A novel Gal(β1-4)Gal(β1-4)Fuc(α1-6)-core modification attached to the proximal *N*-acetylglucosamine of keyhole limpet haemocyanin (KLH) N-glycans

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KLH (keyhole limpet haemocyanin), the oxygen-carrying molecule of the marine snail *Megathura crenulata*, is often used as an adjuvant or as a hapten carrier for immunizations with peptides, oligosaccharides or other low-molecular-mass organic compounds. KLH exhibits several carbohydrate determinants, at least some of which are immunogenic: it shares an antigenic Fuc(α1-3)GalNAc- determinant with schistosomes and contains unique Gal(β1-6)Man- structural motifs on its N-glycans. This study reveals the presence of N-glycans with unusual $\pm$Gal(β1-4)Gal(β1-4)Fuc- units (α1-6)-linked to the reducing end *N*-acetylglucosamine residue. The following novel structures of KLH N-glycans were deduced by linkage analysis, exoglycosidase digestion, matrix-assisted laser-desorption ionization-tandem MS and nano-LC-ESI-IT-MS (where LC stands for liquid chromatography, ESI for electrospray ionization and IT for ion trap): Man(α1-6)[$\pm$Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Fuc(α1-6)]GlcNAc and Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Gal(β1-4)Fuc(α1-6)]GlcNAc. The Gal(β1-4)Fuc- and Gal(β1-4)Gal(β1-4)Fuc- core modifications are expected to be immunogenic, similar to other non-mammalian-type core modifications, and to contribute to the immunostimulatory properties of KLH.

Key words: ESI, internal fucose, MALDI-MS/MS, nano-LC, snail haemolymph.

INTRODUCTION

Haemocyanins serve as oxygen-carrying proteins in the haemolymph of many molluscs [1]. Whereas the mammalian oxygen carrier haemoglobin exhibits haem-complexed iron and is packed into red blood cells, haemocyanins contain copper in their oxygen-binding sites and often form oligomers up to the 1000 kDa range.

The most intensely studied haemocyanin is that of the marine snail keyhole limpet (*Megathura crenulata)* [2]. Most work dealing with KLH (keyhole limpet haemocyanin) does not address its role as an oxygen-transporting molecule, but relates to the properties of KLH as a potent immunostimulant and hapten carrier [3], which is reflected by several thousands of biomedical publications. KLH has been used, e.g. as a hapten carrier for covalently attached peptides [4,5] and carbohydrate-based antigens [6–10], mostly with the scope of developing anti-cancer therapy. Likewise, glycolipids have been adsorbed to KLH for immunizations [11]. Furthermore, KLH has been shown to be a potent adjuvant that stimulated peptide-specific cellular responses [12] and has been tested as an adjuvant in immunotherapy against primary liver cancer [13]. In addition, KLH in its natural, i.e. unconjugated form, has been shown to be effective against bladder cancer [14,15].

Concerning the glycosylation of this protein, two structural motifs have recently been described: (i) KLH exhibits a variety of unique N-glycans with Gal(β1-6)Man- structural units [16]; and (ii) a Fuc(α1-3)GalNAc- antigenic determinant found in glycoconjugates of the parasite *Schistosoma mansoni* is also present on KLH and seems to be responsible for cross-reactivity of schistosome infection sera with KLH [17]. In addition, immunization of rabbits with KLH leads to high titres of antibodies cross-reacting with schistosomal antigens [18]. Indications for further unusual carbohydrate epitope(s) on KLH were given by the detection of 4-substituted fucose in linkage analysis of total KLH glycans [16]. A recent study has shown in an *in vivo* model for granuloma formation that induction of a cellular immune response by KLH is dependent on its glycosylation (Koen Van de Vijver, personal communication). Hence, knowledge of specific structural features of KLH glycosylation is needed to study the contribution of these carbohydrate determinants to KLH immunogenicity.

In the present study, we describe a group of unusual KLH N-glycans characterized by a 4-substituted core fucose. As KLH glycans with an internal fucose could not be released in reasonable amounts by PNGase F (peptide N-glycosidase F) treatment in a former study [16], in the present study we followed a different method for KLH denaturation and enzymic deglycosylation, which allowed the isolation and structural characterization of novel KLH N-glycans containing core fucose 4-substituted by a single β-galactose or, alternatively, by a digalactosyl unit.

EXPERIMENTAL

Release and purification of N-glycans

To aliquots (900 μl) of a solution of KLH (100 mg in 18 ml PBS; Sigma), 90 μl of 10 % SDS and 4.5 μl of 2-mercaptoethanol were added. The samples were incubated for 10 min at 100 °C, allowed to cool to room temperature (25 °C) and 9 mg of Chaps was added. After incubation with 5 m-units of PNGase F (Roche Diagnostics, Mannheim, Germany) overnight at 37 °C, samples were fractionated by gel filtration on a Superdex 75 HiLoad column (16 × 600 mm; Amersham Biosciences, Uppsala,

Abbreviations used: AB, 2-aminobenzamide; ESI, electrospray ionization; IT, ion trap; KLH, keyhole limpet haemocyanin; LC, liquid chromatography; MALDI, matrix-assisted laser-desorption ionization; MS/MS, tandem MS; PNGase F, peptide N-glycosidase F; RP, reverse-phase.

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Sweden) at a 1 ml/min flow of 25 mM NH_4HCO_3 (pH 8.0). Fractions (5 ml) were collected and released glycans were detected by carbohydrate constituent analysis. Fractions containing released glycans were pooled and applied to a self-packed porous graphitized carbon column (8 mm × 50 mm; Carbograph; Alltech, Deerfield, IL, U.S.A.). After washing the carbon column with water, glycans were eluted with 25 % aqueous acetonitrile. Fractions of 1 ml were collected and assayed for the presence of oligosaccharides by MALDI (matrix-assisted laser-desorption ionization)-MS. Glycan-containing fractions were pooled and freeze-dried. Fractions from gel filtration which contained protein were pooled, freeze-dried, taken up with 50 mM ammonium bicarbonate and trypsinized. The resulting (glyco)peptides were desalted using an RP (reverse-phase)-cartridge as described earlier [16], taken up with 50 mM ammonium acetate (pH 5), and subjected to PNGase A treatment (Roche Diagnostics). The sample was again applied to an RP cartridge, and the released N-glycans were collected as the flow-through, subjected to AB (2-aminobenzamide) labelling, and analysed by analytical normal-phase HPLC using fluorescent detection for oligosaccharides. The total glycan-release procedure with successive PNGase F and PNGase A treatment was performed in parallel on horseradish peroxidase (Sigma), which served as a positive control to confirm PNGase A activity.

