CHARACTERIZATION OF A NEW ISOMER OF LIPID-LINKED HEPTASACCHARIDE FORMED DURING IN VITRO BIOSYNTHESIS OF MAMMARY GLYCOPROTEINS

Inder K. VIJAY and Gary H. PERDEW
Department of Dairy Science, University of Maryland, College Park, MD 20742, USA

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1. Introduction

Incubation of microsomes from the lactating bovine mammary tissue with UDP–GlcNAc and GDP–Man results in the biosynthesis of lipid-linked oligosaccharides $Man_n(GlcNAc)_2$, $n = 1–9$ [1]. Pulse and chase kinetics indicated these to be interrelated as precursor–products for the biosynthesis of asparagine-linked glycoproteins in this tissue. Structural analyses showed that among these, $Man(GlcNAc)_2$ through $Man_3(GlcNAc)_2$ and $Man_9(GlcNAc)_2$ species were monoisomeric [1]; however, 2 isomers of $Man_4(GlcNAc)_2$ and 3 isomers of $Man_5(GlcNAc)_2$ could be identified [2].

The resolution of isomers among hexa- and heptasaccharides was facilitated by the specificity of endo D and endo H towards oligomannosylchitobiose substrates [3,4]. The heptasaccharide cleaved by endo D was characterized as Manα1→2Manα1→3Manα1→6-(Manα1→3)Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc. Structural data on the octa- through decasaccharide indicated that an additional isomer might also be present in the endo-D-cleaved heptasaccharide. Using controlled acetolysis, in which only incipient cleavage of the α-1,6 linkages occurs and aided by the availability of an α-1,2-specific mannosidase, we now report the characterization of another isomer within the heptasaccharide, $Man_5(GlcNAc)_2$.

Abbreviations: Man, mannose; GlcNAc, *N*-acetylglucosamine; $(GlcNAc)_2$, *N,N'*-diacetylchitobiose; subscript OH and OT refer to $NaBH_4$- and NaB^3H_4-reduced oligosaccharides; endo, endo-β-*N*-acetylglucosaminidase; CHO, Chinese hamster ovary; All sugars are of the D-configuration

2. Materials and methods

These were as in [1], with the exception of minor additions and changes noted below.

The [^{14}C]mannose-labeled heptasaccharide isolated from a large scale in vitro incubation run without exogenous dolichol phosphate [2] was digested with endo D as before. The cleaved fragment $Man_5GlcNAc$ was separated from the resistant $Man_5(GlcNAc)_2$ by gel filtration on column of Bio Gel P-4 [1] and is the source material for these results.

2.1. *Digestion with α-1,2-mannosidase*

This enzyme was isolated from *Aspergillus saitoi* [5,6]. It had no α-1,3 and α-1,6 mannosidase activity since it failed to digest [^{14}C]mannose-labeled Manα1→3(Manα1→6)Manβ1→4(3)GlcNAcβ1→4(3)-GlcNAc. [^{14}C]Mannose-labeled $Man_5GlcNAc$ (~30 000 cpm) was treated with 100 ng enzyme in 0.05 M sodium acetate, pH 5.0 at 37°C under toluene. Additional enzyme was added after 24 and 48 h and the incubation continued for 72 h. After desalting [1], the products were chromatographed on Schleicher and Schüll paper 589C in ethyl acetate/pyridine/acetic acid/water (5:5:1:3 by vol.) for 33 h.

2.2. *Acetolysis*

This was conducted as before except that the incubation temperature was 30°C and the reaction was run for either 3 h (incipient acetolysis) or for 7 h (extended acetolysis). Incipient acetolysis revealed no cleavage of α-1,2, α-1,3 and β-1,4 linkages in Manα1→2Manα1→2Manα1→3Manβ1→4$GlcNAc_{OT}$ [1] and [^{14}C]mannose-labeled $Man_9GlcNAc_{OH}$ characterized earlier [1]. Only a trace release of free mannose

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was detectable upon extended acetolysis of the controls.

3. Results

In [2], the release of mannose and mannobiose upon acetolysis of the endo D-susceptible heptasaccharide was interpreted to be a result of overdegradation of this saccharide. A re-examination revealed that over a wide range of time courses of the acetolysis reaction at 35°C, the relative proportion of the mannose and mannobiose released from endo D-susceptible heptasaccharide was unchanged. A variation of different time and temperature combinations indicated that incipient acetolysis could be observed as early as 3 h upon incubation at 30°C; extended acetolysis provided a much higher cleavage of α-1,6 linkages without significant overdegradation resulting from the cleavage of α-1,2, α-1,3 and β-1,4 linkages. The availability of α-1,2-specific mannosidase provided an additional tool for a more rigorous structural analysis.

