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Primary structure of the neutral carbohydrate chains of hemocyanin from *Panulirus interruptus*

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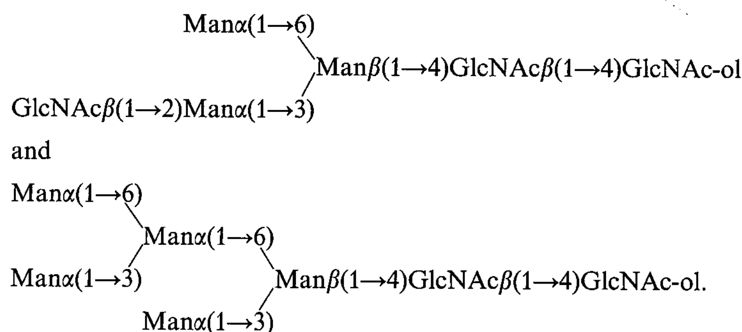
The *N*-glycosidic carbohydrate chains of *Panulirus interruptus* hemocyanin, consisting of only mannose and *N*-acetylglucosamine residues, were liberated by hydrazinolysis of a pronase digest of the purified glycoprotein. The carbohydrate chains were fractionated by high-voltage paper electrophoresis, yielding mainly neutral oligosaccharide-alditols. These were further separated by high-performance liquid chromatography and characterized by 500-MHz ^{1}H-NMR spectroscopy as



The hemocyanin of the spiny lobster *Panulirus interruptus* is the first hemocyanin of which the crystal structure has been determined [1, 2]. This glycoprotein is a hexamer with a molecular mass of 450 kDa [3], which is composed of three types of subunits called a, b and c. The amino acid sequence of subunit a has been determined [4], showing only one glycosylation site at Asn^{172}. The crystallographic data derived from a mixture of subunit a and b in roughly equal amounts demonstrate that the carbohydrate moiety is present at the periphery of the protein, having no interaction with the amino acid side chains [1, 2].

In this study the isolation and characterization of the carbohydrate chains of *P. interruptus* hemocyanin will be reported.

MATERIALS AND METHODS

Isolation of P. interruptus *hemocyanin*

Hemocyanin was isolated from *P. interruptus* hemolymph according to the method of Kuiper et al. [3]. The protein was stored at −20 °C in the presence of sucrose at a sucrose/protein ratio of 2.5:1 (w/w).

Preparation of a pronase digest

The lyophilized glycoprotein/sucrose mixture (1.5 g, corresponding to 0.4 g hemocyanin) was dialyzed to remove sucrose and subsequently made copper-free, denatured and digested by pronase as described earlier for hemocyanin from *Helix pomatia* [5], yielding 12 mg glycopeptide material.

Carbohydrate analysis

A sample of the glycopeptide mixture, containing 50 nmol carbohydrate, was subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85 °C) followed by GLC of the trimethylsilylated (re-*N*-acetylated) methyl glycosides on a capillary CPsil5 WCOT fused silica column (0.34 mm × 25 m; Chrompack) [6].

Hydrazinolysis procedure and fractionation of carbohydrate chains

The thoroughly dried glycopeptide was suspended in 1 ml anhydrous hydrazine and heated for 8 h at 100 °C. After evaporation of hydrazine, the material was re-*N*-acetylated and reduced as described [7], yielding 8 mg of a mixture of oligosaccharide-alditols. For the reduction with NaB^3H_4, 1 mg of the sample was dissolved in 200 µl 0.08 M NaOH and treated with $NaBH_4$ containing 1.7 mCi NaB^3H_4 in 200 µl *N, N*-dimethylformamide (specific activity, 341 Ci/mol; New England Nuclear). The remaining part was reduced with NaB^2H_4. To facilitate the detection of oligosaccharide-[1-

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Abbreviations. Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; GlcNAc-ol, *N*-acetyl-D-glucosaminitol; HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography.

^{2}H]alditols during the purification and fractionation procedures, 0.32 µCi (5%) of the oligosaccharide-[1-^{3}H]alditols were added. Paper electrophoresis (Whatman 3MM paper; 70 V/cm; 90 min) was carried out using a pyridine/acetic acid/water buffer (3:1:387, v/v), pH 5.4. The oligosaccharide-alditols were recovered from the paper by elution with water.