Labelling and fractionation of N-glycans

From a mixture containing 30 μl of glacial acetic acid and 100 μl of DMSO, 100 μl was used to dissolve 4.8 mg of AB (Sigma) and 6.3 mg of $NaCNBH_3$ (Fluka). This mixture (100 μl) was added to 300 μg of KLH glycans and incubated for 2 h at 65 °C. The sample was then applied to an RP cartridge (500 mg of Bakerbond octadecyl; Baker, Phillipsburg, NJ, U.S.A.). After a 5 ml wash with water, AB-labelled glycans were eluted with 2 ml of 50 % methanol and dried under N_2.

AB glycans were fractionated by normal-phase HPLC on a TSK-Amide 80 column (4 mm × 250 mm; Tosohaas, Montgomeryville, PA, U.S.A.) at 0.4 ml/min. Solvent A was 50 mM formic acid adjusted to pH 4.4 with ammonia solution. Solvent B was 20 % of solvent A in acetonitrile. The following gradient conditions were used: at time $t = 0$ min, 100 % solvent B; $t = 152$ min, 52.5 % solvent B; $t = 155$ min, 0 % solvent B; $t = 162$ min, 0 % solvent B; and $t = 163$ min, 100 % solvent B. The total run time was 180 min. Samples were injected into 80 % acetonitrile [19]. Fluorescence was detected at 360 nm/425 nm.

For a two-dimensional separation, KLH AB-labelled glycans were first separated on a CarboPac PA-100 (4 mm × 250 mm; Dionex, Sunnyvale, CA, U.S.A.) at 1 ml/min. Solvent A was 500 mM NaOH, solvent B was 500 mM sodium acetate and solvent C was water. Starting conditions were 25 % of solvent A and 0 % solvent B. After a 10 min isocratic run, an 80 min linear gradient was applied to 25 % solvent A and 20 % solvent B. Oligosaccharides were detected amperometrically and collected manually. Fractions were re-chromatographed on an RP column (Sephasil Peptide C_{18}, 5 μm; 4.6 mm × 100 mm; Amersham Biosciences) at 1 ml/min. Solvent A was water and solvent B was 20 % aqueous acetonitrile. The column was equilibrated at 5 % solvent B, and a 35 min gradient to 35 % solvent B was applied. Oligosaccharides were detected by fluorescence (360 nm/425 nm) and collected manually.

Nano-LC (liquid chromatography) ESI (electrospray ionization)-IT (ion trap)-MS

AB glycans were separated on a Pep-Map column (75 μm × 100 mm; Dionex/LC Packings, Amsterdam, The Netherlands) using an Ultimate nano-LC system (LC Packings). The system was directly coupled with an Esquire 3000 ESI-IT-MS (Bruker Daltonik, Bremen, Germany) equipped with an on-line nanospray source operating in the positive-ion mode. For ESI (1200–2500 V), capillaries (360 μm outer diameter, 20 μm inner diameter with 10 μm opening) from New Objective (Cambridge, MA, U.S.A.) were used. The solvent was evaporated at 150 °C with a nitrogen stream of 6 l/min. Ions with m/z from 50 to 3000 were registered. The column was equilibrated with eluent A [water/acetonitrile, 19:1 (v/v), containing 0.1 % formic acid] at a flow rate of 100 nl/min. After injecting the sample, the column was run isocratically for 5 min, followed by a linear gradient to 30 % eluent B [water/acetonitrile, 1:19 (v/v), containing 0.1 % formic acid] for 15 min and a final wash with 100 % solvent B for 5 min. The eluate was monitored by measuring the absorption at 320 nm.

Exoglycosidase treatment

AB oligosaccharides (2–20 pmol) were treated with α-mannosidase from jack beans (100 m-units; Sigma), α-fucosidase from bovine kidney (20 m-units; Sigma) or β-galactosidase from Streptococcus 6646 K (2 m-units; Seikagaku, Falmouth, MA, U.S.A.) in 50 μl of 50 mM sodium acetate buffer (pH 5.0). AB oligosaccharides were extracted by using a Zip-Tip (Millipore, Billerica, MA, U.S.A.), eluted with 50 % methanol on to the target plate and analysed by MALDI-MS, nano-LC-ESI-IT-MS or normal-phase HPLC.

MALDI-MS/MS (tandem MS)

MALDI-MS(/MS) results were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker) equipped with a LIFT-MS/MS facility. 6-Aza-2-thiothymine (5 mg/ml; Sigma) was used as a matrix; spectra were acquired in the positive-ion mode. For fragment ion analysis in the tandem time-of-flight (TOF-TOF) mode, precursors were accelerated to 6 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell, and their masses were analysed after the ion reflector passage. To obtain proton adducts besides the routinely observed sodium adducts, 10 nmol of ammonium bicarbonate was added per spot.

Carbohydrate constituent analysis

Samples were hydrolysed in 20 μl of 4 M aqueous trifluoroacetic acid (Sigma) at 100 °C for 4 h, and dried under a stream of nitrogen. Monosaccharides were converted into their anthranilic acid derivatives by reductive amination, resolved by RP-HPLC and detected by fluorescence [20].

Methylation analysis

AB glycans were permethylated and hydrolysed (4 M trifluoroacetic acid, 4 h, 100 °C). Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analysed by capillary GLC-MS using electron-impact ionization [21].

