CARBOHYDRATE ANALYSIS OF HEMOCYANINS

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Hemocyanins (Hcs) are the high-molecular-mass copper-containing oxygen carriers, freely dissolved in the hemolymph of arthropods and molluscs. There are, however, no strong indications that their Hcs have one common ancestor molecule. Probably they evolved independently from tyrosinase (Markl, 1986; Lerch *et al.*, 1986). Most Hcs seem to be glycoproteins, but there exist remarkable differences in carbohydrate content and monosaccharide composition between arthropodan and molluscan Hcs (Van Kuik, 1987). Detailed information, mainly based on monosaccharide analysis, methylation analysis, and 500-MHz ^{1}H-NMR spectrometry, has become available on the primary structure of the carbohydrate chains of the Hcs of the scorpion *Androctonus australis* (Debeire *et al.*, 1986), the spiny lobster *Panulirus interruptus* (Van Kuik *et al.*, 1986a, 1987a), the terrestrial snail *Helix pomatia* (Hall *et al.*, 1977; Van Kuik *et al.*, 1985), and the freshwater snail *Lymnaea stagnalis* (Hall *et al.*, 1977; Van Kuik *et al.*, 1986b, 1987b). Here, we will present a survey of our present-day knowledge concerning the carbohydrate analysis of Hcs.

CARBOHYDRATE ANALYSIS OF ARTHROPODAN HEMOCYANINS

Table 1 presents a survey of the monosaccharide composition and the carbohydrate content of Hcs from various arthropodan species (Van Kuik, 1987), showing this content to be usually low. For those species containing >0.1% carbohydrate, only D-mannose (Man) and *N*-acetyl-D-glucosamine (GlcNAc) are observed, indicating the occurrence of asparagine-linked glycans of the oligomannose type (Berger *et al.*, 1982). Because of our finding that the carbohydrate content of *Eurypelma californicum* Hc is <0.1%, the reported data for the Hcs from the Araneae *E. californicum*, *Eurypelma helluo*, and *Cupiennius salei* [carbohydrate content:

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Table 1. Monosaccharide composition (nmol sugar/mg protein) and carbohydrate content (%, w/w) of arthropodan Hcs

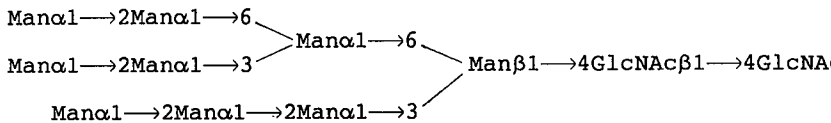
Class *Species*		Monosaccharide Man	 GlcNAc	%
Merostomata (Xiphosura)				
Limulus polyphemus				< 0.1
Arachnida (Scorpiones)				
Androctonus australis		34.8	7.9	0.8
Arachnida (Araneae)				
Eurypelma californicum				< 0.1
Crustacea (Decapoda)				
Panulirus interruptus	(*a*)	29.6	11.3	0.8
	(*b*)	39.7	16.1	1.0
	(*c*)	35.9	15.2	1.0
Astacus leptodactylus	(1×6)	31.9	22.0	1.1
	(2×6)	6.0	1.9	0.2
Chilopoda (Scutigeromorpha)				
Scutigera coleoptrata		182.0	78.0	4.9

1.5-2%; monosaccharide composition in mol sugar per 70 000 g Hc: arabinose (1), fucose (Fuc) (1), Man (1-2), glucose (5-6), and GlcNAc (2-3)] (Markl *et al.*, 1976) have to be considered with care. In addition to the data summarized in Table 1, for the decapod *Homarus americanus* (lobster) a carbohydrate content of 0.9% has been found, whereas the monosaccharide analysis yielded 2-3 mol Man and 0.5 mol GlcNAc per 75 000 g (Waxman, 1975).

