Structural study of the asparagine-linked oligosaccharides of lipophorin in locusts

Eriko Nagao and Haruo Chino

Biochemical Laboratory, Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan

Abstract The complete structure of oligosaccharides from locust lipophorin was studied. The asparagine-linked oligosaccharides were first liberated from the protein moiety of lipophorin by digestion with almond glycopeptidase (N-oligosaccharide glycopeptidase, EC 3.5.1.52). Two major oligosaccharides (E and F), separated by subsequent thin-layer chromatography, were analyzed by methylation analysis and ^{1}H-NMR. Based on the experimental data, the whole structure of oligosaccharide E was identified as Manα1→2Manα1→6(Manα1→2Manα1→3)Manα1→6(Manα1→2Manα1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc. The data also revealed that oligosaccharide F is identical with oligosaccharide E in the structure, except for one glucose residue that is linked to the nonreducing terminal Manα1→2 residue. —**Nagao, E., and H. Chino.** Structural study of the asparagine-linked oligosaccharides of lipophorin in locusts. *J. Lipid Res.* 1987. **28:** 450–454.

Supplementary key words methylation analysis • nuclear magnetic resonance

Lipophorin which exists in the hemolymph of most insect species, is a unique lipoprotein that serves as a reusable shuttle to transport a variety of lipid classes, including diacylglycerol, cholesterol, hydrocarbons, and carotenoids between tissues (1). Lipophorin also contains carbohydrates, mainly mannose (about 3% relative to the amount of protein) and N-acetylglucosamine, which are covalently associated with the apoproteins (2). The carbohydrate chains may serve as recognition sites when lipophorin loads and unloads a particular lipid at a specific tissue. In order to test this possibility, it is necessary to elucidate the structure of these carbohydrate chains.

We have reported previously (3) that there are six molecular species, two major (E, F) and four minor (A, B, C, D), of oligosaccharides linked with an asparagine residue of the apoprotein of locust lipophorin. The following compositions were proposed: $Man_5GlcNAc_2$ for A, $Man_6GlcNAc_2$ for B, $Man_7GlcNAc_2$ for C, $Man_8GlcNAc_2$ for D, $Man_9GlcNAc_2$ for E, and $Glc_1Man_9GlcNAc_2$ for F, respectively. The present study was designed to further elucidate the structure of the two major oligosaccharides (E and F), using methylation analysis and ^{1}H-NMR. The results indicate that oligosaccharide E has a high-mannose type structure identical to that of bovine thyroglobulin oligosaccharide, whereas oligosaccharide F has one glucose residue linked to the nonreducing terminal Manα2 residue.

MATERIALS AND METHODS

Animals

Adult locusts (3–5 weeks after final molt), *Locusta migratoria,* were taken from colonies maintained in this laboratory.

Chemicals

Pepsin was purchased from Worthington Biochemical Corporation. Almond glycopeptidase was prepared from almond nuts as described by Takahashi and Nishibe (4) with a slight modification. This enzyme acts specifically on the linkage (β-asparatyl-glucosamine linkage) between asparagine and N-acetylglucosamine (5). All other chemicals were of analytical grade and solvents were redistilled as appropriate. Double glass-redistilled water was used throughout.

Purification of lipophorin

Hemolymph was collected by the "flushing method" (6) from male and female adult locusts, and was centrifuged at 10,000 *g* for 5 min to remove the hemocytes. Lipophorin was purified from the freshly collected hemolymph according to the method of Chino and Kitazawa (2).

Liberation and isolation of oligosaccharides from lipophorin

All the procedures were essentially similar to those described previously (3). The lipids were first removed

Abbreviations: NMR, nuclear magnetic resonance: TLC, thin-layer chromatography.