To estimate the size of the sugar chains, the neutral oligosaccharide-alditols were passed via two connected columns (2 × 100 cm each) of Bio-Gel P-4 (under 400 mesh; Bio-Rad), eluted with water (30 ml/h, 2.5-ml fractions) at 55 °C [8]. Oligosaccharide-alditols were separated preparatively by HPLC using a Perkin-Elmer series 3 liquid chromatograph, equipped with a Rheodyne injection valve. A column (4 × 250 mm) of Lichrosorb-NH_2 (5 µm, Merck) was used. Elution was performed isocratically by a mixture of acetonitrile/water (76:24, v/v) for 120 min at room temperature at a flow rate of 1 ml/min. Fractions of 1 ml were collected and assayed by absorbance at 205 nm and by scintillation counting [9].

500-MHz ^{1}H-NMR spectroscopy

Oligosaccharide-alditols were repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, Aldrich) with intermediate lyophilization. ^{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 at 500 MHz in the Fourier-transform mode at a probe temperature of 27 °C. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [10]. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone in ^2H_2O ($\delta = 2.225$ ppm) [11].

RESULTS AND DISCUSSION

Sugar analysis of the purified hemocyanin from *Panulirus interruptus* revealed the presence of only Man and GlcNAc in the molar ratio of 2.4:1.0. This ratio has not been corrected for the amount of Asn-linked GlcNAc that is not cleaved under the conditions used for methanolysis [6]. For structural analysis, the carbohydrate chains were released from a pronase digest of the glycoprotein by hydrazinolysis. After re-*N*-acetylation and reduction the carbohydrate material was subjected to high-voltage paper electrophoresis. As is evident from Fig. 1 the mixture of oligosaccharide-alditols consists mainly of neutral material and for 10% of acidic material.

The elution profile (Fig. 2) of the neutral oligosaccharide-alditols on Bio-Gel P-4 showed essentially one peak at a position corresponding to that of a glucose oligomer consisting of about nine residues.

The neutral oligosaccharide-alditols, obtained by paper electrophoresis, were separated by HPLC (Fig. 3) yielding two major fractions, denoted **c** (50%) and **f** (40%), and five minor fractions (10%) denoted **a, b, d, e** and **g**. These fractions were subjected to 500-MHz ^{1}H-NMR spectroscopy. Relevant NMR parameters of the major fractions **c** and **f**, together with those of reference compounds are compiled in Table 1.

The structural-reporter-group regions of the ^{1}H-NMR spectrum of fraction **c** are presented in Fig. 4A. All separately observable anomeric signals are of equal intensity, which points to the presence of a single compound in this fraction. Comparison of the chemical shifts of the reporter groups of

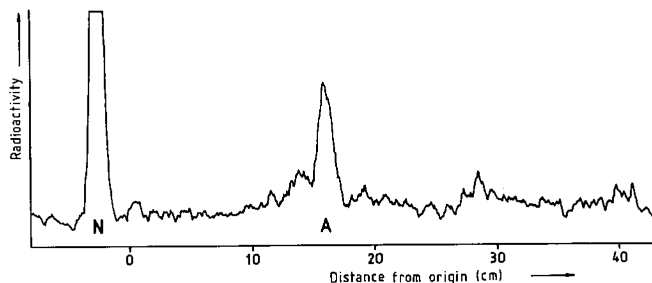


Fig. 1. *High-voltage paper electrophoresis of the ^{3}H-labelled hydrazinolysate of* P. interruptus *hemocyanin.* N, neutral fraction; A, acidic fraction

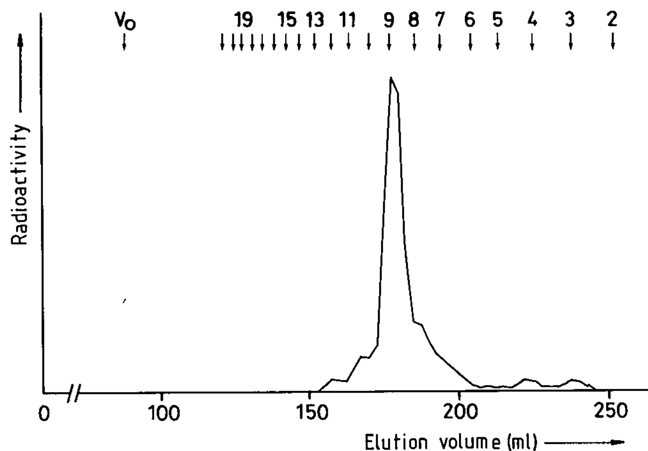


Fig. 2. *Elution profile of ^{3}H-labelled oligosaccharide-alditols derived from* P. interruptus *hemocyanin on Bio-Gel P-4.* The column was eluted with bidistilled water at 55 °C. Fractions of 2.5 ml were collected at a flow rate of 30 ml/h and assayed for ^{3}H radioactivity. The arrows at the top indicate the elution positions of glucose oligomers generated by a dextran hydrolysis. The numbers at the top indicate the glucose units