After reduction of the [^{14}C]mannose-labeled $Man_5GlcNAc$ with $NaBH_4$, a portion was subjected to incipient acetolysis (fig.1A). In addition to the radioactive peaks corresponding to mannose, mannobiose, mannotriose and $Man_2GlcNAc_{OH}$ (the latter 2 comigrate on paper but can be readily resolved by gel filtration [2]), a peak of radioactivity migrating as $Man_4GlcNAc_{OH}$ along with the undegraded $Man_5GlcNAc_{OH}$ was obtained. These results can be rationalized if one assumes that the $Man_5GlcNAc_{OH}$ contains 2 isomers (saccharides VII-1 and VII-2 in fig.1). Of these, VII-2 is the previously characterized endo D-susceptible heptasaccharide. For the saccharide VII-1, the Manα1→6Man linkage farther from the β-mannose would appear to be more readily cleaved upon incipient reaction than the α-1,6 link to β-mannose and thus would give rise to a $Man_4GlcNAc_{OH}$ as an acetolysis intermediate.

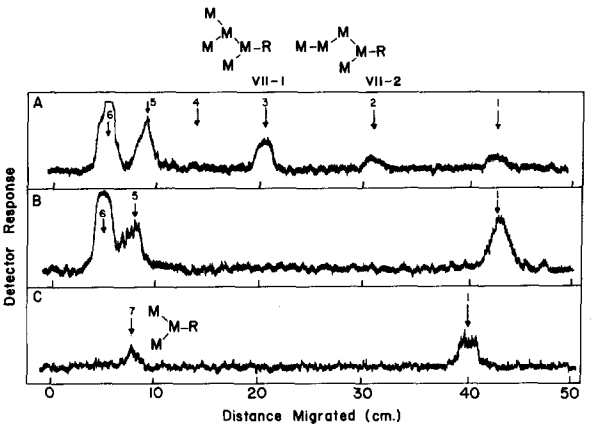

Fig.1. Products from the analysis of [^{14}C]mannose-labeled $Man_5GlcNAc_{OH}$ obtained by $NaBH_4$ reduction of endo D-cleaved $Man_5(GlcNAc)_2$: (A) incipient acetolysis; (B) α-1,2-specific mannosidase digestion; (C) shows products obtained from a control incubation of [^{14}C]mannose-labeled Manα1→2Manα1→2Manα1→3(Manα1→6)Manβ1→4(3)GlcNAcβ1→4(3)-GlcNAc with α-1,2-mannosidase. The standards are: (1) mannose; (2) mannobiose; (3) mannotriose and $Man_2GlcNAc_{OH}$; (4) $Man_3GlcNAc_{OH}$; (5) $Man_4GlcNAc_{OH}$; (6) the starting saccharide $Man_5GlcNAc_{OH}$; (7) $Man_3(GlcNAc)_2$.

Another portion of $Man_5GlcNAc_{OH}$ was treated with α-1,2-specific mannosidase (fig.1B). Nearly 50% of the saccharide was digested to give rise to mannose and a fragment that chromatographed as $Man_4GlcNAc_{OH}$. Taken together with the data from incipient acetolysis, these results indicate that radioactivity in the endo D-susceptible heptasaccharide is distributed almost equally between isomer VII-2 and an isomer that appears to be saccharide VII-1. A control incubation of α-1,2-mannosidase with [^{14}C]mannose-labeled Manα1→2Manα1→2Manα1→3(Manα1→6)-Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (see [3]) released mannose and a product that comigrated with Manα1→3(Manα1→6)Manβ1→4(3)GlcNAcβ1→4(3)-GlcNAc (fig.1C).