from lipophorin with chloroform–methanol 2:1 (v/v). The delipidated lipophorin (about 10 mg for each run) was digested with 0.2 mg of pepsin in 1 ml of 0.01 N HCl (pH 2.0) at 37°C for 16 hr and then heated at 100°C for 30 min. The digested sample was evaporated to dryness by a nitrogen stream. The residue was dissolved in 0.5 ml of 100 mM citrate–phosphate buffer (pH 5.0) and incubated with almond glycopeptidase. After incubation with almond glycopeptidase at 37°C for 16 hr, the reaction mixture was passed successively through two different columns (0.5 × 2.5 cm) of Dowex 50-X8 (H^+) and Dowex 1-X8 (CO_3^{2-}) to obtain the neutral fraction. The neutral fraction was evaporated to dryness by a nitrogen stream, dissolved in a small volume of water, and then applied to a thin-layer chromatography (TLC) plate. The silica gel 60 TLC plates (Merck Art. 5553) were developed with n-propanol–acetic acid–water 3:3:2 (v/v/v) (7). The spots were visualized with charring after spraying with orcinol–H_2SO_4 reagent. Two major oligosaccharide spots (E and F), separated on a silica gel plate, were scraped and extracted with water. An aliquot of the extracts was concentrated as appropriate and rechromatographed by TLC. Each oligosaccharide always appeared as a distinct single spot, thus confirming the previously reported homogeneity (3).

Methylation analysis

The pooled oligosaccharide samples were methylated by the method of Cicanu and Kerek (8). The finely powdered NaOH (10 mg) and methyl iodide (50 μl) were added to a solution of the sample (30–40 μg) in methyl sulfoxide (100 μl). The mixture was stirred for 1 hr in a sealed vial at room temperature. Water (1 ml) and chloroform (1 ml) were then added, and the chloroform layer was washed with 1 ml water three times and dried under a nitrogen stream. The methylated samples thus obtained were then hydrolyzed, reduced, and acetylated as described by Stellner, Saito, and Hakomori (9). The resulting alditol acetates were analyzed by gas–liquid chromatography–mass spectrometry (JOEL JMS-DX 300) with a 3% OV-17 on Gas-chrom Q column (2 mm × 1 m); the injection temperature was 250°C, and the column temperature was from 150°C to 250°C at 4°C/min. Conditions for the mass spectrometry were: ionizing energy, 30 eV; separator temperature, 250°C; and accelerating voltage, 1.35 KV.

NMR analysis

The pooled samples [oligosaccharide E (0.30 mg) and oligosaccharide F (0.75 mg)] were exchanged with 99.98% ^2H_2O (Merck) and concentrated by lyophilization. This process was repeated three times. The samples were then dried over P_2O_5 in vacuo and resuspended in 0.4 ml of 99.995% ^2H_2O (Merck) with 3-(trimethylsilyl)-propionic acid-d_4 sodium as an internal marker, using 5-mm tubes (Wilmad Glass Co.). The compounds were examined by ^{1}H-NMR spectroscopy (JOEL GX-400) at 400 MHz at 40°C and 70°C. Spectra were recorded by using 32K data blocks and a sweep width of 6,000 Hz. Pulses of 45° were used with a cycle delay time of 4.0 sec. With the final concentrations of oligosaccharide (0.75 mM for E and 1.88 mM for F), 250 sweeps were utilized for adequate signal-to-noise ratio. Assignments of linkage, anomeric configuration, and substitution of compounds in various spectra were made by comparison of chemical shifts of anomeric hydrogens (Cl–H) in closely related or identical compounds (10–12).

RESULTS AND DISCUSSION

Methylation analysis

As previously reported (3), $Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$ were proposed as the sugar compositions of oligosaccharides E and F, respectively. The methylation analysis was performed in order to obtain detailed information of the structure of these two oligosaccharides. **Table 1** summarizes the results of methylation analysis of two oligosaccharides and demonstrates that oligosaccharide E contains nonreducing terminal (unsubstituted) mannose, 2-O-substituted mannose, and 3,6-di-O-substituted mannose in the approximate proportions of 3:4:2, and that the oligosaccharide F contains unsubstituted mannose and/or glucose, 2-O-substituted mannose and/or glucose, 3-O-substituted mannose and 3,6-di-O-substituted mannose in the approximate proportions 3:4:1:2.

