.1	4.8	3.1	5.6
6-A	MGnXF	0.9	1	2.8	0.6	1.1	1.1	1.3
7	Man_6	3.3	2.2	1.1	0.5	1.6	1.9	1.8
8	GnGnXF	3.9	4.9	4.9	0.8	2.1	0.6	2.9
9-A/B	Man_7[b]	2.5	3.9	2.2	0.3	2.6	1.1	2.1
10	Man_8	0.7	0.9	1.8	1	3.7	1.6	1.5
11	Man_9	1.2	1.4	2.5	1.2	1.3	3.2	1.8

[a] Structures are depicted in Table 3.
[b] Isomer structures are not shown separately.

tomatoes and the high amount of free sugars and polysaccharides (Seymour, Colquhoun, DuPont, Parsely, & Selvendran, 1990; York, Kumar Kolli, Orlando, Albersheim, & Darvill, 1997) complicated the effective isolation of the protein fraction. However, the employed low-temperature acetone powder method proved to be very efficient for this application: several by-products are effectively removed, the resulting powder can be stored for several months at −20°C and, although not of relevance in the present study, protein denaturation is minimized due to the extremely low temperature during isolation, yielding extracts of high immunochemical activity (Vieths, Schöning, & Baltes, 1992). In the further sample preparation, two steps shall be briefly addressed: borohydride reduction, which was employed in order to prevent residual free sugars from subsequent derivatization which proved its necessity in the case of grass pollen extracts where several artefactic peaks appeared on the chromatogramms without this step (F. Altmann, personal communication). Second, *N*-glycosidase A from almonds was used for the deglycosylation of the glycopeptides, which is important, since the (more) frequently used *N*-glycosidase F from *Flavobacterium meningosepticum*

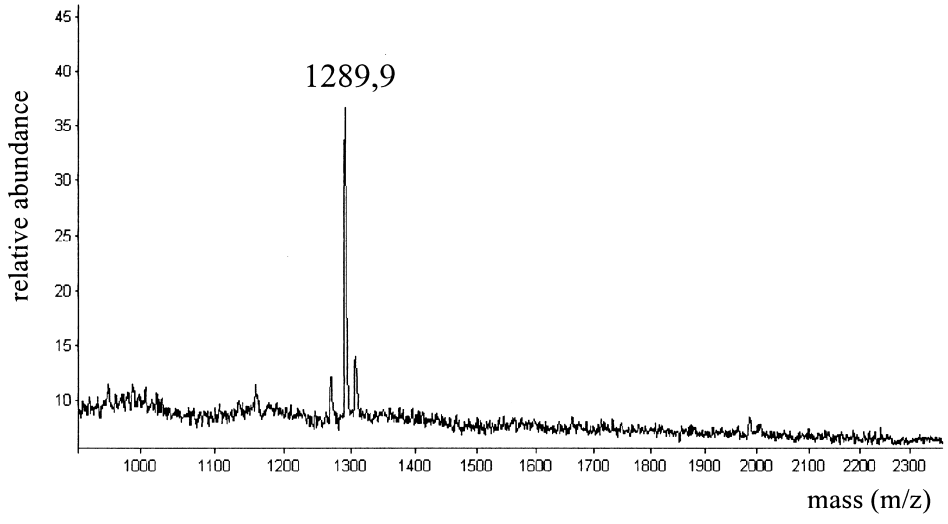


Fig. 2. MALDI analysis of the collected HPLC fraction No. 5, representing MMXF. About 5 pmol of oligosaccharide were dried in vacuo, resuspended in water and mixed with matrix-solution (2%, 2,5-dihydroxybenzoic acid in water containing 30% acetonitrile and 0.5 mM NaCl); an aliquot of 1 μl was analyzed. The spectrum was drawn from 20 summarized shots.

Table 5
Mass determination of tomato fruit *N*-glycans by MALDI. Purified HPLC-fractions of the pyridylaminated *N*-glycans were analyzed (see Fig. 2); the calculated and the observed mass values are compared

Fraction	Designation[a]	Calculated average mass (Dalton)[b]	Observed mass (Dalton)[b]
1-A	00F	849.8	849.7
1-B	00XF	965.8	965.5
1-C	M0X	981.9	982.2
2	MM	1011.9	1011.7
3-A	M0XF	1128.1	1128.1
3-B	MMX	1144.1	1144.2
4	MMF	1158.1	1159.7
5	MMXF	1290.2	1289.9
6-A/B	MGnXF/GnMXF	1493.4	1493.6
7	Man_6	1498.9	1498.4
8	GnGnXF	1696.6	1697.6
9	Man_7	1660.5	1660.7
10	Man_8	1822.6	1822.2
11	Man_9	1984.8	1983.7

[a] The corresponding structures are depicted in Table 3.
[b] Masses represent the $[M + Na]^+$-ion.

cannot cleave glycans containing fucose linked α1–3 to Asn–GlcNAc (Tretter, Altmann, & März, 1991; Altmann, Schweiszer, & Weber, 1995).