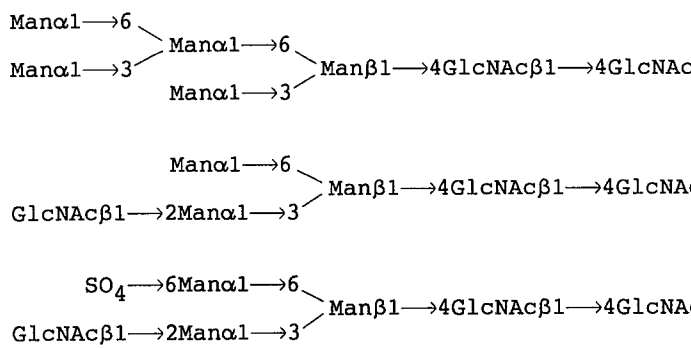
The carbohydrate content of the 1×6-mer Hc of *Astacus leptodactylus* is essentially the same as that of the 1×6-mer of *Panulirus*, but the *Astacus* 2×6-mer Hc contains considerably less carbohydrate. This is in agreement with the proposal (Nakashima *et al.*, 1986) that the carbohydrate chains prevent 2×6-mer formation. The absence of carbohydrate in the large Hcs from *Limulus polyphemus* (8×6-mer) and *E. californicum* (4×6-mer) is in accordance with this theory; the presence of carbohydrate in *Androctonus* 4×6-mer Hc, however, is in contrast with it. For the three-dimensional structure of the Hc of *Panulirus*, it has been shown that the glycan moiety protrudes into a solvent region, and does not, except for the asparagine-bound GlcNAc unit, make

contacts with the amino-acid side chains (Gaykema *et al.*, 1984). The centipede *Scutigera coleoptrata* has an unusually high carbohydrate content (4.9%) for an arthropodan Hc. The deviation of the chilopodan Hc from other arthropodan Hcs is also reflected by the quaternary structure, which is composed of six hexamers (Mangum *et al.*, 1985).

Detailed analysis of the carbohydrate moiety of the *Androctonus* Hc, released by hydrazine treatment and, after re-*N*-acetylation, converted into the corresponding oligosaccharide-alditol, demonstrated the presence of a $Man_9GlcNAc_2$ structure (Debeire *et al.*, 1986), as follows:



The carbohydrate chains of the *Panulirus* Hc were also analysed after conversion into their oligosaccharide-alditols, revealing the occurrence of $Man_5GlcNAc_2$, $GlcNAcMan_3GlcNAc_2$, and sulfated $GlcNAcMan_3GlcNAc_2$ structures (molar ratio, 4:5:1) (Van Kuik *et al.*, 1986a, 1987a), as follows:



A comparison of the carbohydrate structures indicates the presence of deglucosylated oligomannose-type chains in *Androctonus* Hc and of strongly trimmed chains in *Panulirus* Hc. Moreover, in the latter species a unique sulfated structure occurs.

Table 2. Monosaccharide composition and carbohydrate content (%, w/w) of molluscan Hcs

Class / *Species*	Monosaccharide								%
	Fuc	Xyl	3MeMan	3MeGal	Man	Gal	GalNAc	GlcNAc	
Gastropoda (Pulmonata)									
*Helix pomatia**	40.9	42.7		115.0	136.4	23.6	47.3	69.1	9.0
*Lymnaea stagnalis**	15.5	9.9	15.5	4.2	42.4	18.3	17.0	33.9	3.0
Gastropoda (Prosobranchia)									
*Megathura crenulata**	36.9				63.3	49.3	34.0	44.3	4.3
*Busycon carica**	23.1				116.4	6.7	12.8	44.7	3.9
Buccinum undatum†	1.1				5.1	0.5	0.9	3.3	4.0
Neptunea antiqua†	0.8				5.1	0.8	0.8	3.1	4.0
Colus gracilis†	1.1				5.8	0.7	2.7	1.1	4.0
Cephalopoda (Decabrachia)									
*Loligo forbesi**	1.2				63.0	9.8	9.0	45.0	2.5
*Sepia officinalis**	8.5				77.7	8.5	5.5	39.2	2.7
Cephalopoda (Octobrachia)									
*Octopus vulgaris**					89.4	8.3	2.8	26.0	2.3
Bivalvia (Protobranchia)									
*Acila castrensis**	28.9		37.0		49.5	63.4	21.1	41.8	5.2
Amphineura (Chitonida)									
*Mopalia muscosa**	15.1				126.4	22.1	4.8	36.5	3.8
*Nuttalina fluxa**	31.8				116.0	33.2	29.5	53.4	5.0
*Stenoplax conspicua**	25.6		9.9		70.2	9.4	5.0	28.0	2.4

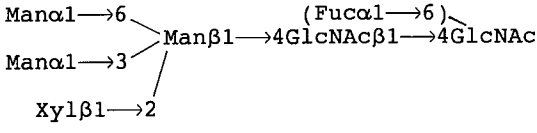
*nmol sugar/mg protein.
†mol sugar/functional unit (50 000 g), data taken from Hall & Wood (1976).

