Discovery and Structural Characterization of Fucosylated Oligomannosidic N-Glycans in Mushrooms

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L-Fucose is a common constituent of Asn-linked glycans in vertebrates, invertebrates, and plants, but in fungal glycoproteins, fucose has not been found so far. However, by mass spectrometry we detected N-glycans and O-glycans containing one to six deoxyhexose residues in fruit bodies of several basidiomycetes. The N-glycans of chanterelles (Cantharellus cibarius) contained a deoxyhexose chromatographically identical to fucose and sensitive to α-L-fucosidase. Analysis of individual glycan species by tandem MS, glycosidase digestion, and finally 1H NMR revealed the presence of L-fucose in α1,6-linkage to an α1,6-mannose of oligomannosidic N-glycans. The substitution by α1,6-mannose of α1,2-mannosyl residues of the canonical precursor structure was yet another hitherto unknown modification. No indication for the occurrence of yet other modifications, e.g. bisecting N-acetylglucosamine, was seen. Besides fucosylated N-glycans, short O-linked mannan chains substituted with fucose were present on chanterelle proteins. Although undiscovered so far, L-fucose appears to represent a prominent feature of protein-linked glycans in the fungal kingdom.

The glycosylation capacity of fermentable yeasts and filamentous fungi has been well studied because of their potential biotechnological importance. In contrast to the members of other multicellular, euakaryotic organisms, i.e. green plants and metazoa, these fungi formed no complex-type N-glycans but rather oligomannosidic N-glycans often with characteristic extensions such as α1,3-mannose chains, phosphomannose or α-galactofuranose residues. This difference comfortably explains that fucose has so far not been found in fungal glycoproteins, as in animals and plants fucose occurs attached to N-acetylgalactosamine (GlcNAc) or galactose residues in complex-type glycans only (1, 2). In fungi, fucose has only been detected in fungal polysaccharides (3–5) and in activated N-glycans containing one α1,6-linked mannan chains (6). With the exception of morels and truffles belonging to the ascomycetes, most of the fungal species with macroscopic fruiting bodies well known as - often edible - mushrooms are found in the phylum basidiomycota. Glycoproteins of three species of this group, i.e. Agaricus bisporus (8), Schizophyllum commune (9) and Coprinopsis cinerea (10) have been studied and found to contain exclusively oligomannosidic N-glycans. C. cinerea additionally contained glycans with a bisecting α1,4-GlcNAc residue (10).

In this work, we studied the N-glycans of naturally growing mushrooms, in particular chanterelle (Cantharellus cibarius) by matrix assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Surprisingly, many species exhibited oligomannosidic N-glycans, with a deoxyhexose, which, for chanterelles, was shown to be fucose α1,6-linked to mannose by tandem MS (MS/MS), glycosidase digestion, and last but most importantly, high field 1H NMR. In addition, fucosylated O-glycans, more exactly O-mannans, were detected in chanterelles and penny buns (porcini, Boletus edulis).

EXPERIMENTAL PROCEDURES

Preparation of N-Glycans for NMR Analysis—Chanterelles (5 kg fresh weight; purchased from a local supermarket) were homogenized in distilled water and digested overnight at 37 °C with pepsin (0.1 mg/ml) in 5% (v/v) formic acid and 0.05% (v/v) 2-mercaptoethanol. After centrifugation for 10 min at 12,000 rpm and 4 °C, the supernatant was applied to Dowex 50W-X2 with 2% (v/v) acetic acid. (Glyco)peptides were then treated with pepsin. The glycopeptides were then treated with 0.05% (v/v) 2-mercaptoethanol. After centrifugation for 10 min at 12,000 rpm and 4 °C, the supernatant was applied to Dowex 50W-X2 with 2% (v/v) acetic acid. (Glyco)peptides were eluted with 0.6 M ammonium acetate, pH 6. The orcinol-positive fractions were subjected to two different gel filtrations (Sephadex G-25 and G-50 in 1% acetic acid). Collected fractions were lyophilized, dissolved in 0.1 M citrate phosphate buffer, pH 5.0, and incubated at 95 °C for 10 min to inactivate pepsin. The glycopeptides were then treated with peptide N-glycosidase A (12 milliunits/50 mg of lyophilized glycopeptides = 100 g fresh chanterelles) at 37 °C overnight. The digest was purified by passage over C18 solid phase extraction cartridges (1 g, Bakerbond SPE; Fisher Scientific) equilibrated with 2% acetonitrile in water. Released glycans in the flow-through were desalted by size exclusion chromatography on Sephadex G15 (General Healthcare, Vienna). Orcinol-positive fractions were again lyophilized.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5 and NMR data.
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3 The abbreviations used are: MS/MS, tandem mass spectrometry; ESI, electrospray mass ionization; PA, pyridylaminated; PGC chromatography, porous, graphitic carbon chromatography; RP, reversed phase.
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Preparation of Fluorescently Labeled N-Glycans—The dried glycans were subjected to pyridylation by the modified method of the original overnight procedure (11). 2-Aminopyridine (5.86 mmol; Sigma-Aldrich) was dissolved in acetic acid and added to the dried glycans (2–6 mg). The sample was incubated at 90 °C for 1 h and allowed to cool, and acetone (water-free) was added. After vortexing for 30 s and centrifuging at 13,000 rpm for 5 min, the solvent was decanted. This procedure was repeated twice. After very brief drying under vacuum, reduction was accomplished by adding dimethylborane (water-free) was added. After vortexing for 30 s and centrifuging the dried glycans (2–6 mg). The sample was incubated at 90 °C for 1 h and allowed to cool, and acetone was added. The dabsylated glycopeptide Ser-(GnGnF6-)Asn served as control substrate for α-L-fucosidase digestion. Both exoglycosidase digestions were performed at 37 °C overnight and analyzed by PGC-LC-ESI-MS or MALDI-TOF MS. The control glycopeptide was obtained from human IgG by digestion with Pronase and β-galactosidase.