An extended acetolysis of the $Man_4GlcNAc_{OH}$ fragment in (fig.1A), presumably derived from saccharide VII-1, gave mannose, mannobiose, $Man_2GlcNAc_{OH}$ and the undegraded $Man_4GlcNAc_{OH}$ as products (fig.2A). These results are consistent with the structure expected from an acetolysis intermediate of isomer VII-1 in which there is only one α-1,6 link with the β-mannose. An extended acetolysis of the $Man_4GlcNAc_{OH}$ fragment in fig.1B, arising from Manα1→2Manα1→3Manα1→6(Manα1→3)Manβ1→4(3)-$GlcNAc_{OH}$ due to the action of α-1,2-mannosidase, also gave identical products (fig.2B). An extended acetolysis of $Man_5GlcNAc_{OH}$ species resistant to α-1,2-mannosidase digestion in fig.1B released mannose, mannobiose, $Man_2GlcNAc_{OH}$ along with the undegraded $Man_5GlcNAc_{OH}$ as major products (fig.2C). Upon methylation of the mannobiose, 2,3,4,6-tetra- and 2,4,6-*O*-methyl mannose were obtained as sole products (fig.3). The extremely high degree of uneven labeling of the mannose residues,

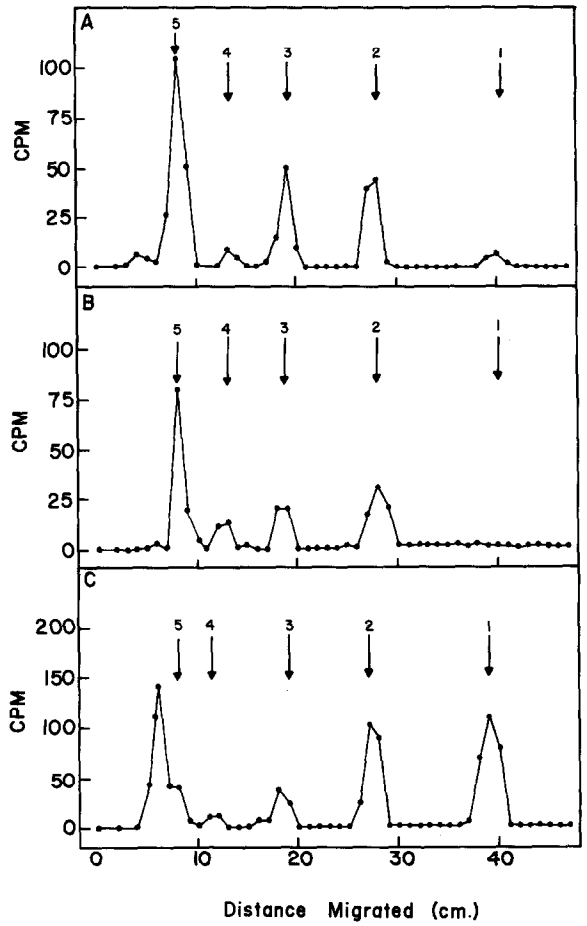

Fig.2. Paper chromatograms of the [^{14}C]mannose-labeled products obtained upon extended acetolysis of: (A) peak identified as $Man_4GlcNAc_{OH}$ in fig.1A; (B) peak identified as $Man_4GlcNAc_{OH}$ in fig.1B; (C) peak identified as the α-1,2-mannosidase resistant $Man_5GlcNAc_{OH}$ in fig.1B. The arrows indicate the position of standards: (1) mannose; (2) mannobiose; (3) $Man_2GlcNAc_{OH}$; (4) $Man_3GlcNac_{OH}$; (5) $Man_4GlcNAc_{OH}$. The slowest peak of radioactivity represents the undegraded starting saccharide.

due to the presence of endogenous lipid-linked oligosaccharides [1], is clearly noticeable.

Incubations of [^{14}C]mannose-labeled $Man_3(GlcNAc)_2$ and $Man_9(GlcNAc)_2$, characterized previously and a mixture of lipid-linked Man_3- to $Man_9(GlcNAc)_2$ [1] with the mammary microsomal preparation under the conditions used for the in vitro biosynthesis of these intermediates did not release any mannose (not shown), indicating the absence of interfering membrane-bound α-mannosidase(s).