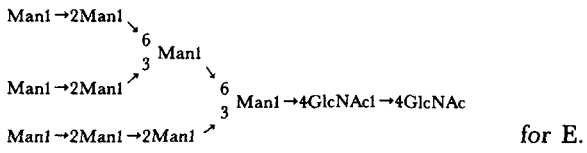
Combining the above data with the known pathway of

TABLE 1. Methylation analysis of oligosaccharides E and F

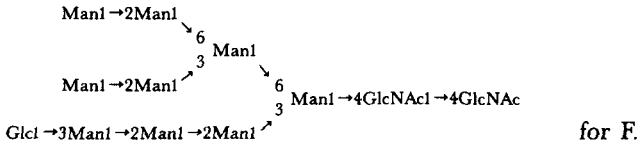
Methylated Sugars	Molar Ratio[a] E	F
Glucitol and/or mannitol		
2,3,4,6-Tetra-O-methyl(1,5-di-O-acetyl)	3.0	3.0
3,4,6-Tri-O-methyl(1,2,5-tri-O-acetyl)	4.0	3.5
Mannitol		
2,4,6-Tri-O-methyl(1,3,5-tri-O-acetyl)	trace[b]	1.2
2,4-Di-O-methyl(1,3,5,6-tetra-O-acetyl)	2.2	1.7
2-N-Methylacetamido-2-deoxyglucitol[c]		
3,6-Di-O-methyl(1,4,5-tri-O-acetyl)	1.1	1.4

[a] 2,3,4,6-Tetra-O-methylmannitol and/or glucitol set to 3.0.
[b] Less than 0.3.
[c] The recovery of these fragments was generally low, as compared with that of neutral alditol acetate. The theoretical molar ratios calculated from the proposed oligosaccharide compositions ($Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$) should be 2.0.

biosynthesis of asparagine-linked oligosaccharide (13), the following structure may be proposed for the E and F oligosaccharides:



for E.



for F.

NMR analysis

In order to provide further experimental basis for the structures of oligosaccharides deduced from methylation analysis, the oligosaccharides were examined by ^{1}H-NMR spectroscopy at 400 MHz. The results are illustrated in **Fig. 1** and the chemical shifts of resonances arising from the C1-H hydrogens were assigned as shown in **Table 2.** Examination of the anomeric region of the spectrum reveals the presence of a relatively homogeneous high-mannose type oligosaccharide and each mannose spectrum of oligosaccharide E is identical with the ^{1}H-NMR spectra

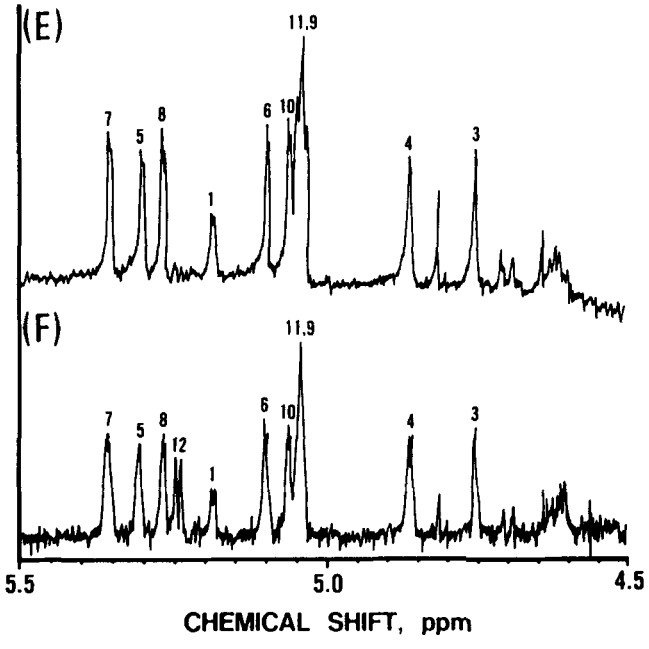
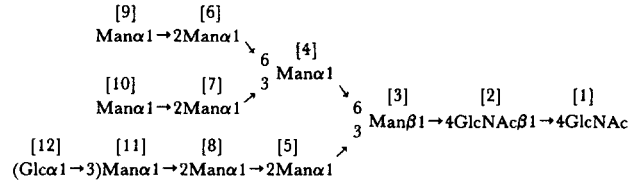


Fig. 1. ^{1}H-NMR spectra at 70°C and 400 MHz of oligosaccharides E ($Man_9GlcNAc_2$) and F ($Glc_1Man_9GlcNAc_2$) from locust lipophorin (see the Methods section for detail). Numerals beside peaks refer to the residue numbers in brackets in Table 2.