The methodology for the structural characterization, using two-dimensional HPLC in combination with exoglycosidase digestions, was first introduced by Tomiya et al. (1988) and has been employed for glycoprotein–glycans from various sources (Takahashi et al., 1986; Kubelka et al., 1994; Ohsuga et al., 1996).

Complex-type structures, most of them truncated and exhibiting α1–3 linked fucose and/or β1–2 linked xylose, as well as oligomannosidic glycans were detected. Most of these structures have been reported to occur in other plant glycoproteins; $MMXF^3$, addressed to be the typical vacuole-type plant *N*-glycan (Sturm, 1995) and the most abundant oligosaccharide in tomato fruit, has also been found in horseradish peroxidase (Kurosaka et al., 1991), a protease inhibitor from barbados pride (*Caesalpinia Pulcherrima* Sw.) seeds (Hase et al., 1986), *Sophora japonica* lectin (Fournet et al., 1987) and ricin D from the seeds of castor plants (*Ricinus communis*) (Kimura et al., 1988). $M0XF^3$, another structure which occurs in relatively high amounts in tomato (17–22%), has been reported for both pineapple stem bromelain (Van Kuik et al., 1986) and barley peroxidase (Johannson, Rasmussen, Harthill, & Welinder, 1992). Small α1,3-fucosylated and/or β1,2-xylosylated glycans that are minor fractions in tomato, have recently also been found in other plants; MMX was found to be the most abundant *N*-glycan in rice α-amylase from germinated seedlings (Hayashi et al., 1990) and also represents the complex oligosaccharide side chain of the bean storage protein phaseolin (Sturm, van Kuik, Vliegenthart, & Chrispeels, 1987). M0X accounts for 15% of the *N*-glycans of barley(1–3,1–4)-β-D-glucan 4-glucanohydrolase isoenzyme EII (Harthill, & Thomsen, 1995), whereas MMF^3 was found to be a minor fraction of soybean peroxidase (Gray, Yun Yang, Hull, Venzke, & Montgomery, 1996). Three structures that exhibit outer-arm GlcNAc residues and represent 6–14% of the *N*-glycans in tomato fruit, have been previously reported for lactase from sycamore cells (Harthill, & Thomsen, 1995). $GnMXF^3$ and $MGnXF^3$ were also found to be present in red kidney bean Fe(III)–Zn(II) purple acid phosphatase (Stahl et al., 1994).

Oligomannosidic structures, accounting for only 3–9% of the *N*-glycans in tomato fruit, are wide-spread among plants (e.g. soybean agglutinin (Dorland, van Halbeek, Vliegenthart, Lis, & Sharon, 1981), phaseolin of the common bean (Sturm et al., 1987), rice α-amylase (Hayashi et al., 1990), α-amylase inhibitor from white kidney bean (*Phasolus vulgaris)* (Yamaguchi, Funaoka, & Iwamoto, 1992), but are also found in insect, yeast and mammalian glycoproteins.

α-1,3-Fucosylation of the innermost *N*-acetylglucosamine residue, a typical feature of *N*-glycans from plants but also from insects, was found in 8 of the 16 tomato *N*-linked oligosaccharides, accounting for 86–90% of the *N*-glycan pool in tomato fruits. Such fucosyl residues as well as β1,2-linked xylosyl residues are not present in mammalian glycoproteins and therefore render plant glycoproteins immunogenic (Wilson et al., 1998). In fact, even IgE antibodies may be directed against this epitope. Owing to their widespread occurrence, α1,3-fucosylated *N*-glycans (with or without xylose) constitute a highly cross-reactive carbohydrate determinant detected in a wide variety of plant as well as insect allergens (Tretter et al., 1993; Garcia-Casado

et al., 1996; Jankiewicz, Aulepp, Altmann, Foetisch, & Vieths, 1998).