RESULTS

Analysis of the entire N-glycan pool

KLH was heat-denatured after the addition of a reducing agent and detergent, followed by PNGase F-release of N-glycans.

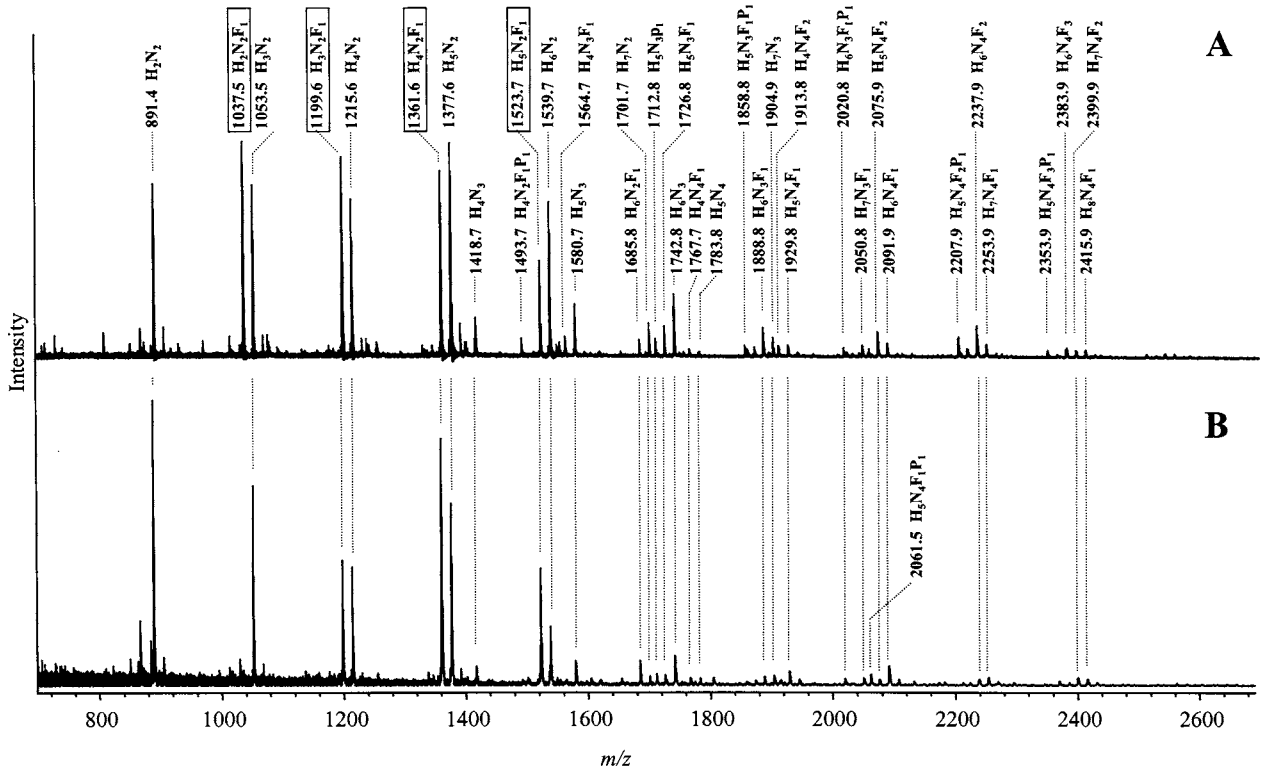

Figure 1 MALDI-MS of the total pool of PNGase F-released KLH N-glycans before and after α-fucosidase treatment

PNGase F-released, AB-labelled N-glycans of KLH were registered as sodium adducts before (**A**) and after (**B**) treatment with α-fucosidase from bovine kidney. Peaks are marked with measured mass and deduced glycan composition. Of the four major fucose-containing species (highlighted by boxes) only the species at *m/z* 1037 completely disappeared after α-fucosidase treatment, whereas the others were largely resistant to enzymic defucosylation (**B**). H, hexose; N, *N*-acetylhexosamine; F, fucose; P, pentose.

Oligosaccharides were purified by gel filtration, and glycan-containing fractions were identified by carbohydrate constituent analysis and pooled. Retrieved protein fractions were trypsinized, and the resulting glycopeptides were subjected to PNGase A treatment, which did not release N-glycans in detectable amounts (results not shown). Oligosaccharide pools obtained by PNGase F treatment were applied to a carbon column and eluted in 25 % acetonitrile. Linkage analysis of this pool revealed the presence of 4-substituted fucose (results not shown), which has been observed in KLH samples in earlier studies [16,18]. Furthermore, the following *N*-acetylhexosamine species were detected (relative abundance given in parenthesis): terminal GlcNAc (0.1), 4-substituted GlcNAc (1.0), 3-substituted GlcNAc (0.2), 3-substituted GalNAc (0.3), 3,4-disubstituted GlcNAc (0.9) and 4,6-disubstituted GlcNAc (0.5). This pattern of *N*-acetylhexosamine species resembled the pattern observed for total KLH (glycopeptides) as well as hydrazinolysis-released glycans [17,18], suggesting an efficient release of KLH N-glycans. PNGase F-released oligosaccharides were then labelled with AB and analysed by MALDI-MS (Figure 1A). In the mass range up to 1.6 kDa, high-intensity signals were obtained for species with deduced compositions of $H_{2\text{-}6}N_2F_{0\text{-}1}$ (where H is hexose, N is *N*-acetylhexosamine and F is fucose), whereas the mass range up to 2.5 kDa displayed a variety of minor, mainly fucosylated structures and some of them contained a pentose residue.