TABLE 2. Chemical shifts of anomeric protons for $(Glc_1)Man_9GlcNAc_2$ from lipophorin in ^{1}H-NMR spectra at 400 MHz



Residue No.	Temperature	Oligosaccharide E	Oligosaccharide F
	°C		
7	40	5.383	5.381
	70	5.361	5.360
5	40	5.320	5.319
	70	5.308	5.307
8	40	5.291	5.286
	70	5.272	5.271
12	40		5.250
	70		5.245
1	40	5.186	5.182
	70	5.190	5.188
6	40	5.122	5.121
	70	5.102	5.101
10	40	5.057	5.057
	70	5.064	5.063
11	40	5.044	5.037
	70	5.049	5.043
9	40	5.039	5.037
	70	5.043	5.043
4	40	4.865	4.861
	70	4.866	4.864
3	40	4.755	4.758
	70	4.752	4.751

reported by Byrd et al. (10) for the oligosaccharide from bovine thyroglobulin and by Viegenthart, Dorland, and van Halberk (12) for that from bovine lactotransferrin. The resonance at 4.752 ppm is characteristic of the C-1 proton of β-linked Man residue 3 and the three resonances appearing at 5.043, 5.064, and 5.049 ppm correspond to C-1 protons of terminal $\alpha(1\rightarrow2)$-linked Man residues 9, 10, and 11, respectively. The resonances at 4.866, 5.308, 5.102, 5.361, and 5.272 ppm can also be assigned to the C-1 protons of C-3 and C-6 substituted $\alpha(1\rightarrow3)$-linked Man residue 4, C-2 substituted $\alpha(1\rightarrow3)$-linked Man residue 5, C-2 substituted $\alpha(1\rightarrow6)$-linked Man residue 6, C-2 substituted $\alpha(1\rightarrow3)$-linked Man residue 7, and C-2 substituted $\alpha(1\rightarrow2)$ Man residue 8, respectively. The resonance appearing at 5.190 ppm is attributed to the C-1 proton of the α anomer of N-acetylglucosamine residue 1, while the signals of residue 2 and β anomer of residue 1 were not detected because the resonances were obscured by H^2HO signal.

Fig. 1 and Table 2 demonstrate that, except for signals

11 and 12, each signal in the spectrum of oligosaccharide F corresponds to that observed for the oligosaccharide E. Signal 11 in the spectrum of oligosaccharide F shifts slightly upfield, compared with signal 11 in the spectrum of oligosaccharide E. An additional signal 12 is also seen in the spectrum of oligosaccharide F at 5.245 ppm with a coupling constant (3.6 Hz) characteristic of α-linked glucose and at a similar position to the signal of α-linked glucose observed for known glucose-containing oligosaccharides by Tsai et al. (11). The observed shift of resonance signal 11 of oligosaccharide F from 5.049 ppm to 5.043 ppm and the appearance of a new resonance peak at 5.245 ppm suggest a structure of oligosaccharide F in which α-glucose is attached to mannose residue 11 of oligosaccharide E.

On the basis of the carbohydrate composition, the methylation analysis, and the ^{1}H-NMR spectroscopy, the structures presented in **Fig. 2** are proposed as the most probable structures of oligosaccharides E and F isolated from locust lipophorin.

The proposed structures suggest that the synthetic pathway of asparagine-linked oligosaccharides of other organisms may function also for insect lipophorin. The biosynthesis of the asparagine-linked carbohydrate units of glycoproteins in vertebrates involves an N-glycosylation step in which a glucose-containing polymannose-di-N-acetylchitobiose oligosaccharide ($Glc_3Man_9GlcNAc_2$) is transferred from a dolichyl pyrophosphoryl carrier to nascent protein (13). The first phase of N-linked oligosaccharide processing entails the removal of glucose residues from the precursor oligosaccharide soon after its transfer to protein. Glucose residues are then removed and the protein-linked oligosaccharide can be processed, either partially, to yield the high mannose species or, more completely, to provide oligosaccharides with a $Man_3GlcNAc_2$ core containing peripheral N-acetylglucosamine, galactose, sialic acid, or fucose residues.