As far as tomatoes are concerned, Petersen et al. (1996) reported the reactivity of IgE antibodies of grass pollen allergic individuals to tomato extracts. Lectin blotting of proteins and ELISA inhibition experiments using glycopeptides of known glycan structures suggested the presence of carbohydrate IgE epitopes with an α1,3-fucose residue. The characterization of the tomato *N*-glycans presented in this work, revealing a high degree of α1,3-fucosylated structures in the fruit, offers the structural explanation for these findings.

4. Experimental

4.1. Materials

Red tomatoes (variety Notoro) were bought in a retail shop; red and green tomatoes (variety San Marzano) were obtained from RIKILT-DLO, Netherlands.

N-glycosidase A from almonds and endoglycosidase H from *Streptomyces plicatus* were purchased from Boehringer Mannheim. β-*N*-acetylglucosaminidase (jack bean), α-mannosidase (jack bean) and pepsin (from porcine stomach mucosa) were obtained from Sigma. 2-aminopyridine, sodium cyanoborohydride, dithioerythritol, benzamidine and PMSF were obtained from Sigma.

4.2. Precipitation of proteins using a low-temperature acetone powder method

The extracts were prepared in a similar manner to that described by Vieths, Schöning, and Petersen (1994): 200 g of tomato fruit were homogenized in 400 ml of ice-cold acetone on dry ice. Another 400 ml of ice-cold acetone were added and the sample was kept on dry ice overnight with occasional stirring during the first few hours. The resulting precipitate was filtered through sintered glass and washed twice with acetone and once with acetone/diethylether (1:1, v/v) at −20°C. The sediment was lyophilized. 4 g of acetone powder were taken up in 150 ml phosphate buffered saline (10 mM phosphate pH 7.4; 0.15 M NaCl) and stirred at 4°C for 60 min. After centrifugation (20000 rpm, 20 min, 0°C), the supernatant was filtered (0.45 μm), extensively dialyzed against double-distilled water (membrane 3.5 kD cut-off) overnight and lyophilized.

4.3. Ammonium sulfate precipitation

As an alternative to the above-mentioned procedure, ammonium sulfate precipitation was per-formed: to 200 g of tomato fruit 400 ml of extraction buffer (10 mM phosphate pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM benzamidine, 1 mM DTE, 0.1 mM PMSF) was added and the sample was homogenized. Centrifugation was carried out at 20000 rpm (20 min, 4°C). Solid ammonium sulfate was added to the supernatant to a final concentration of 80% saturation. The mixture was stirred at 4°C for 3 h, centrifuged (20000 rpm, 20 min, 4°C) and the pellet was dialyzed against double-distilled water and lyophilized.

4.4. Purification of N-glycans

The lyophilisate was dissolved in 5% v/v formic acid and digested with pepsin (2%, w/w) for 48 h at 37°C. (Another 1% w/w pepsin was added after 24 h). After centrifugation (10000 rpm, 10 min), the supernatant was adjusted to pH 2.5 and chromatographed on an ion-exchange SP650-M column, 1 × 5 cm (Toso Haas), equilibrated with 5% acetic acid. After application of the sample, the column was rinsed with 40 ml 5% acetic acid. The glycopeptide fraction was eluted with 1 M ammonia (collection of 2 ml fractions). Carbohydrate-positive fractions, detected using the orcinol–sulfuric acid reagent according to Winzler (1955), were pooled and lyophilized. The sample was taken up in 1 ml water; the pH was adjusted to 9 with ammonia. 100 μl of a 2% w/v sodium borohydride solution was added and the sample was incubated at room temperature overnight. After addition of acetic acid and evaporation, this step was repeated twice with 1% v/v acetic acid in methanol and twice with MeOH.

The sample was dissolved in 100 μl citrate/phosphate pH 5.0 and digested with 0.5 mU of *N*-glycosidase A for 37°C for 48 h. Oligosaccharides were separated from residual glycopeptides and peptides using a reverse-phase cartridge as described by Dell et al. (Dell et al., 1993). The glycans were labelled with 2-aminopyridine according to Hase, Ibuki, and Ikenaka (1984). To remove the excess of reagent, a gelfiltration step was performed using a 1 × 40 cm Sephadex G15 column, equilibrated with 1% w/v acetic acid.