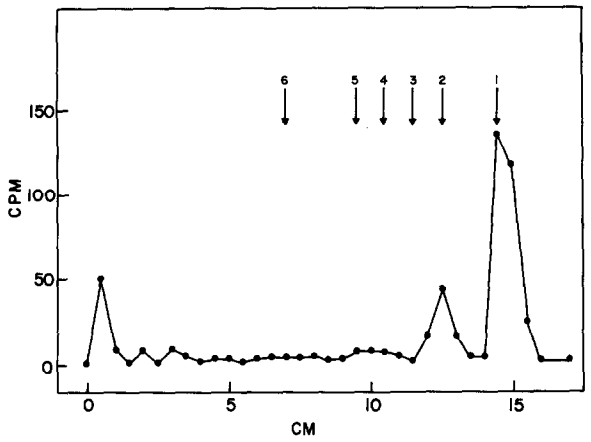

Fig.3. Thin-layer chromatography of methylated species from the product identified a mannobiose in fig.2C. The standards are: (1) 2,3,4,6-tetra-*O*-methyl mannose; (2) 2,4,6-tri-*O*-methyl-mannose; (3) 2,3,6-tri-*O*-methyl-mannose; (4) 2,3,4-tri-*O*-methyl-mannose; (5) 3,4,6-tri-*O*-methyl-mannose; (6) 2,4-di-*O*-methyl mannose.

4. Discussion

It is now established that lipid-linked oligosaccharides of the type $Glc_3Man_9(GlcNAc)_2$ serve as precursors for the en bloc glycosylation of nascent chains of asparagine-linked glycoproteins [7]. An ordered sequence for the in vivo assembly of the multibranched $Glc_3Man_9(GlcNAc)_2$ and its truncated form $Glc_3Man_5(GlcNAc)_2$ in CHO cells was proposed [8]. In vitro studies with the lipid-linked assembly of similar oligosaccharides revealed the presence of multiple isomers of several intermediates [2]. This study characterizes an unidentified lipid-linked heptasaccharide, Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4-(3)GlcNAcβ1→4(3)GlcNAc. This represents ~6–7% of the heptasaccharide synthesized by bovine mammary membranes.

Isomers VII-1 and VII-2 are present in roughly equal amounts and show a high degree of polarity in labeling in which bulk of the label is present in a mannose residue at the non-reducing end. These isomers could arise from the common precursor *Manα1→3**-Manα1→6(Manα1→3)Manβ1→4(3)GlcNAcβ1→4(3)-GlcNAc by either an α-1,2 addition to the *Man residue or an α-1,6 addition to the **Man residue of the hexasaccharide characterized in [2].

We were unable to detect a membrane-bound α-mannosidase in the bovine mammary membranes

under the conditions used to synthesize lipid-linked oligosaccharides. Also, pulse-chase kinetics carried over 3 h provided an excellent precursor–product relationship between different lipid-linked intermediates [1]. Thus, multiple isomers of lipid-linked oligosaccharides similar to saccharide VII-1 reported here could arise from alternate pathways of biosynthesis or result from lack of absolute specificity of mannosyltransferases for the acceptor substrates. The results in [9] indicated that the mannosyltransferases in lymphoma mutant cell membranes were not very specific for the acceptor substrate. Whether these in vitro synthesized isomers arise from 'flexibility' of the mannosyltransferases, relieved from the constraints of intracellular compartmentation, or result from multiple pathways of assembly, it seems important to realize the possibility of such multiisomeric products for defining the enzymology of glycoprotein biosynthesis. We are investigating the in vivo assembly of lipid-linked oligosaccharides in the bovine mammary tissue to better understand the basis of multiple isomers observed in vitro.

Certain features of the acetolysis technique and the oligosaccharide structure should be pointed out. The relative rate constants for the acetolysis of disaccharides Manα1→2Man, Manα1→3Man and Manα1→6Man were shown to be 1, 14 and 286, respectively [10]. However, biosynthetic high mannose oligosaccharides often have two Manα1→6Man linkages [11]. It need not be assumed that the relative rates of cleavage of the two α-1,6 linkages in these saccharides are the same. It would appear that the outer α-1,6 link is more readily accessible and cleaved than the inner α-1,6 linkage which is sterically somewhat blocked from the acetolysis reagents. In support of this, conformational models of high mannose saccharides in asparagine-linked glycoproteins indicate that the outer α-1,6-linked mannose is extended out while the inner α-1,6-mannose is somewhat folded back on the chitobiose unit [12]. Thus, incipient acetolysis, as described here, can generate an intermediate which can be further acetolysed for structural analysis. Not only does such an analysis prevent over-degradation but also it allows a more definitive interpretation of the fragmentation pattern. This should be a useful adjunct to the usual acetolysis procedure, designated here as extended acetolysis.

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