Glycoconjugate Journal (1998) **15:** 89–91

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HPLC method for the determination of Fuc to Asn-linked GlcNAc fucosyltransferases

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Mammalian cells often contain an enzyme which transfers fucose onto the reducing terminal GlcNAc (GlcNAc-1) of N-glycans with an α1,6-linkage. In plants, on the other hand, the fucose is transferred to GlcNAc-1 with an α1,3-linkage. Insect cells can exhibit both enzymatic activities. Hitherto, the activity of these fucosyltransferases has been determined by the incorporation of radioactively labelled fucose into an acceptor glycopeptide. This assay, however, cannot discriminate these two activities. Here we report on the use of dansylated glycoasparagine for the specific determination of 1,3- and 1,6-fucosyltransferases. The two possible products and the substrate are separated on a reversed phase column and detected by fluorescence.

***Keywords*: Glycosyltransferases, fucosyltransferases, asparagine-linked oligosaccharides**

***Abbreviations*: Fuc-T C3, GDP-fucose, β-N-acetylglucosamine (Fuc to Asn-linked GlcNAc) α1,3-fucosyltransferase; Fuc-T C6, GDP-fucose, β-N-acetylglucosamine (Fuc to Asn-linked GlcNAc) α1,6-fucosyltransferase; GnGn-dNS, $GnGnF^3$-dNS, $GnGnF^6$-dNS and $GnGnF^3F^6$-dNS, dansylated glycopeptides (see Figure 1).**

Introduction

Fucosyltransferases catalysing the transfer of fucose to the asparagine-bound GlcNAc in N-glycans appear to be almost ubiquitous in eukaryotic organisms. In mammals and many other animals, fucose is transferred to the 6-position of the GlcNAc residue [1], whereas in plants the fucose is found in 3-linkage [2]. In insects, both activities can be present so giving rise to a mixture of structures with α1,3-, α1,6- and even doubly fucosylated core-GlcNAc [3–6]. In contrast to the fucosyltransferases acting on the non-reducing terminal branches of complex oligosaccharides, the fucosyltransferases acting on the core-GlcNAc have received very little attention. The systematic names for these two transferases are GDP-fucose: β-N-acetylglucosamine (Fuc to Asn-linked GlcNAc) α1,3-fucosyltransferase and GDP-fucose: β-N-acetylglucosamine (Fuc to Asn-linked GlcNAc) α1,6-fucosyltransferase. To avoid these bulky terms while still dinstinguishing these fucosyltransferases from those acting on the non-reducing terminus, we suggest the abbreviated names core-α1,3-fucosyltransferase (or Fuc-T C3) and core-α1,6-fucosyltransferase (or Fuc-T C6).

The activities of Fuc-T C3 and Fuc-T C6 are typically assayed by incubating an acceptor glycopeptide with radioactively labelled GDP-fucose, enzyme and other components such as cations and detergents. The remaining GDP-fucose is then removed by an anion-exchange resin and the remaining radioactivity is counted. The activity can be calculated from the difference between a sample with and a sample without the acceptor. In the case of tissues containing both activities, this assay is unsatisfactory because the two activities cannot be discriminated. Since up to three different products can arise (Figure 1), a chromatographic separation is required. With pyridylaminated oligosaccharides, fucosylation of the reducing GlcNAc exerts significant and specific effects on the elution position on a reversed phase column [3–8]. However, pyridylaminated oligosaccharides are not accepted by the core-oriented fucosyltransferases [9].

Here we describe a promising substrate for the specific, non-radioactive determination of the activity of Fuc-T C3 and Fuc-T C6 by reversed phase HPLC.

Materials and methods

Fuc-T C3 was partially purified from mung bean sprouts as previously described [9]. Chicken heart microsomes were used as a source of Fuc-T C6. The activities of these preparations were measured by incubation with asialo-, agalacto-glycopeptides from bovine fibrin [9]. The structure of the products obtained was verified as previously described [3].