Mass Spectrometry—Electrospray ionization mass spectrometry (ESI-MS) was performed on a Q-TOF Ultima Global instrument (Waters-Micromass, Manchester, UK). Analysis of O-glycans and borohydride-reduced N-glycans was performed by positive-ion LC-ESI-MS with a PGC column as described (20). For MS/MS experiments, fucosylated N-glycans were infused directly in a solution of 50% acetonitrile containing 0.1% formic acid and 0.5 mm sodium hydroxide (21). Free oligosaccharides of various mushrooms were analyzed by MALDI-TOF MS on an Ultraflex II instrument (Bruker, Bremen, Germany) using 2% 2,5-dihydroxybenzoic acid as matrix.

NMR Spectroscopy—Isolated oligosaccharides were lyophilized and dissolved in D2O (99.996% D2O; Sigma-Aldrich) to concentrations of ~120 μg 600 μl−1. Solutions were transferred into 5-mm high precision NMR sample tubes (Promochem, Wesel, Germany). All spectra were recorded on a Bruker DRX-600 AVANCE spectrometer (Bruker, Rheinstetten, Germany) at 600.13 MHz (1H) using the Bruker Topspin 1.3 software. Man6Fuc and Man5Fuc were measured at 298.1 K and 308.0 K, all other samples only at 298.1 K. Chemical shifts were referenced to external acetone (δH 2.225 ppm). The one-dimensional proton spectra were recorded with presaturation, acquisition of 32,000 data points, and a relaxation delay of 1.0 s. After zero filling to 64,000 data points and Fourier transformation, spectra were performed with a range of 7,200 Hz. To determine two-dimensional homonuclear COSY, TOCSY (100-ms mixing time), and ROESY (400-ms mixing time) spectra, 384 experiments with 2,048 data points and appropriate number of scans, each were recorded using standard Bruker programs. After linear forward prediction to 512 data points in the f2 dimension and sinusoidal multiplication in both dimensions, they were Fourier transformed to two-dimensional spectra with a range of 6,000 Hz in both dimensions (see also supplemental NMR Data).

RESULTS

Mass Profiles of Fungal N-Glycans—Pure research interest led us to subject chanterelles to our standard scheme of N-glycan preparation (22). Surprisingly, the MALDI-TOF MS profile deviated considerably from that of common mushroom (A. bisporus), which was recently shown to contain only oligomannosidic N-glycans (8). In chanterelles, additional glycans were seen, whose masses could be explained by the presence of one or more deoxyhexose residues (Fig. 1). The number of deoxyhexose residues, later shown to be fucose,
varied from 1 to 6 and correlated with the number of hexose units (Fig. 1 and supplemental Fig. 1).

Mass spectrometric N-glycan profiling of protein extracts of fruit bodies from 25 species of Agaricomycotina revealed either plain oligomannosidic N-glycans or such structures with one to several fucose residues (Table 1 and supplemental Figs. 2 and 3). Commercially cultivated fungi other than champignon such as oyster mushroom (*Pleurotus ostreatus*) and shitake (*Lentinula edodes*) also exhibited plain oligomannosidic N-glycans only. The same applied to the magic mushrooms *Psilocybe semilanceata* and *Panaelus cyanescens* and many other mushrooms of our adventitious collection. However, many other species contained fucosylated N-glycans, and notably most or all of these fungi live in symbiotic (ectomycorrhizal) or parasitic association with green plants. The host plants include angiosperm trees and conifers and tend to prefer acidic soil poor in nutrients (Table 1). The degree of fucosylation varied from 13 to 80% depending on the species. In all mushrooms, the dominant glycan structures comprised five to nine mannoses, but larger glycans were usually present indicative of elongation of the original Glc3Man5 precursor. The N-glycans of porcini (*B. edulis*), showed a fucosylation pattern similar to that of chanterelles but with a bias toward multiply fucosylated N-glycan structures (supplemental Fig. 2).