The present results indicate that the third glucose residue is not removed from the precursor of oligosaccharide, which results in the structure proposed for oligosaccharide F in this report. It has recently been reported (14) that the oligosaccharides of acid phosphatase from *Tetrahymena pyriformis* contain glucose residues linked with the nonreducing terminal Manα1→2 residue and have the composition of $Glc_{1-3}Man_5GlcNAc_2$. The presence of a glucose-containing oligosaccharide in lipophorin may provide interesting phylogenic problems concerning the biosynthesis of asparagine-linked oligosaccharides.

Very recently, Ryan et al. (15) and Osir et al. (16) studied the asparagine-linked oligosaccharides of hemolymph proteins, arylphorin and vitellogenin, from the tobacco hornworm (*M. sexta*), and identified the major oligosaccharide structures using ^{1}H-NMR (250 MHz). The structures proposed are identical with that of oligosaccharide E reported in this paper. They also observed the set of unidentifiable peaks in the spectra of the oligosaccharide from vitellogenin. The peaks appear at a position that is closely similar to the signal of α-linked glucose observed for oligosaccharide F and exhibit the

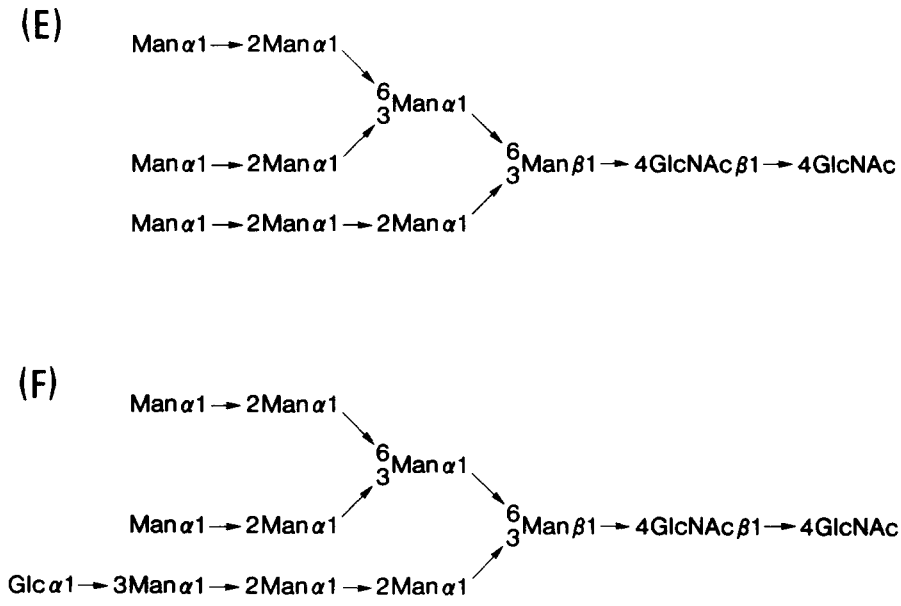


Fig. 2. Structures of oligosaccharides E ($Man_9GlcNAc_2$) and F ($Glc_1Man_9GlcNAc_2$).

coupling constant (3.75 Hz) characteristic of α-linked glucose. However, the authors doubted the presence of glucose-containing oligosaccharide in vitellogenin. The present study implies that the *M. sexta* vitellogenin may have glucose-containing oligosaccharide, and that such an oligosaccharide may be a common component of glycoproteins in insects.

The authors wish to thank Mr. N. Fujii (JOEL, Tokyo) for performing the NMR and Dr. R. G. H. Downer (University of Waterloo, Canada) for reading the manuscript. This study was supported in part by a research grant (60440004) from the Japanese Ministry of Education.

Manuscript received 24 September 1986 and in revised form 8 December 1986.

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