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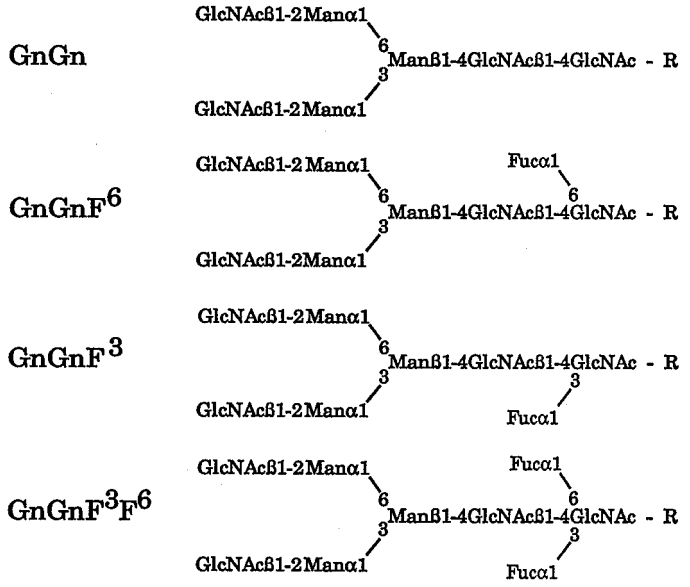

Figure 1. Abbreviations and structures of glycopeptides with differently fucosylated core-GlcNAc. R, dansyl-Asn-Ser.

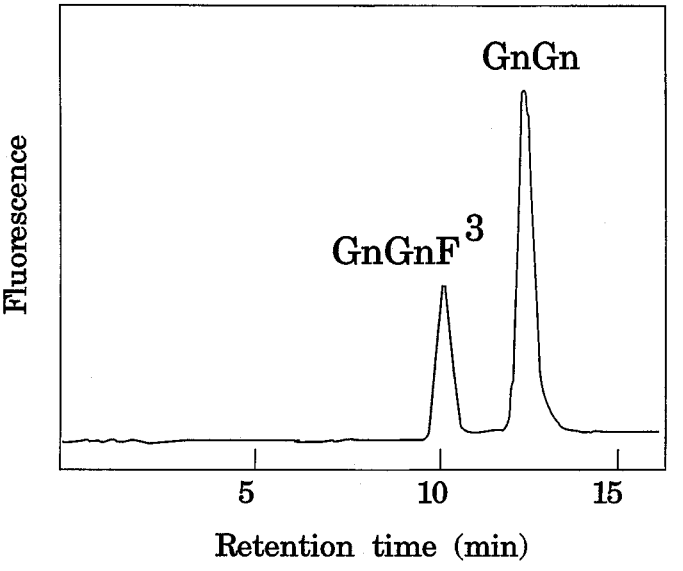

Figure 2. HPLC profile of a typical activity determination of mung bean α1,3-fucosyltransferase on a 5 µm ODS column. The peak labels refer to dansylated glyco-dipeptides as shown in Figure 1.

The acceptor substrate used for HPLC analysis was prepared from human IgG by extensive digestion with pronase, chemical desialylation and finally enzymatic degalactosylation [3]. Dansylation and separation by reversed phase HPLC were performed as described [10]. Incubations with fucosyltransferases were performed with 5 nmol of acceptor substrate in a volume of 20 µl and otherwise as described [9].

For the analytical HPLC separations, 50 mM potassium phosphate at pH 2.0 was used as buffer A and 70% (v/v) acetonitrile in water as buffer B. Separations were achieved by isocratic elution with 10% B at a flow rate of 1.5 ml min^{-1} for the 4 × 250 cm 5 µm Hypersil ODS column and 12% B at 0.6 ml min^{-1} for the 3 × 250 cm 3 µm Hypersil ODS column. The dansyl-peptides were detected by fluorimetry at wavelengths of 315 and 550 nm.

Results

Preliminary trials to find a Fuc-T C3 and C6 substrate suitable for HPLC analysis showed: (1) reduction of the innermost GlcNAc, as is the case with pyridylaminated oligosaccharides, abolishes the ability to act as a substrate; (2) the peptide moiety must be small to minimize the chance of proteolytic degradation; and (3) the overall affinity of the compound to the reversed phase matrix must be low in order to allow separation of the differently fucosylated glycans. The latter requirement stands in the way of the necessity to introduce a chromogenic or fluorogenic tag since, for example, the dabsyl group is very hydrophobic. Even dansylated glycoasparagines were found to be too hydrophobic. A solution to this problem is to use a dansylated glycopeptide with a hydrophilic amino acid.