Comparative separation of reduced N-glycans from chanterelles, porcini (*B. edulis*) and others by PGC-LC-ESI-MS showed that these mushrooms contained the same isoforms albeit in different abundance as shown on the example of Man7Fuc (Fig. 2). Man7Fuc glycans of penny buns and chanterelles that had comparable elution times also had comparable collision-induced decay MS/MS spectra (data not shown).

### Analysis of the Monosaccharide Composition

To elucidate the nature of the deoxyhexose, the monosaccharides of chanterelle N-glycans were at first analyzed as anthranilic acid derivatives. This revealed the presence of mannose, GlcNAc and fucose or rhamnose as these deoxyhexoses co-elute. To solve this ambiguity, the monosaccharides were labeled with 3-methyl-1-phenyl-2-pyrazolin-5-one (17), which resulted in a clear identification of fucose. This identification was strongly corroborated by digestion with α-L-fuco-
sidase, which also demonstrated the l-configuration of this fucose (see later).

Preparation of Homogeneous Isoforms of Chanterelle N-Glycans—PA-labeled N-glycans were subjected to size separation by normal phase HPLC (supplemental Fig. 4). The monofucosylated species Man4Fuc, Man5Fuc, Man6Fuc each gave one dominant peak on reversed phase, whereas Man7Fuc was fractionated into three isomers (supplemental Fig. 4). The fractions were then subjected to carbon HPLC, which did not lead to further subfractionation. Structural Characterization of Chanterelle N-Glycans by Mass Spectrometry and Glycosidase Digestions—MS/MS experiments with Man4Fuc, the smallest fucosylated N-glycan, were at first conducted with the protonated ion. However, the fragments indicated that fucose was linked to the first GlcNAc as well as to one of the mannoses. Proton adducts of fucosylated N-glycans are prone to fucose rearrangement, which gives rise to misleading fragments and therefore, the sodium adduct was selected for analysis by collision-induced decay (23, 24). This yielded fragments unambiguously indicating the fucose to be linked to a mannose residue (Fig. 3).

α-Mannosidase from jack bean could remove three mannoses from PA-labeled Man6Fuc, suggesting that the fucose ter-
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FIGURE 3. MS/MS spectrum of Man4Fuc from chanterelles. The [M+Na]^+ ion of PA-labeled Man4Fuc (m/z 1319.35) was selected. Schemes label fragments that indicate the fucose to be linked to a mannose residue.

The reduced and 2-aminopyridyl protected carbohydrate unit was identified as 2-acetylamino-2-deoxy-D-glucitol, which had originally been the D-Glc unit (1), indicating by a NOE between the characteristic H-1 signal of β-D-GlcNac [2] at 4.66 ppm and the H-4 at approximately 3.85 ppm. The consecutive saccharide unit is a β-D-Manp [3], linked to C-4 of β-D-GlcNac [2]. Its indicative proton signal is below the overwhelming HDO signal at 298 K (25). Therefore, the structure and interglycosidic linkages have been determined from Man6Fuc and Man5Fuc by two-dimensional NMR spectra recorded at 308 K (Fig. 4, inset). The β-D-Manp [3] carries two further saccharide units. A α-D-Manp [D1] is bound at position 2 causing the characteristic low field shift of H-1 in saccharide [B] (25, 29). On position 6 a α-1-Fucp [D2] is bound. Anomeric form and pyranose ring closure of the fucose was determined by 1H chemical shifts (30–32), and its absolute configuration was deduced from glycosidase digestion (see above).