For the four major fucosylated species, fragment-ion patterns were obtained from protonated precursors by nano-LC-ESI-IT-MS/MS and MALDI-MS/MS. This revealed for the $\mathbf{H_2N_2F_1}$ precursor at *m/z* 1015, a fragmentation indicative of core fucosylation (N_1F_1AB ion at *m/z* 488; see Table 1, fraction 2). For the precursors at *m/z* 1177 ($\mathbf{H_3N_2F_1}$), 1339 ($\mathbf{H_4N_2F_1}$) and 1501 ($\mathbf{H_5N_2F_1}$), unusual core modifications were indicated by ions at *m/z* 812 and/or 650, which could be interpreted as the protonated Y-type ions $H_2N_1F_1$AB and $H_1N_1F_1$AB respectively (Figure 2; bold type is used to indicate glycan species, whereas compositions of fragment ions are given in roman type). Nano-LC-ESI-IT-MS/MS of the sodium adducts at *m/z* 1199 ($\mathbf{H_3N_2F_1}$), 1361 ($\mathbf{H_4N_2F_1}$) and 1523 ($\mathbf{H_5N_2F_1}$) yielded similar fragment ions at *m/z* 672 and 834 (results not shown).

Treatment of KLH AB-labelled N-glycans with α-fucosidase from bovine kidney exhaustively removed the core fucose from $\mathbf{H_2N_2F_1}$, as monitored by MALDI-MS (Figure 1B), and led to the production of $\mathbf{H_2N_2}$, as indicated by disappearance of the corresponding sodium adduct at *m/z* 1037. Signals ($[M + Na]^+$) at *m/z* 1199, 1361 and 1523, however, were not affected by enzymic defucosylation (Figure 1B), which corroborated the assumption of an unusual core modification as indicated by MS fragmentation.

N-glycan fractionation and elucidation of unusual core modifications

For a more detailed study of these unusual structures, the AB-labelled KLH N-glycan pool was fractionated by normal-phase HPLC (Figure 3). Glycans were structurally characterized by carbobohydrate constituent analysis (results not shown), linkage analysis (Table 1), MALDI-MS/MS (Figure 4) and α-mannosidase treatment followed by analytical-scale normal-phase separation and nano-LC-ESI-IT-MS as well as -MS/MS of the individual

Table 1 Structural data obtained for KLH AB-labelled N-glycans

For KLH AB-labelled N-glycans in fractions 1–11, the molecular masses were determined by MALDI-MS. Proton adducts were analysed by MALDI-MS/MS. In the case of non-resolved isobaric structures, MALDI-MS/MS was performed after isolation by 2-dimensional HPLC. Characteristic fragments observed are listed with the deduced composition given in parentheses. Bold type indicates Y-type ions indicative of unusual core modification. AB, 2-aminobenzamide; F, fucose; H, hexose; N, *N*-acetylhexosamine. For linkage analysis (LA), partially methylated monosaccharide derivatives obtained after hydrolysis were analysed by GC-MS. t-Man, terminal mannose (2,3,4,6-tetra-*O*-methylmannitol as alditol acetate); 6-Man, 6-substituted mannose, etc.

Fraction, Species	Determined mass (Da)	Key structural data
1, H_2N_2	$[M+H]^+$ 869.4; $[M+Na]^+$ 891.1	Fragments: 342 (N_1AB); 528 (H_2N_1); 707 (H_1N_2AB) LA: t-Man, 6-Man, 4-GlcNAc
2, $H_2N_2F_1$	$[M+H]^+$ 1015.4; $[M+Na]^+$ 1037.1	Fragments: 342 (N_1AB); 488 (N_1F_1AB); 528 (H_2N_1); 707 (H_1N_2AB); 869 (H_2N_2AB) LA: t-Fuc, t-Man, 6-Man, 4-GlcNAc
3, H_3N_2	$[M+H]^+$ 1031.5; $[M+Na]^+$ 1053.1	Fragments: 342 (N_1AB); 545 (N_2AB); 690 (H_3N_1); 869 (H_2N_2AB); LA: t-Man, 3,6-Man, 4-GlcNAc
4, $H_3N_2F_1$-a	$[M+H]^+$ 1177.2; $[M+Na]^+$ 1199.4	LA*: t-Man, 3,6-Man, 4-GlcNAc
4, $H_3N_2F_1$-b	$[M+H]^+$ 1177.2; $[M+Na]^+$ 1199.4	Fragments: 342 (N_1AB); 528 (H_2N_1); **650 ($H_1N_1F_1$AB)**; 869 (H_2N_2AB); 1015 ($H_2N_1F_1$AB) LA: t-Man, t-Gal, 4-Fuc, 6-Man, 4-GlcNAc
5, H_4N_2-a	$[M+H]^+$ 1193.5; $[M+Na]^+$ 1215.4	Fragments: 342 (N_1AB); 545 (N_2AB); 690 (H_3N_1); 707 (H_1N_2AB); 852 (H_4N_1); 1031 (H_3N_2AB) LA: t-Man, 6-Man, 3,6-Man, 4-GlcNAc
6, H_4N_2-b	$[M+H]^+$ 1193.5; $[M+Na]^+$ 1215.4	Fragments: 342 (N_1AB); 545 (N_2AB); 690 (H_3N_1); 707 (H_1N_2AB); 852 (H_4N_1); 1031 (H_3N_2AB) LA: t-Man, t-Gal, 6-Man, 3,6-Man, 4-GlcNAc
7, $H_4N_2F_1$-c	$[M+H]^+$ 1339.6; $[M+Na]^+$ 1361.2	Fragments: 342 (N_1AB); 528 (H_2N_1); 707 (H_1N_2AB); **812 ($H_2N_1F_1$AB)**; 869 (H_2N_2AB); 1015 ($H_2N_2F_1$AB); 1177 ($H_3N_1F_1$AB) LA: t-Man, t-Gal, 4-Fuc, 4-Gal, 6-Man, 4-GlcNAc
8, $H_4N_2F_1$-a	$[M+H]^+$ 1339.6; $[M+Na]^+$ 1361.2	Fragments: 342 (N_1AB); 488 (N_1F_1AB); 545 (N_2AB); 690 (H_3N_1); 852 (H_4N_1); 1193 (H_4N_2AB) LA*: t-Man, 6-Man, 3,6-Man, 4-GlcNAc
8, $H_4N_2F_1$-b	$[M+H]^+$ 1339.5; $[M+Na]^+$ 1361.4	Fragments: 342 (N_1AB); **650 ($H_1N_1F_1$AB)**; 690 (H_3N_1), 1031 (H_3N_2AB), 1177 ($H_3N_2F_1$AB) LA: t-Man, t-Gal, 4-Fuc, 3,6-Man, 4-GlcNAc
9, H_5N_2	$[M+Na]^+$ 1377.1;	LA: t-Man, 3,6-Man, 4-GlcNAc
10, H_5N_3	$[M+Na]^+$ 1580.1	–
10, $H_5N_2F_1$	$[M+H]^+$ 1501.4; $[M+Na]^+$ 1523.1	Fragments: 342 (N_1AB); 366 (H_1N_1); 488 (N_1F_1AB); 528 (H_2N_1); 545 (N_2AB); **650 ($H_1N_1F_1$AB)**; 690 (H_3N_1); 707 (H_1N_2AB); **812 ($H_2N_1F_1$AB)**; 852 (H_4N_1); 869 (H_2N_2AB); 1014 (H_5N_1); 1031 (H_3N_2AB); 1177 ($H_3N_1F_1$AB); 1193 (H_4N_2AB); 1339 ($H_4N_1F_1$AB)
11, H_6N_2	$[M+Na]^+$ 1539.1	LA: t-Man, 2-Man, 3,6-Man, 4-GlcNAc