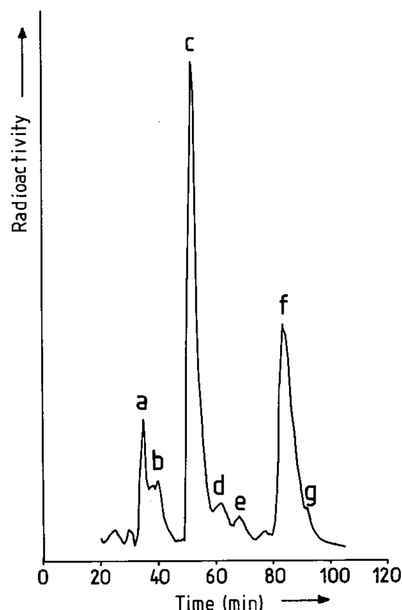
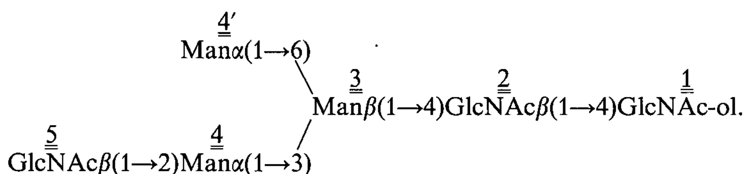


Fig. 3. *HPLC elution profile of ^{3}H-labelled oligosaccharide-[1-^{2}H]alditols derived from* P. interruptus *hemocyanin on 5-µm Lichrosorb-NH_2.* The column was run isocratically with a mixture of acetonitrile/water (76:24, v/v) at room temperature at a flow rate of 1 ml/min. Fractions of 1 ml were collected and assayed for ^{3}H-radioactivity

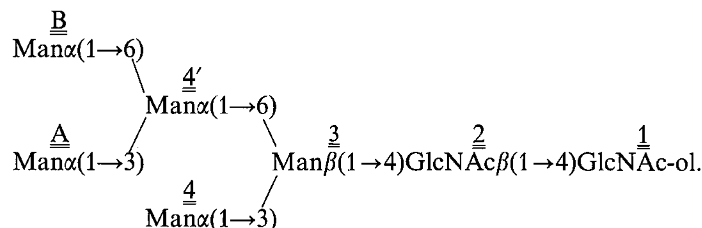
Table 1. [1]*H-chemical shifts of structural-reporter groups of the constituent monosaccharides for the major oligosaccharide-alditols (***c** *and* **f***) derived from* Panulirus interruptus *hemocyanin, together with those for two reference compounds (R1 and R2)*
R1 is compound 1 from hen ovomucoid [12], R2 is compound 62 from urine from a patient with Gaucher's disease [11]. Chemical shifts are given at 27 °C, downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O

Reporter group	Residue	Chemical shift in			
		R1	c	R2	f
		4′ \\ 3-2-1-ol / 4	4′ \\ 3-2-1-ol / 5-4	B \\ 4′ / \\ A 3-2-1-Asn / 4	B \\ 4′ / \\ A 3-2-1-ol / 4
		ppm			
NAc	1-ol	2.055	2.055	2.012	2.055
	2	2.076	2.077	2.060	2.063
	5	–	2.055	–	–
H-1	1	–	–	5.071	–
	2	4.637	4.636	4.606	4.631
	3	4.78	4.789	4.781	4.78
	4	5.103	5.118	5.099	5.099
	4′	4.915	4.918	4.872	4.871
	5	–	4.551	–	–
	A	–	–	5.093	5.089
	B	–	–	4.908	4.906
H-2	1-01	4.244	4.239	–	4.232
	3	4.259	4.255	4.251	4.256
	4	4.067	4.186	4.077	4.076
	4′	3.974	3.975	4.144	4.146
	A	–	–	4.066	4.059
	B	–	–	3.985	3.980