Dansylated pronase glycopeptide from human IgG exhibited two peaks closely eluting at moderate concentrations of acetonitrile. Both peaks contained serine as the only (non- dansylated) amino acid which accordingly forms the C-terminus. The majority of the N-glycans from IgG are 6-fucosylated at the reducing GlcNAc [11]. Incubation of the second, larger peak with α-fucosidase from bovine kidney changed its elution time to that of the smaller peak. Apparently, the two compounds (GnGn-dNS and $GnGnF^6$-dNS; for structures see Figure 1) are separated on the basis of the absence or presence of 6-linked fucose. Indeed, incubation of GnGn-dNS with Fuc-T C6 led to the re-appearence of $GnGnF^6$-dNS. Then, GnGn-dNS as well as $GnGnF^6$-dNS were incubated with Fuc-T C3 to obtain the 3-fucosylated products $GnGnF^3$-dNS and $GnGnF^3F^6$-dNS, respectively. In both cases, 3-fucosylation lead to a reduction of elution time. By using GDP-[^{14}C]-fucose as the donor substrate for Fuc-T C3, the product peak was shown to contain fucose. The dansylated dipeptide therefore appeared to be suitable for the determination of Fuc-T C3 activity using a conventional 5 µm HPLC column (Figure 2). Although it remains to be shown, we are optimistic that the analysis time can be considerably reduced to allow the analyses of many samples. The increase of elution time exerted by α1,6-fucosylation is roughly half the difference caused by α1,3-fucosylation. While this makes determination of Fuc-T C6 slightly more difficult, it leads to a difference in retention of GnGn-dNS and $GnGnF^3F^6$-dNS. Therefore, all four N-glycans possibly occuring in a sample containing both Fuc-T C3 and Fuc-T6 can be separated by HPLC. However, while a 5 µm column did not provide baseline separation of all four compounds, this

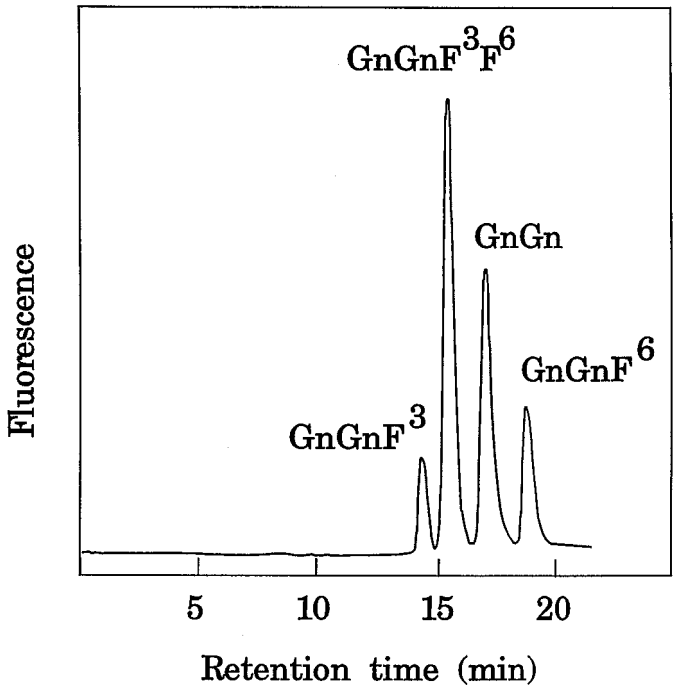

Figure 3. Separation of dansylated glyco-dipeptides with differently fucosylated core-GlcNAc on a 3 μm ODS column. Peak labels refer to the glycan structures shown in Figure 1. The mixture shown here was obtained by combining two samples where either GnGn-dNS or $GnGnF^6$-dNS had been incubated with Fuc-T C3.

was achieved by using a 3 μm column of the same length (Figure 3).

Finally, the new chromatographic activity assay was compared to the conventional method which uses GDP-[^{14}C]-fucose and anion-exchange resin to separate product from remaining donor substrate. Under otherwise identical conditions the rate of incorparation of fucose into GnGn-dNS as measured by HPLC was 84% of that obtained with the conventional method using non-dansylated glycopeptide. In terms of the minimal enzyme activity detectable, the HPLC method appeared to be at least as sensitive as the radioactive standard procedure.

Recently, an interesting HPLC method for the determination of Fuc-T C6 has been reported [12]. It is currently unknown whether this procedure is suitable for the determination of Fuc-T C3.

Conclusion

Dansylated Asn(CHO)-Ser prepared from human IgG constitutes a substrate for the non- radioactive determination of Fuc-T C3 and Fuc-T C6 activity. Moreover, the two activities can be discriminated, which is a prerequisite for work with samples such as insect cells which can contain both fucosyltransferases.

Acknowledgements

This work was supported by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (PI0611GEN). We thank Thomas Dalik for excellent technical assistance.

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Received 18 October 1996, revised 20 March 1997, accepted 4 April 1997