* Linkage analysis performed after enzymic defucosylation and re-chromatography.

fractions (Figure 3). MALDI-MS/MS of proton adducts revealed structures with unusual core modifications in fractions 4, 7, 8 and 10, as indicated by fragment ions at *m/z* 812 and/or 650.

Fraction 4 was shown to contain the two isobaric species **$H_3N_2F_1$-a** (60%) and **$H_3N_2F_1$-b** (40%). Using the two-dimensional separation system, the two species could be separated, and only the former compound was sensitive to α-fucosidase treatment. **$H_3N_2F_1$-a** was elucidated as Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-AB consistent with [16]. For **$H_3N_2F_1$-b**, MALDI-MS/MS (Figure 4A) revealed a hexose attached to the Fuc(α1-6)GlcNAc-AB moiety (fragment at *m/z* 650). The compound was sensitive to α-fucosidase treatment only after the removal of a galactose residue by β-galactosidase treatment, as analysed by normal-phase HPLC of the cleavage products using fluorescence detection (results not shown). This indicated a β-galactose capping of the core fucose. Together with the linkage analysis results (Table 1), the structure was deduced as Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Fuc(α1-6)]GlcNAc-AB (schematically represented in Figure 4A).

Fraction 7 contained the species **$H_4N_2F_1$-c** for which MALDI-MS/MS revealed a major ion at *m/z* 812 indicating the attachment of two hexoses to the Fuc(α1-6)GlcNAc-AB core region. This species was sensitive to Streptococcus 6646 K β-galactosidase, which removed two galactose residues (Figure 5). Only after this β-galactosidase treatment, could the core fucose be enzymically removed (results not shown). Together with the linkage analysis results (Table 1), **$H_4N_2F_1$-c** glycans could be structurally characterized as Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Gal(β1-4)Fuc(α1-6)]GlcNAc-AB (schematically represented in Figure 4D).

Fraction 8 comprised the two isobaric species, **$H_4N_2F_1$-a** and **$H_4N_2F_1$-b**, only the former being sensitive to α-fucosidase treatment. The two isobaric species, **$H_4N_2F_1$-a** (60%) and **$H_4N_2F_1$-b** (40%), differed in retention properties in the two-dimensional separation system and were thus purified on an analytical scale for MALDI-MS/MS (Figures 4B and 4C). Together with linkage analysis and exoglycosidase treatment, **$H_4N_2F_1$-a** was elucidated as Man(α1-6)Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-AB consistent with [16]. For **$H_4N_2F_1$-b**, MALDI-MS/MS (Figure 4C) revealed structural similarities to **$H_3N_2F_1$-b** (Figure 4A), as both species exhibited major ions at *m/z* 650. On the basis of MS, linkage analysis, β-galactosidase and α-fucosidase treatment, the structure was defined as Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Fuc(α1-6)]GlcNAc-AB (Figure 4C). MALDI-MS/MS of **$H_5N_2F_1$** (fraction 10) indicated the occurrence of galactosylated core structures (Table 1). However, full structural characterization was hindered by the relatively low abundance of these species.

Taken together, the characterization of KLH AB-labelled N-glycans by MS, linkage analysis, enzymic cleavage and HPLC revealed two unusual types of core modifications. Whereas two glycans were shown to exhibit a Gal(β1-4)Fuc(α1-6)-modification of the innermost GlcNAc (Figures 4A and 4C), a further N-glycan exhibited an extension of this motif by a second galactose, namely Gal(β1-4)Gal(β1-4)Fuc(α1-6)- (Figure 4D).

$Man_{2\text{-}6}GlcNAc_2$ and Gal(β1-6)Man-exhibiting N-glycans

Using the set of methods mentioned above, the structures of **H_2N_2** (fraction 1), **$H_2N_2F_1$** (fraction 2) and **H_3N_2** (fraction 3)