fraction **c** with those of $Man_3GlcNAcGlcNAc$-ol (reference compound R1, Table 1), reveals that the structure of the oligosaccharide-alditol present in fraction **c** consists of the pentasaccharide-alditol structure of reference compound R1, extended with GlcNAc-5 $\beta(1\rightarrow2)$-linked to Man-4. The presence of GlcNAc-5 is evident from its H-1 doublet at δ =4.551 ppm and its *N*-acetyl signal at δ = 2.055 ppm. The latter coincides with the *N*-acetyl signal of GlcNAc-1-ol, which can be deduced from the double intensity of the *N*-acetyl singlet at δ = 2.055 ppm, as compared to the spectrum of R1 [12]. The apparent attachment of GlcNAc-5 to the pentasaccharide-alditol R1 does not affect the chemical shifts of most structural-reporter groups. Only H-1 and H-2 of Man-4 show significant downfield shifts ($\Delta\delta$ = 0.015 ppm and $\Delta\delta$ = 0.119 ppm, respectively). The structural parameters of Man-4′ (H-1 and H-2) are not influenced. The pentasaccharide-alditol R1 does not affect the chemical shifts found for a similar extension of an oligosaccharide structure from new-born human meconium (step from compound 24 to 26 [11]). Based on the ^{1}H-NMR data the structure of fraction **c** is



The ^{1}H-NMR spectral features of fraction **f** are shown in Fig. 4B. The equal intensity of the α-anomeric proton signals indicates the presence of a pure compound. The chemical shifts of H-1 and H-2 of the various Man residues (Table 1) are essentially identical to those of the corresponding residues in $(Man)_5(GlcNAc)_2Asn$ (reference compound R2, Table 1). This illustrates that both primary structures are identical with respect to the Man residues. Furthermore, the spectrum of fraction **f** shows two *N*-acetyl signals in a ratio 1:1, but at positions different from those reported for R2. By comparison with the data of $(Man)_5(GlcNAc)_2Asn$ (Table 1), the *N*-acetyl signal in the spectrum of **f** at δ = 2.063 ppm is assigned to GlcNAc-2. The second *N*-acetyl signal, at δ = 2.055 ppm, is attributed to GlcNAc-1-ol (compare reference compound R1). The presence of the reduced chitobiose unit is further evidenced by the chemical shifts of H-2 of GlcNAc-1-ol and H-1 of GlcNAc-2 (δ = 4.232 ppm and δ = 4.631 ppm, respectively; cf. [13]). Therefore, the structure of fraction **f** is



The minor fractions **a**, **b**, **d**, **e** and **g** are present in very low quantities (less then 10% of total neutral carbohydrate material), which resulted in poor quality ^{1}H-NMR spectra. They contain mainly hydrazinolysis artefacts of the structures

