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Primary Structure of the Xylose-containing *N*-Linked Carbohydrate Moiety from Ascorbic Acid Oxidase of *Cucurbita pepo medullosa*

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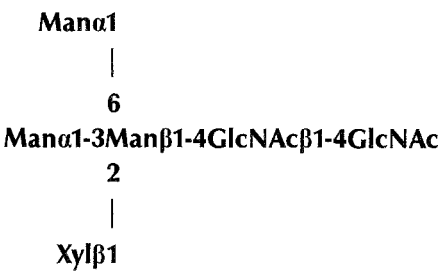
Ascorbic acid oxidase (E.C.1.10.3.3) from the green zucchini squash (*Cucurbita pepo medullosa*) is a copper-containing glycoprotein which catalyzes the reaction: L-ascorbic acid + 1/2 O_2 → L-dehydroascorbic acid + H_2O. The carbohydrate content of the purified plant glycoprotein amounted to 3% (w/w), and monosaccharide analysis revealed the carbohydrate moiety to be of the *N*-glycosidic type. The carbohydrate chains were released from the apoenzyme by digestion with PNGase-F immobilized on Sepharose 4B. After fractionation on Bio-Gel P-2 and purification on Mono-Q, the neutral oligosaccharide was investigated by 500-MHz ^{1}H-NMR spectroscopy. The primary structure of the *N*-linked carbohydrate chain was established to be:



Abbreviations: AAO, ascorbic acid oxidase; PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; Xyl, xylose; GLC, gas-liquid chromatography; FPLC, fast protein liquid chromatography; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Ascorbic acid oxidase (E.C.1.10.3.3) is a copper-containing enzyme widely found in plants. With ceruloplasmin and laccase it belongs to the group of copper proteins known as "blue oxidases" [1]. It exhibits a high degree of specificity towards L-ascorbic acid and certain related compounds [2]. AAO from green zucchini squash (*Cucurbita pepo medullosa*) is a dimer with a molecular mass of 140 kDa containing eight copper atoms per molecule. In view of the glycoprotein nature of this enzyme and the possible biological significance of the carbohydrate chains, the characterization of the oligosaccharide moieties was undertaken.

Materials and Methods

AAO from *C. pepo medullosa* purified according to [3] was a gift of Prof. Dr. L. Avigliano (University of L'Aquila, Italy). PNGase-F (E.C.3.5.1.52) was purchased from Boehringer Mannheim, W. Germany; CNBr-activated Sepharose 4B from Pharmacia, Uppsala, Sweden; and Bio-Gel P-2 (minus 400 mesh) from Bio-Rad Labs, Richmond, CA, USA.

Preparation of Copper-free Enzyme

AAO (18 mg), dissolved in 5 ml bidistilled H_2O, was dialyzed against 1 l 0.1 M potassium phosphate buffer, pH 7.2, containing 25 mM KCN, for 24 h with three intermediate changes. Subsequently, cyanide was removed by dialysis against 1 l 0.08 M potassium phosphate buffer, pH 6.0, for 48 h with three intermediate changes in the presence of 10 mM EDTA and three intermediate changes without EDTA. The final solution was dialyzed against 1 l bidistilled H_2O for 24 h with three intermediate changes, and lyophilized. All dialysis steps were carried out at 4°C.

Monosaccharide Analysis

Purified glycoprotein (1 mg) was subjected to methanolysis (1 M methanolic HCl, 24 h, 85°C), followed by GLC of the trimethylsilylated (re-*N*-acetylated) methyl glycosides on a capillary CP Sil 5 CB WCOT fused silica column (0.34 mm × 25 m; Chrompack) using a Perkin-Elmer gas-chromatograph Model 8410 [4, 5].

PNGase-F Digestion

The lyophilized apo-form of the enzyme (17 mg) was dissolved in 1.5 ml 50 mM Tris/HCl buffer, pH 8.4 containing 1% SDS (w/v), 1% 2-mercaptoethanol (v/v) and 10 mM EDTA, and kept for 50 min at 37°C. Then the sample was loaded on a CNBr-activated Sepharose 4B-immobilized PNGase-F column (0.7 × 8 cm) and incubated for 48 h at room temperature. PNGase-F (15 U) was coupled to CNBr-activated Sepharose 4B (1 g) as described [6]. The reaction products were eluted with 5 bed volumes of 50 mM Tris/HCl buffer, pH 8.4, containing 10 mM EDTA, and lyophilized.