These seven saccharide units represent our smallest isolated oligosaccharide Man4Fuc and build the conserved backbone of all further isolated oligosaccharides (Fig. 4). Man5Fuc further carries a α-D-Manp [4] linked to position 3 of the β-D-Manp [3]. This type of branching of the carbohydrate skeleton has been reported earlier for very similar oligosaccharides isolated from other sources (25–28). The indicative 1H NMR signal of the α-D-Manp [4] anomeric proton has a resonance at approximately 5.1 ppm. Man6Fuc carries an additional α-D-Manp [C] bound to α-D-Manp [4] in a 1→2 linkage. This unit causes an indicative low field shift of H-1 in unit [4] (Fig. 4), which has also been reported earlier (25, 29). The further three isolated Man7Fuc oligosaccharides had different substitution patterns of further mannoses. Man7Fuc-a has the Man6Fuc skeleton and additionally carries an α-D-Manp [E1] at position 6 of α-D-Manp [D1]. Man7Fuc-c also has the Man6Fuc skeleton with a further α-D-Manp [A] in 1→3 linkages to α-D-Manp [4']. Man7Fuc-b carries both units [E1] and [A], but not the mannose [C] leading to a high field shift of H-1 signal to 5.09 ppm in α-D-Manp [4], comparable with those in Man5Fuc. Spectral regions with indicative signals of all six compounds are shown in Fig. 4, and the structural features are presented Fig. 4.

Detection of GDP-Fucose—Chanterelle extracts contained a compound isobaric with GDP-fucose and eluting at the same position on porous graphitic carbon. As the carbon column exerts a high selectivity with regard to the nature of the sugar and the linkage of phosphates (33), it is likely that chanterelles contain exactly the same GDP-fucose as mammals and plants. This agrees with the previous detection of GDP-fucose and the relevant enzymes for its synthesis in the filamentous fungus Mortierella alpina (6), which belongs to a phylum very distant to the Ascomycota investigated here. Common mushrooms (A. bisporus) also contained GDP-fucose despite the lack of fucosylated N-glycans.

O-Glycan Analysis of Chanterelle—O-Glycans were released from glycoproteins of chanterelles, champignons, and penny buns by reductive β-elimination from gel bands. Analy-
ysis of these glycans by PGC-ESI-MS revealed them to consist of hexoses plus one or two deoxyhexoses in chanterelle and porcini and only hexoses in champignons (Fig. 5). Fragmentation analysis of the different peaks showed the reducing sugar to be a hexose (data not shown). The first deoxyhexose is linked to the reducing sugar and the second deoxyhexose to the next hexose. Monosaccharide analysis with anthranilic acid identified the sugars as mannose and deoxyhexose, i.e. fucose as demonstrated by its sensitivity to \(\alpha\)-fucosidase (data not shown). Anthranilic acid derivatization of the glycans ob-

FIGURE 4. Proton NMR measurements of the isolated oligosaccharides. The frequency region with indicative signals of all saccharide units is shown, and these protons are highlighted in the structure scheme. Spectra have been recorded at 298.0 K. Additional measurements of Man5Fuc and Man6Fuc have been made at 308.0 K to cause a high field shift of the HDO signal. H-1 proton signals of \(\beta\)-Man [3] can hence be detected and are shown in the inset. Selected indicative \(^1\)H shifts of the isolated oligosaccharides are given in the supplemental NMR Data. The drawing at the bottom shows a fictive Man8Fuc, including all saccharide units occurring in the measured compounds. The distinctive units of each isolated oligosaccharide are specified. Proton atoms labeled in bold caused indicative \(^1\)H NMR signals. The numbering of saccharide units refers to Van Halbeek et al. (25).
tained by nonreductive $\beta$-elimination (18) corroboration man-
nose as the reducing sugar (data not shown).

**DISCUSSION**

Fucose has not yet been found as a constituent of fungal
glycopeptides (34–36), even though not less than 10 of the 25
somewhat arbitrarily selected mushroom species contained
obvious amounts of fucosylated N-glycans in their fruiting
bodies. At least chanterelles and penny buns additionally con-
tained fucosylated O-glycans. Some recent studies have dealt
with mushroom N-glycans (8–10). Incidentally, all three spe-
cies belong to the Agaricales order. Also, no other of the sev-
eral investigated species of this order contained fucosylated
glycopeptides.

The linkage of fucose to mannosae has so far not been seen in
eukaryotic glycoproteins with the possible but enigmatic excep-
tion of the parasite glycans listed as entry 8486 in the glycome
data base. The biosynthesis of this unusual structural element
remains mysterious, as our efforts at demonstrating
in vitro enzymatic activity remained unsuccessful. Possible reasons
for this failure may be lack of the transferase in overground
fungal organs, its rapid deterioration during storage, or the
need for some labile cofactor or other unknown requirement.
Apparently, fucosae addition does not require the assistance of
GlCNAC-transferase I, which initiates the generation of fucosyl-
ated complex-type N-glycans in animals or plants. This
assumption is supported by the total absence of homologs to
mammalian GlCNAC-transferase I among the so far known
fungal proteins.

The data may point at a preferential occurrence of fucosyl-
lated glycopeptides in fungi that form mycorrhiza. More pro-
nounced are the absence of fucose in cultivable fungi and its
presence in species preferring acidic habitats. These observa-
tions may point at a functional significance of this novel type
of fucosylation.

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