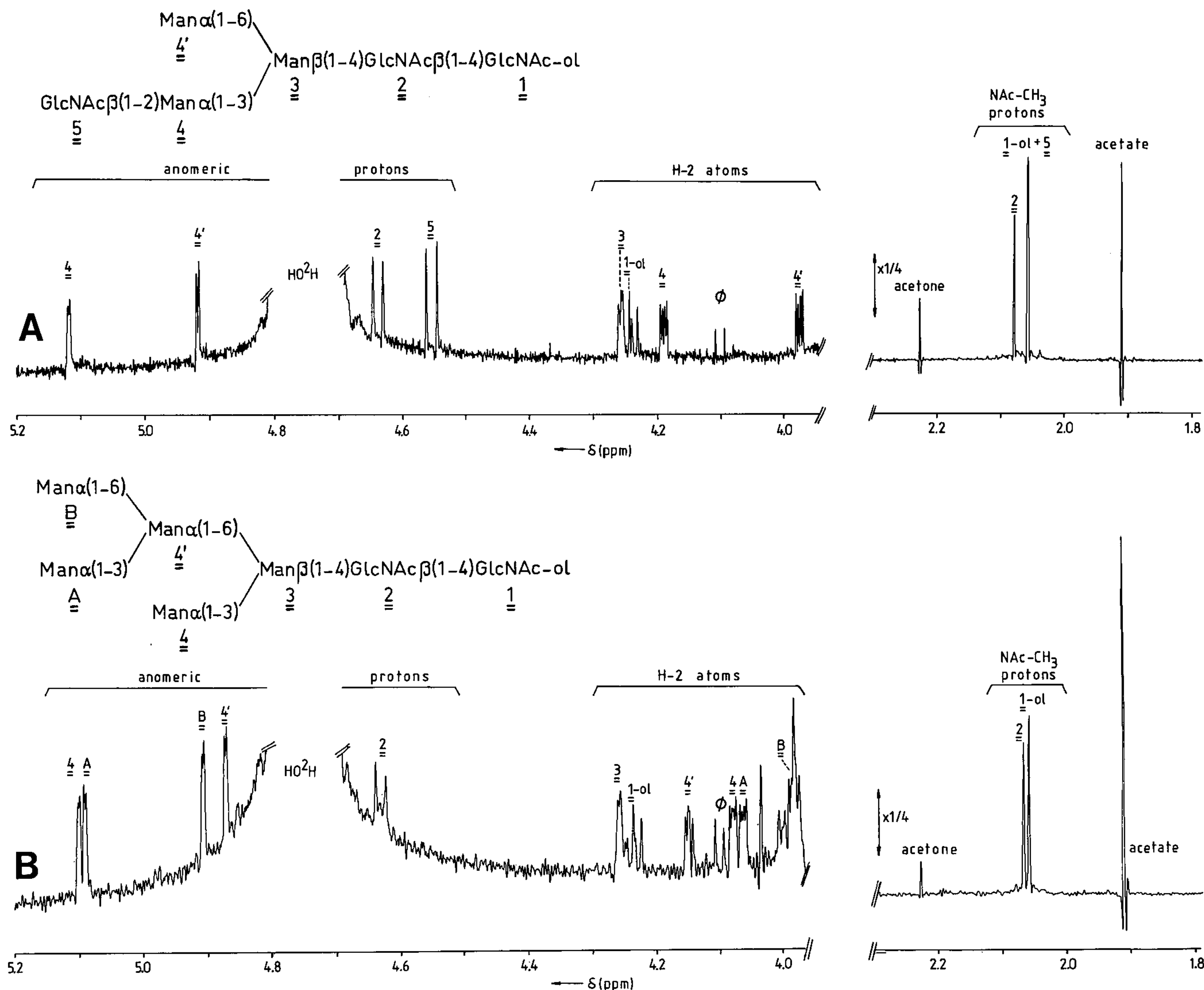


Fig. 4. *Structural-reporter-group regions of the resolution-enhanced 500-MHz* ^{1}*H-NMR spectra* (^2H_2O *at 27°C) of oligosaccharide-[1-*^{2}*H]alditol fraction* **c** *(trace A) and* **f** *(trace B) from* P. interruptus *hemocyanin.* The numbers and letters in the spectra refer to the corresponding residues in the structure. The relative intensity scale of the *N*-acetyl regions differs from that of the other part of the spectra as indicated. ∅ means impurity

found in fractions **c** and **f**. In those minor fractions GlcNAc-1-ol is modified or absent. The precise nature of these modifications could not be elucidated. The acidic fraction A, obtained after high-voltage paper electrophoresis, is still under investigation. The total amount of sugar in *P. interruptus* hemocyanin is established to be 1 – 2% (w/w).

There seems to be a wide variety in the carbohydrate structures of the hemocyanins from molluscs and arthropods. The mollusc *Helix pomatia* has as rare sugar constituents Xyl and 3-*O*-methyl-Gal, whereas the mollusc *Lymnaea stagnalis* has Xyl, 3-*O*-methyl-Gal and 3-*O*-methyl-Man [14]. The GalNAc residue found in both species seems to be bound in an *N*-glycosidic carbohydrate structure (cf. [5]). The carbohydrate structures of arthropod hemocyanins, studied so far, appeared to have the more 'classical type' of *N*-glycosidic structure. The hemocyanin of the scorpion *Androctonus australis* has $(Man)_9(GlcNAc)_2$ as its sugar chain [15] while the carbohydrate structures of *P. interruptus* hemocyanin, discussed above, are intermediate structures between oligomannoside and *N*-acetyllactosamine type. It is possible that the type of sugar chain provides information on the phylogenetic relationship of the various species.

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