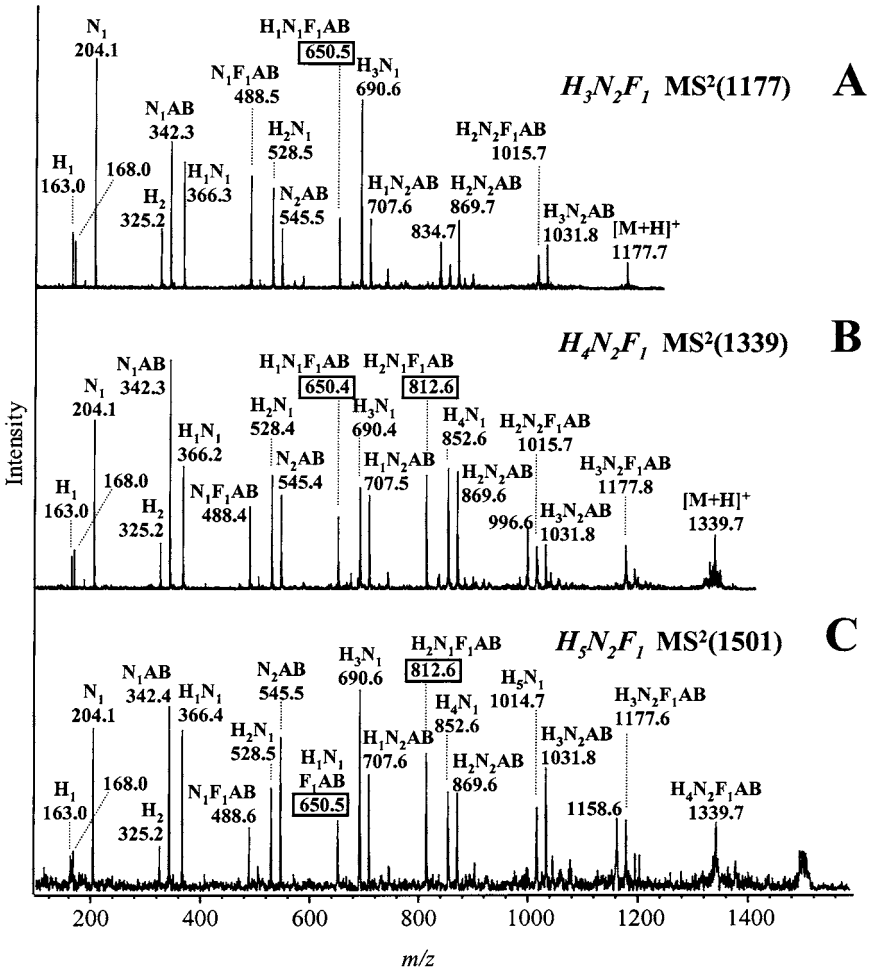

Figure 2 MALDI-MS/MS analysis of some fucose-containing species out of the total pool of released KLH N-glycans

Proton adducts of the KLH AB-labelled N-glycans of composition (**A**) $\mathbf{H_3N_2F_1}$ (*m/z* 1177), (**B**) $\mathbf{H_4N_2F_1}$ (*m/z* 1339) and (**C**) $\mathbf{H_5N_2F_1}$ (*m/z* 1501) were subjected to fragment ion analysis. Peaks are labelled with measured mass and deduced glycan composition. Fragment ions at *m/z* 650 or 812 indicative of unusual core-modifications are boxed. H, hexose; N, *N*-acetylhexosamine; F, fucose.

were deduced as Man(α1-6)Man(β1-4)GlcNAc(β1-4)GlcNAc-AB, Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-AB and Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-AB respectively, which is in agreement with the former findings [16]. $\mathbf{H_4N_2}$**-a** (fraction 5) was shown to represent the tetramannosidic structure Man(α1-6)Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-AB. Linkage analysis of $\mathbf{H_4N_2}$**-b** (fraction 6) revealed equal amounts of terminal mannose and terminal galactose in addition to 6-substituted mannose, 3,6-disubstituted mannose and 4-substituted GlcNAc. These results are consistent with the N-glycan structure Gal(β1-6)Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-AB, which has been elucidated before [16].

For $\mathbf{H_5N_2}$ (fraction 9) and $\mathbf{H_6N_2}$ (fraction 11), linkage results (Table 1) were consistent with the oligomannosidic structures Man(α1-6)[Man(α1-3)]Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-AB and Man(α1-6)[Man(α1-3)]Man(α1-6)[Man(α1-2)Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-AB, respectively. Both structures have already been elucidated [16]. Taken together, these results confirm the former finding of oligomannosidic structures as well as Gal(β1-6)Man-substituted N-glycans [16].

DISCUSSION

In the present study, novel KLH N-glycans were characterized which exhibit an unusual core modification containing a 4-substituted fucose residue. Interestingly, structures with 4-substituted fucose were very inefficiently released in former experiments in which KLH was reduced, alkylated and trypsinized before PNGase F-treatment, as evidenced by the detection of only trace amounts of 4-substituted fucose in linkage analysis. Consequently, glycans containing internal fucose were not characterized in the former study [16]. In the present study, PNGase F-release was performed on KLH denatured by application of detergents and reducing agent as well as a heat step, which resulted in a more efficient release of the unconventionally core-modified N-glycans. Similarly, efficient release of certain glycans by PNGase F treatment of an insect-derived protein has been reported to depend on protein denaturation, resulting in very different glycan populations depending on variations in protein pretreatment [23].

The novel KLH N-glycans, which exhibit a core fucosylation that resisted fucosidase treatment (Figure 1B), were isolated and structurally elucidated. The structural analysis revealed two unusual types of core substitution. Although two glycans were