4.5. Fractionation of pyridylaminated oligosaccharides by two-dimensional HPLC

A Shimadzu module system, consisting of a system controller, a pump and a fluorescence detector was used. Separations of the pyridylamino-oligosaccharides using two different HPLC columns were performed at room temperature. Glucose oligomers were used in both systems for calibration and as internal standards. Elution volumes of the samples were expressed in glucose units according to Lee, Lee, Tomiya, and Takahashi (1990). Fractionation according to size was

accomplished on a MicroPak AX-5 column (4 × 300 mm, VARIAN); the eluents were as follows: (A) 3% w/v acetic acid, triethylamine pH 7.3: acetonitrile 35:65, v/v and (B) 3% w/v acetic acid, triethylamine pH 7.3: acetonitrile 50:50, v/v. A linear gradient of 0–60%B in 30 min at a flow rate of 1 ml/min was applied. Further separation was achieved by reverse-phase HPLC on a 5 μm hypersil ODS column (Shandon). The column was equilibrated with 0.1 M ammonium acetate pH 4.0 and eluted with a linear gradient of 0–7.5% (by vol.) methanol in 25 min at a flow rate of 1.5 ml/min. In both systems, pyridylaminated oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively.

4.6. Exoglycosidase digestions

Generally, about 50–100 pmol of oligosaccharide were incubated for 20 h at 37°C.

The following buffers and enzyme quantities were used: 50 mU α-mannosidase in 50 mM sodium acetate, pH 4.2 containing 0.1 mM $ZnCl_2$ to achieve complete removal of α-linked mannose residues; 5 mU of α-mannosidase to selectively remove α1,3-linked mannose residues; 2 mU β-*N*-acetylhexosaminidase (for partial hydrolysis) or 10 mU of the enzyme for complete hydrolysis, using 0.1 M sodium citrate, pH 5.0; 1 mU endoglycosidase H in 0.1 M sodium citrate/phosphate, pH 5.0.

4.7. Preparation of reference oligosaccharides

Pyridylaminated oligosaccharides used as reference substances were prepared from the following sources: Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc (MM) from bovine fibrin (Staudacher, Kubelka, & März, 1992); Manα1–6(Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4GlcNAc (MMX) from zucchini ascorbate oxidase (Altmann, 1998); Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc (MMF^3) from honeybee venom phospholipase A_2 (Kubelka et al., 1993); Manα1–6(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc ($M0XF^3$) from pineapple stem bromelain (Van Kuik et al., 1986); Manα1–6(Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc ($MMXF^3$) from horseradish peroxidase (Kurosaka et al., 1991); GlcNAcβ1–2Manα1–6(GlcNAcβ1–2Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc ($GnGnXF^3$) from hornbeam pollen (Wilson, & Altmann, 1998); $Man_6GlcNAc_2$ [M6.1][1], $Man_7GlcNAc_2$ [M7.1], [M7.2], and $Man_8GlcNAc_2$ [M8.1] from soybean 7*S*-glycoprotein (Neeser, Vedovo, Mutsaers, & Vliegenthart, 1985). The structures of the reference oligosaccharides were confirmed by methylation analysis (data not shown). For column calibration, pyridylaminated isomaltooligosaccharides were prepared from partially hydrolyzed dextran.

4.8. Matrix-assisted laser desorption/ionization – time of flight – mass spectrometry

MALDI–TOF–MS data was obtained using a Dynamo instrument (Thermo Bioanalysis, UK), operating in the positiv-ion linear mode.

About 5–10 pmol of pyridylaminated tomato *N*-glycans (individual reverse-phase HPLC fractions) were repeatedly dried under nitrogen to remove buffer components; the samples were finally dissolved in 6 μl water and 3 μl matrix solution (2% 2,5-dihydroxybenzoic acid in water containing 30% acetonitrile and 0,5 mM NaCl). Aliquots of 1 μl were applied on the target and allowed to dry. Mass spectra were obtained by averaging 20 single spectra. External mass calibration was performed with pyridylaminated $GlcNAc_2Man_3GlcNAc_2$ ($MNa^+ = 1418{,}2$ Da) which was prepared from bovine fibrin as described (Altmann, Schwihla, Staudacher, Glössl, & März, 1995).

4.9. HPLC-analysis of OPA-aminosugars

An aliquot of the glycopeptide fraction was hydrolyzed with 4 M TFA and aminosugar analysis by reverse-phase chromatography was performed as described by Altmann (1992).

Acknowledgements

We thank Hubert Noteborn, RILILT-DLO for supplying us with the tomato samples and Thomas Dalik for performing the aminosugar analyses. This work was financed by the EU-project AIR3-CT94-2311.

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[1] Codes for oligomannose structures according to Tomiya et al. (1991).

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