Fractionation on Bio-Gel P-2

After PNGase-F digestion, the lyophilized eluate was dissolved in 1.2 ml bidistilled H_2O and fractionated on a Bio-Gel P-2 column (1 × 20 cm) at a flow rate of 7.5 ml/h. The elution was monitored by refractive index detection using a Bischoff refractometer. The fractions obtained were analyzed for carbohydrate by the phenol/sulfuric acid assay [7].

FPLC

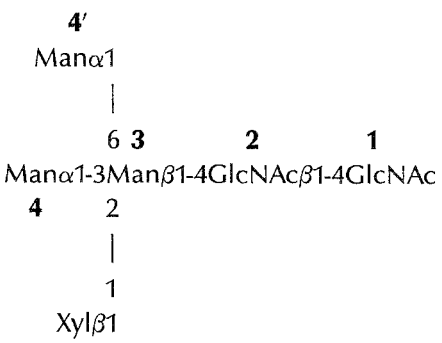
Medium-pressure anion-exchange chromatography of lyophilized Bio-Gel P-2 fractions was performed on a Pharmacia Fast Protein Liquid Chromatography apparatus, equipped with a Liquid Chromatography Controller LCC-500 and two P-500 pumps. Samples, dissolved in 0.7 ml H_2O (Lichrosolv, Merck, Darmstadt, W. Germany), were fractionated on a Mono Q HR 5/5 column at a flow rate of 1.75 ml/min, using a linear concentration gradient from 0-500 mM NaCl in 8 ml H_2O (Lichrosolv, Merck). The elution was monitored at 214 nm by a Pharmacia UV-1/214 detector connected to a dual-chart recorder. Each fraction was checked by the phenol/sulfuric acid assay [7]. Carbohydrate-containing fractions were desalted on Bio-Gel P-2 using H_2O as eluent.

500-MHz ^{1}H-NMR Spectroscopy

Purified oligosaccharide was repeatedly exchanged in ^2H_2O, finally using 99.96 atom % ^2H_2O (Aldrich, Milwaukee, WI, USA) at room temperature. Resolution-enhanced 500-MHz ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating in the Fourier-transform mode at a probe temperature of 27°C. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentanesulfonate (DSS), but were actually measured by reference to internal acetone in ^2H_2O (δ = 2.225 ppm) [8].

Results

Ascorbic acid oxidase isolated from *C. pepo medullosa* behaved as a single band when analyzed on SDS-PAGE (data not shown). Monosaccharide analysis [5] of the purified enzyme revealed the presence of mannose, *N*-acetylglucosamine and xylose in the molar ratio of 3.0 : 1.73 : 0.87, suggesting the occurrence of *N*-linked carbohydrate chains only. For structural analysis the oligosaccharide part was released from the apoenzyme by PNGase-F digestion. Fractionation of the digest on Bio-Gel P-2 yielded one carbohydrate-positive fraction, which was further subfractionated on Mono Q, resulting in a neutral carbohydrate-containing fraction only. This fraction was subjected to 500-MHz ^{1}H-NMR spectroscopy (Fig. 1). Relevant ^{1}H-NMR parameters, together with those of suitable reference compounds, are listed in Table 1.