CARBOHYDRATE ANALYSIS OF MOLLUSCAN HEMOCYANINS

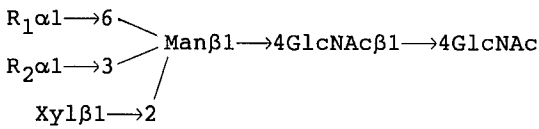
Table 2 presents a summary of monosaccharide analysis data and carbohydrate contents of Hcs from various molluscan species (Van Kuik, 1987). The carbohydrate content of molluscan Hc is higher

than that of most arthropods investigated. It is remarkable that the monosaccharide composition of molluscan Hc exhibits more variation than that of arthropods. Especially, the Hcs of *Helix* and *Lymnaea* contain unusual monosaccharides for animal glycoproteins, namely D-xylose (Xyl), 3-*O*-methyl-D-mannose (3MeMan), and 3-*O*-methyl-D-galactose (3MeGal). Xylose is a typical constituent of proteoglycans and plant glycoproteins (for a survey, see Kerékgyártó *et al.*, 1989). 3-*O*-Methyl-mannose was also detected in the Hcs of *Acila castrensis* and *Stenoplax conspicua*. All species contained Man, D-galactose (Gal), *N*-acetyl-D-galactosamine (GalNAc), and GlcNAc. In general, the presence of the latter series of monosaccharides suggests the occurrence of both asparagine-linked (Man) and serine/threonine-linked (GalNAc) carbohydrate chains (Berger *et al.*, 1982). However, as will be discussed below, for the Hcs of *Helix* and *Lymnaea* only *N*-linked carbohydrate chains could be demonstrated to occur, indicating that GalNAc is a constituent of the latter type of chains. Other examples of GalNAc-containing *N*-linked chains have been established now in other animal glycoproteins, namely in the glycohormones lutropin and thyrotropin (Baenziger & Green, 1988). It has to be noted that the literature data available for the Hcs of *Busycon canaliculatum* (Man, GlcNAc) (Waxman, 1975) and *Octopus vulgaris* (Fuc, Man, GlcNAc) (Albergoni *et al.*, 1972) do not fit the monosaccharide analysis data reported in Table 2.

The primary structures of the carbohydrate chains of the Hcs of *Helix* and *Lymnaea* have been studied in more detail, after release by hydrazine and conversion into re-*N*-acetylated oligosaccharide-alditols. The low-molecular-mass structures of the *Helix* Hc comprise fucosylated (major) and non-fucosylated (minor) forms of $XylMan_3GlcNAc_2$ (Van Kuik *et al.*, 1985), as follows:



The structures of the analysed carbohydrate chains of Hc of *Lymnaea* can be summarized in the following general way (Van Kuik *et al.*, 1986b, 1987b):



(a) R_1 = R_2 = 3MeMan
(b) R_1 = 3MeGalβ1⟶3GalNAcβ1⟶4GlcNAcβ1⟶2Man, R_2 = 3MeMan
(c) R_1 = Man, R_2 = 3MeGalβ1⟶3GalNAcβ1⟶4GlcNAcβ1⟶2Man
(d) R_1 = R_2 = 3MeGalβ1⟶3GalNAcβ1⟶4GlcNAcβ1⟶2Man
(e) R_1 = Fucα1⟶2Galβ1⟶3GalNAcβ1⟶4GlcNAcβ1⟶2Man,
R_2 = 3MeMan
(f) R_1 = Man,
R_2 = Fucα1⟶2Galβ1⟶3GalNAcβ1⟶4GlcNAcβ1⟶2Man
(g) R_1 = R_2 = Fucα1⟶2Galβ1⟶3GalNAcβ1⟶4GlcNAcβ1⟶2Man
(h) R_1 = 3MeGalβ1⟶3GalNAcβ1⟶4GlcNAcβ1⟶2Man
R_2 = Fucα1⟶2Galβ1⟶3GalNAcβ1⟶4GlcNAcβ1⟶2Man

Although no details have been reported up to now for the biological function of carbohydrate chains in molluscan Hc, it has been shown that structures containing a β1⟶2-linked Xyl residue attached at the β-Man unit of the carbohydrate core are highly immunogenic in mammalian species (Kaladas *et al.*, 1983; Faye & Chrispeels, 1988). It has to be stated that in plant glycoproteins the frequently found xylosylated trimannosyl-*N*,*N'*-diacetylchitobiosyl core structure can occur with a 1⟶3-linked instead of a 1⟶6-linked α-Fuc residue, attached at the asparagine-bound GlcNAc unit (for a review of literature data, see Kerékgyártó *et al.*, 1989).

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