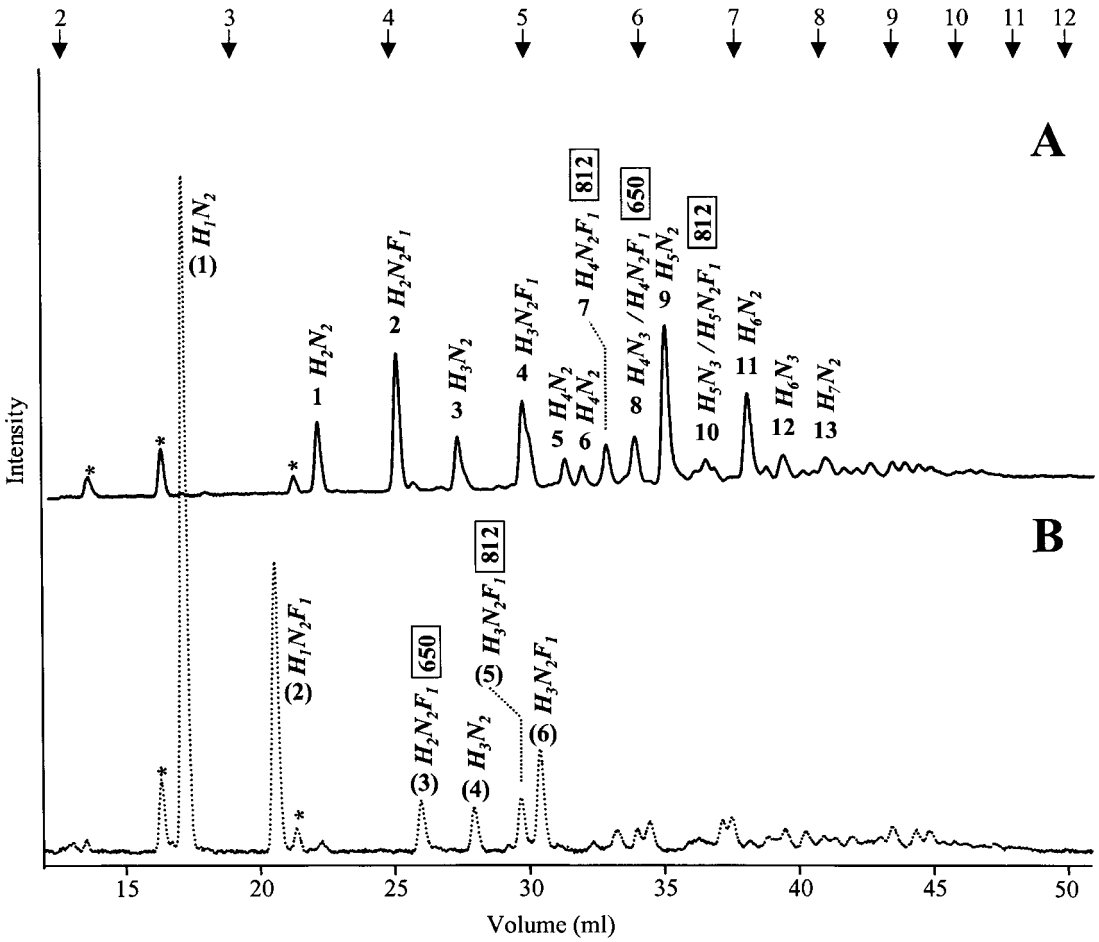

Figure 3 Normal phase HPLC fractionation of AB-labelled KLH N-glycans before and after α-mannosidase treatment

KLH AB-labelled N-glycans were fractionated by normal-phase chromatography before (**A**) and after (**B**) α-mannosidase treatment. The obtained glycans were subjected to MALDI-MS/MS (**A**) or nano-LC-ESI-IT-MS/MS (**B**) of their proton adducts, and fragment ions indicative of an unusual core modification are given in boxes. *, contaminant.

shown to carry a Gal(β1-4)Fuc(α1-6)-modification of the innermost GlcNAc (Figures 4A and 4C), one other N-glycan exhibited an extension of this motif by a second galactose, namely Gal(β1-4)Gal(β1-4)Fuc(α1-6) (Figure 4D). The unconventional Gal(β1-4)Fuc(α1-6) core modification has so far only been described for a monoantennary N-glycan derived from octopus rhodopsin [24] and a core difucosylated N-glycan from squid rhodopsin [25]. The extended structural motif comprising the digalactosyl unit attached to the fucose is described here for the first time. Although the substructure Gal(β1-4)Gal(β1) is found as a terminal or internal element in various glycoconjugates of bacteria, plants and animals, with more than 100 entries in the carbohydrate database SweetDB (http://www.dkfz-heidelberg.de/spec2/sweetdb), its attachment to fucose as elucidated in the present study has not been found before. In fish, the Gal(β1-4)Gal(β1) motif has been found as the terminal element in the antennae of N-glycans [26], whereas it has not yet been described for mammals.

It has been well documented that core modifications may confer immunogenic properties on N-glycans. Core (β1-2)-xylose and (α1-3)-fucose residues, relatively common in plant and insect glycoproteins [27–29], have a strong contribution in IgE binding to plant glyco-allergens [30] and, similarly, allergic reactions to bee venom may be induced by core (α1-3)-fucose-carrying glycans [31]. In addition, several helminth species, including schistosomes [32] and *Haemonchus contortus* [33], produce unusual core-modified N-glycans that have been implicated in the glycan-induced immune responses particularly observed in parasitic helminth infections [34,35].

To obtain a better molecular background for understanding the complex glycan-related immunogenic properties of KLH, it is of importance to have detailed knowledge of the structure of KLH glycans. The Fuc(α1-3)GalNAc element, previously identified on KLH N-glycans [17], is the primary cross-reactive epitope with schistosome antigens and, recently, potentially immunogenic Gal(β1-6)Man-substituted N-glycans [16] of KLH were described. In view of the observed immunogenicity of N-glycans with unusual, non-mammalian core modifications, it is probable that the novel Gal(β1-4)Gal(β1-4)Fuc(α1-6) substitution identified in this study also contributes to the immunostimulatory properties of KLH. Clearly, many interesting aspects of KLH glycosylation have now been revealed. In view of the reported binding of peanut agglutinin to KLH [36], and induction of antibodies reactive with Gal(β1-3)GalNAc- (Thomsen-Friedenreich antigen) by KLH [37], questions regarding the structure of KLH glycans remain. Future research applying alternative analytical approaches based on MS of KLH-derived glycopeptides may help in answering these questions.