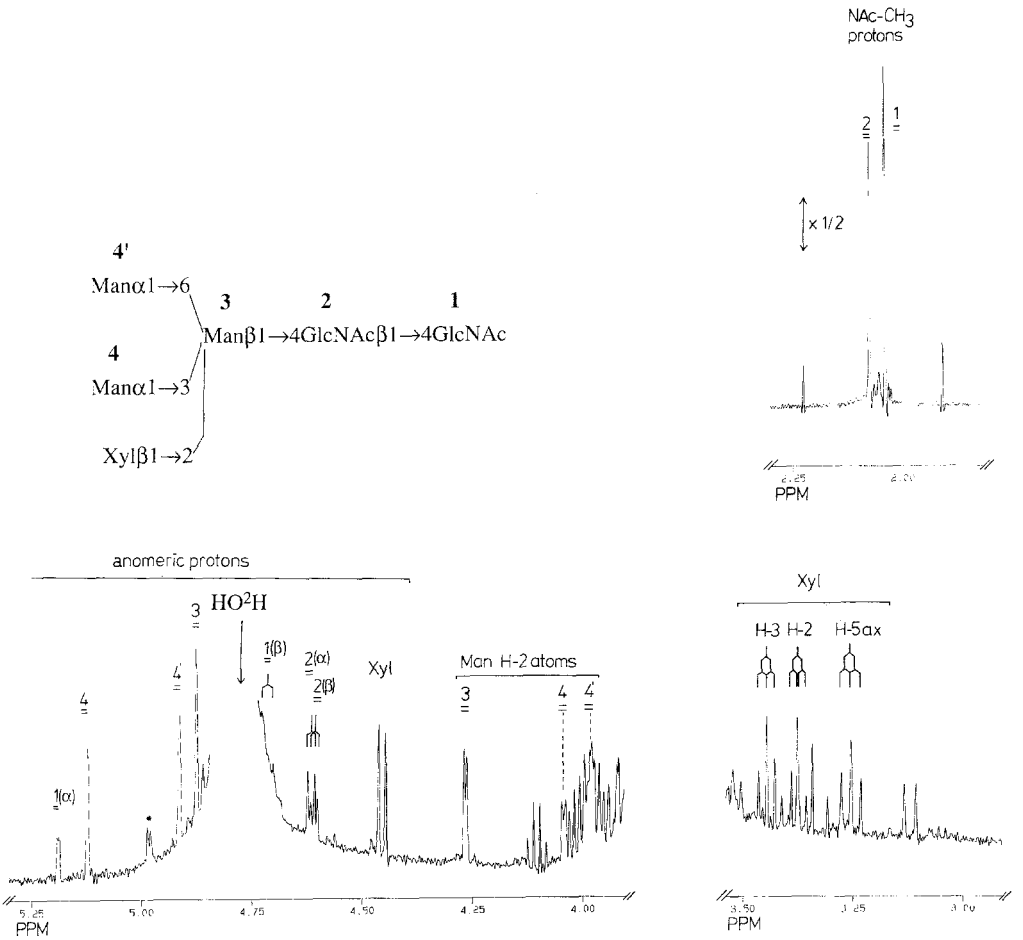
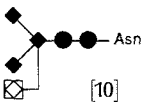
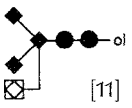
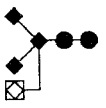


Figure 1. Structural-reporter-group region of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of the carbohydrate chain of ascorbic acid oxidase from *Cucurbita pepo medullosa*. The numbers in the spectra refer to the corresponding residues in the structure. * = unknown contaminant.

The ^{1}H-NMR data of the neutral Mono Q fraction demonstrated the following carbohydrate chain to occur in AAO:

The *N,N'*-diacetylchitobiose structural element is characterized by the following structural reporters. The GlcNAc-**1** H-1α, H-1β and NAc signals are found at δ = 5.189 ppm, δ = 4.703 ppm and δ = 2.039 ppm, respectively. The GlcNAc-**2** H-1 signal is represented by two doublets at δ = 4.615 ppm and δ = 4.607 ppm (2:1), due to the anomerization effect of the reducing GlcNAc-**1** unit, whereas the GlcNAc-**2** NAc signal is present at δ = 2.074 ppm. These structural-reporter-group signals match completely those of the corresponding residues in $Man_3GlcNAc_2$ [9] (see also [6] for ^{1}H-NMR data of bi-antennary oligosaccharide chains from human chorionic gonadotropin and human serotransferrin). The branching mannotriose core is reflected by the characteristic patterns of the Man H-1 and Man H-2 signals (Table 1). The occurrence of the β(1-2)-linked xylose

Table 1. ^{1}H-Chemical shifts of structural-reporter groups of the constituent monosaccharides for the oligosaccharide derived from ascorbic acid oxidase, together with those for reference compounds derived from phaseolin [10] and hemocyanin [11]. Chemical shifts are given at 27°C, in ppm downfield from internal sodium, 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O. Compounds are represented by short-hand-symbolic notation [18]; ●, GlcNAc; ◆, Man; ⊠, Xyl. For numbering of the monosaccharide residues, see text.

Residue	Reporter group	 [10]	 [11]	
GlcNAc-**1**(-ol)	H-1	5.048	N.D.[a]	5.189(α) 4.703(β)
	H-2	N.D.	4.239	N.D.
	NAc	2.010	2.057	2.039
GlcNAc-**2**	H-1	4.608	4.634	4.615(α) 4.607(β)
	NAc	2.070	2.073	2.074
Man-**3**	H-1	4.869	4.883	4.875
	H-2	4.264	4.270	4.265
Man-**4**	H-1	5.121	5.122	5.122
	H-2	4.037	4.039	4.041
Man-**4'**	H-1	4.911	4.913	4.912
	H-2	3.981	3.983	3.982
Xyl	H-1	4.447	4.449	4.453
	H-2	3.373	3.377	3.375
	H-3	3.438	3.437	3.444
	H-5ax	3.248	3.250	3.253

[a] N.D. = Not determined.

residue at this mannotriose is indicated by the highly diagnostic H-1, H-2, H-3 and H-5ax reporter-group signals at δ = 4.453 ppm, δ = 3.375, δ = 3.444 ppm and δ = 3.253 ppm, respectively. As is evident from Table 1, the structural reporters for the Man_3Xyl structural element are in full agreement with the ^{1}H-NMR parameters reported earlier for the corresponding reference compounds $Man_3XylGlcNAc_2Asn$ [10] and $Man_3XylGlcNAc$-GlcNAc-ol [11]. It is interesting to note that the structural differences in the GlcNAc-GlcNAc unit, namely disaccharide, disaccharide-alditol or disaccharide-Asn, have no real influence on the structural-reporter-group signals of the Man_3Xyl part of the carbohydrate chain, suggesting no large differences in conformation of the latter element.

Discussion

For the carbohydrate part of the enzyme ascorbic acid oxidase, only one *N*-linked oligosaccharide structure has been detected. Based on a molecular mass of 140 kDa, the calculated carbohydrate content of 3% (w/w) indicates the presence of four glycan

chains per molecule. A series of recent studies have shown that the xylosylated mannotriosyl-*N,N'*-diacetylchitobiose glycan ($Man_3XylGlcNAc_2$) seems to be a common core element of many plant *N*-glycoproteins. The same structure has been demonstrated to occur in phaseolin, the major storage protein of the common bean *Phaseolus vulgaris*, together with $Man_9GlcNAc_2$ and $Man_7GlcNAc_2$ chains [10]. A fucosylated hexasaccharide, with Fuc α(1-3)-linked at GlcNAc-**1**, has been identified in lectins from five *Erythrina* species [12], from *Sophora japonica* [12, 13], and from *Lonchocarpus capassa* [12]. In the *Erythrina* species the non-fucosylated form occurred as a minor constituent. In ricin and castor bean hemagglutinin from *Ricinus communis* seeds the following oligosaccharides have been found: $Man_3XylGlcNAc(Fuc\alpha1\text{-}3)GlcNAc$, $Man_3XylGlcNAc_2$, $Man_4XylGlcNAc_2$, and $Man_{4\text{-}7}GlcNAc_2$ [14]. On the other hand the protease inhibitor from barbados pride (*Caesalpinia pulcherrima* Sw.) seeds contains the $Man_3XylGlcNAc(Fuc\alpha1\text{-}3)GlcNAc$ chain only [15]. For several other legume lectins the presence of fucose and/or xylose has been shown [12]. In a few cases partial structures have been proposed [12, 16]. In sycamore cell laccase (*Acer pseudoplatanus* L.) the presence of $Man_3XylGlcNAc(Fuc\alpha1\text{-}3)GlcNAc$ in combination with bi-antennary complex type of extensions has been demonstrated, whereby the peripheral *N*-acetylglucosamine residues can be α(1-6)-fucosylated [17]. In pineapple stem bromelain, the α(1-3)-fucosylated form of the core element (but missing Man-**4**) has been detected [18, 19].