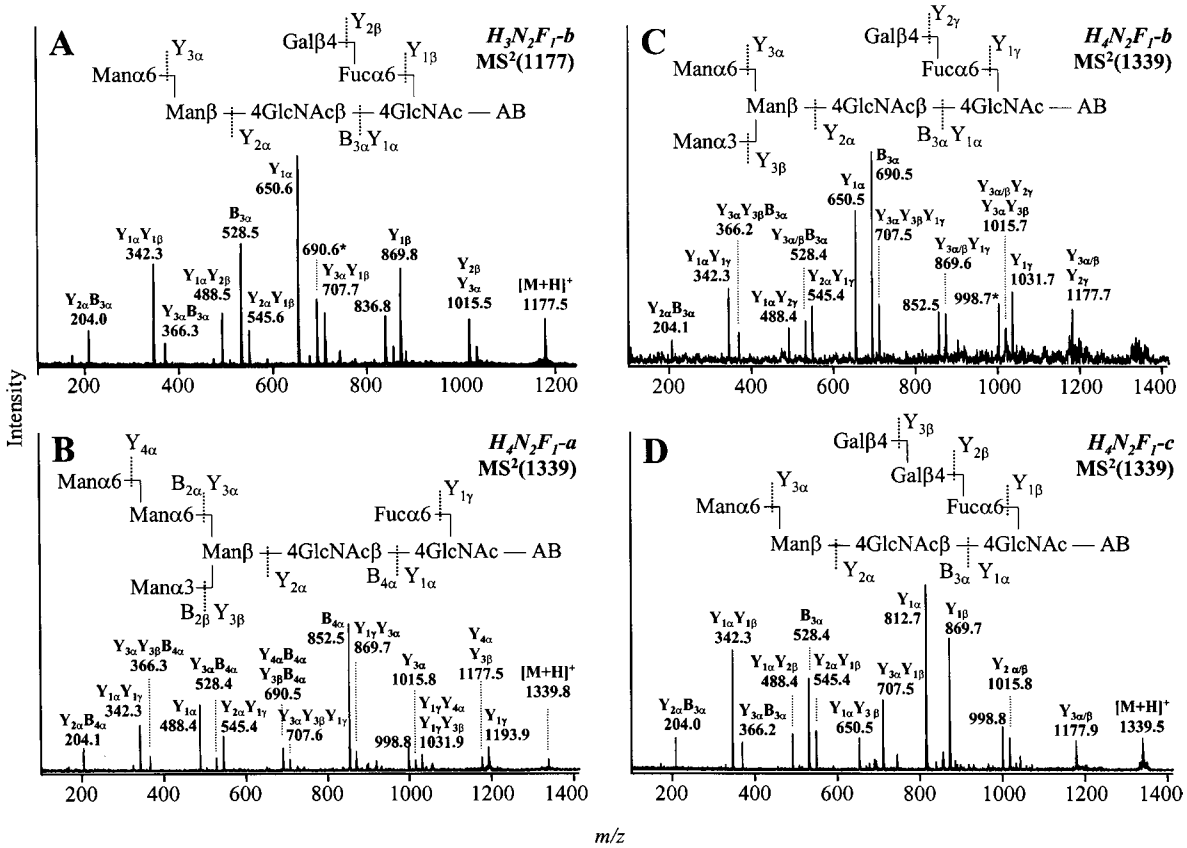

Figure 4 MALDI-MS/MS of individual, isobaric KLH N-glycans

By two-dimensional HPLC fractionation, the individual N-glycan species $H_3N_2F_1$-**b** (**A**), $H_4N_2F_1$-**a** (**B**), $H_4N_2F_1$-**b** (**C**) and $H_4N_2F_1$-**c** (**D**) were obtained and were subjected, similar to their proton adducts, to MALDI-MS/MS. Possible fragmentation pathways are included in the structures. The assignment of fragments is in agreement with the nomenclature introduced by Domon and Costello [22].

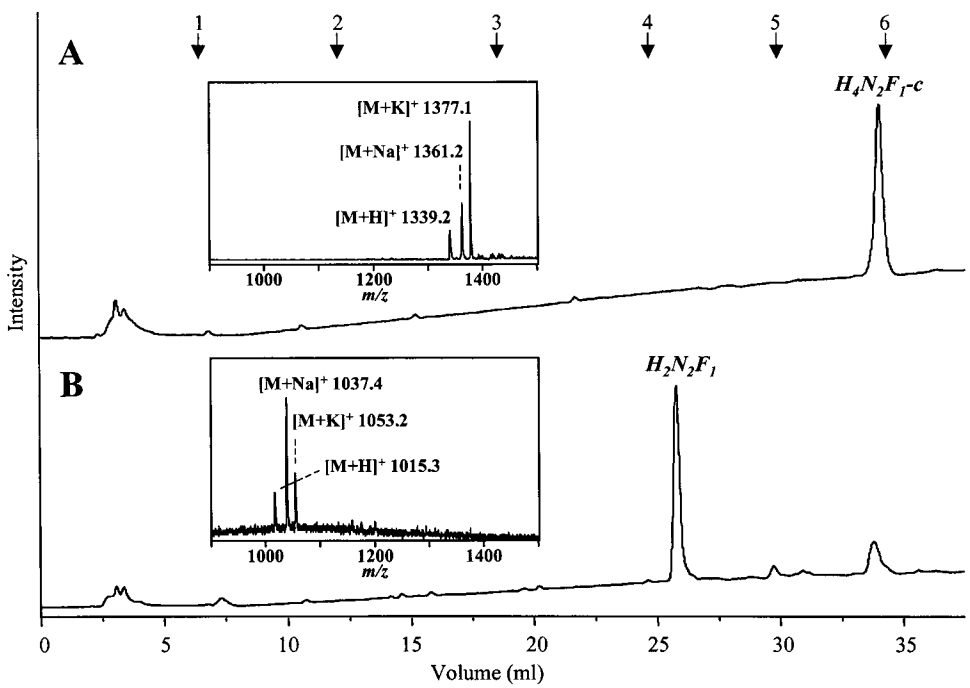

Figure 5 HPLC and MS of $H_4N_2F_1$-c before and after β-galactosidase treatment

$H_4N_2F_1$-**c** was analysed by analytical normal-phase HPLC with fluorescence detection before (**A**) and after (**B**) treatment with Streptococcus 6646 K β-galactosidase. Elution positions obtained for an AB-tagged dextran ladder are indicated by arrows labelled with the number of glucose residues. The insets show the corresponding mass spectra, which were obtained by nano-LC-ESI-IT-MS.

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