Up to now only two examples of glycoproteins of animal origin, containing the $Man_3XylGlcNAc_2$ core element have been found. The low-molecular-mass carbohydrate chains of hemocyanin from the terrestrial snail *Helix pomatia* have been characterized as a hexasaccharide, mainly α(1-6)-fucosylated at GlcNAc-**1** [11]. For the low-molecular-mass carbohydrate chain of hemocyanin from the fresh-water snail *Lymnaea stagnalis* a non-fucosylated core element with terminal 3-*O*-methylmannose instead of mannose has been reported [20], together with unusually complex bi-antennary extensions of this hexasaccharide [21].

The biosynthesis of xylose-containing *N*-linked carbohydrate chains in glycoproteins from plant and animal origin is still subject to speculation. On the basis of the structures established so far and of some biosynthetic experiments, interesting suggestions have been reported for the substrate specificity of the β(1-2)-xylosyltransferase involved [10, 14].

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References

1 Fee JA (1975) in Structure and Bonding, eds. Dunitz JD, Hemmerich P, Holm RH, Ibers JA, Jorgensen CK, Neilands JB, Reinen D, Williams RJP, Springer Verlag, Heidelberg, 23:1-60.
2 Mondovi B, Avigliano L (1984) in Copper Proteins and Copper Enzymes, ed. Lontie R, CRC Press, Boca Raton, Florida, 3:101-18.
3 Avigliano L, Gerosa P, Rotilio G, Finazzi-Agro' A, Calabrese L, Mondovi B (1972) Ital J Biochem 21:248-55.
4 Hall RL, Wood EJ, Kamerling JP, Gerwig GJ, Vliegenthart JFG (1977) Biochem J 165:173-76.
5 Kamerling JP, Vliegenthart JFG (1982) Cell Biol Monogr 10:95-125.
6 Damm JBL, Kamerling JP, Van Dedem GWK, Vliegenthart JFG (1987) Glycoconjugate J 4:129-44.
7 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Anal Chem 28:350-56.
8 Vliegenthart JFG, Dorland L, Van Halbeek H (1983) Adv Carbohydr Chem Biochem 41:209-374.
9 Baussant T, Strecker G, Wieruszeski J-M, Montreuil J, Michalski J-C (1986) Eur J Biochem 159:381-85.
10 Sturm A, Van Kuik JA, Vliegenthart JFG, Chrispeels MJ (1987) J Biol Chem 262:13392-403.
11 Van Kuik JA, Van Halbeek H, Kamerling JP, Vliegenthart JFG (1985) J Biol Chem 260:13984-88.
12 Ashford D, Dwek RA, Welply JK, Amatayakul S, Homans SW, Lis H, Taylor GN, Sharon N, Rademacher TW (1987) Eur J Biochem 166:311-20.
13 Fournet B, Leroy Y, Wieruszeski J-M, Montreuil J, Poretz RD, Goldberg R (1987) Eur J Biochem 166:321-24.
14 Kimura Y, Hase S, Kobayashi Y, Kyogoku Y, Funatsu G, Ikenaka T (1987) J Biochem (Tokyo) 101:1051-54.
15 Hase S, Koyama S, Daiyasu H, Takemoto H, Hara S, Kobayashi Y, Kyogoku Y, Ikenaka T (1986) J Biochem (Tokyo) 100:1-10.
16 Prigent M-J, Montreuil J, Strecker G (1984) Carbohydr Res 131:83-92.
17 Takahashi N, Hotta T, Ishihara H, Mori M, Tejima S, Bligny R, Akazawa T, Endo S, Arata Y (1986) Biochemistry 25:388-95.
18 Ishihara H, Takahasi N, Oguri S, Tejima S (1979) J Biol Chem 254:10715-19.
19 Van Kuik JA, Hoffmann RA, Mutsaers JHGM, Van Halbeek H, Kamerling JP, Vliegenthart JFG (1986) Glycoconjugate J 3:27-34.
20 Van Kuik JA, Sijbesma RP, Kamerling JP, Vliegenthart JFG, Wood EJ (1986) Eur J Biochem 160:621-25.
21 Van Kuik JA, Sijbesma RP, Kamerling JP, Vliegenthart JFG, Wood EJ (1987) Eur J Biochem 169:399-411.