Carbohydrate Research 411 (2015) 37–41

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

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Preparation of asparagine-linked monoglucosylated high-mannose-type oligosaccharide from egg yolk

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ARTICLE INFO

Article history:
Received 23 March 2015
Received in revised form
15 April 2015
Accepted 17 April 2015
Available online 26 April 2015

Keywords:
Monoglucosylated high-mannose-type oligosaccharide
Glycopeptide
Egg yolk
IgY

ABSTRACT

Monoglucosylated high-mannose-type glycan ($Glc_1Man_9GlcNAc_2$: G1M9) is well-known as a key glycoform in the glycoprotein folding process, which is specifically recognized by lectin chaperones calnexin (CNX) and calreticulin (CRT) in the endoplasmic reticulum (ER). In this work, we developed an efficient method for the preparation of G1M9-Asn. The G1M9-Asn was obtained from the IgY-rich fraction derived from hen egg yolk by the digestion with pronase. The α-amino group of asparagine in G1M9-Asn was protected with the 9-fluorenylmethyloxycarbonyl (Fmoc) group and the labeled glycans were subsequently purified using high performance liquid chromatography (HPLC). This method will provide useful substrates for analysis of the glycoprotein folding cycle in the ER.

N-Linked glycosylation is a major post-translational modification of secretory proteins in eukaryotes, which plays important roles in countless biological processes.[1–4] N-Glycosylation of nascent proteins is initiated by the transfer of a high-mannose-type tetradecasaccharide ($Glc_3Man_9GlcNAc_2$) to asparagine residues embedded in an Asn-X-Thr/Ser (X is any amino acid except proline) triad of newly formed polypeptides. It is followed by stepwise digestion by glucosidase I (GI) and glucosidase II (GII), which leads them to mono- ($Glc_1Man_9GlcNAc_2$; G1M9) and non-glucosylated ($Man_9GlcNAc_2$; M9) glycoforms. They are the key elements in the glycoprotein folding cycle constituted by lectin chaperones calnexin (CNX) and calreticulin (CRT), folding sensor enzyme UDP-glucose: glycoprotein glucosyltransferase (UGGT), and GII.[5] To explore molecular mechanism of the folding cycle, facile access to high-mannose-type glycans, especially G1M9 and M9, is important. Our effort has been devoted to establish chemical as well as chemoenzymatic synthesis of high-mannose-type glycans.[6–9]

As chemical synthesis of glycoprotein glycans is labor-intensive, isolation from natural sources is an attractive alternative for certain purposes. In particular, hen egg yolk has been explored as an excellent source of glycoproteins and glycopeptides bearing complex- and high-mannose-type oligosaccharides.[10,11] Recently, Makimura et al.[12] reported preparation of Fmoc-Asn linked M9, through purification from egg yolk, chemical modification and extensive purification, which was used for chemical synthesis of M9 containing glycoproteins.[13]

On the other hand, however, a procedure, which assures the sufficient amount of G1M9-Asn derivatives has not been reported. Instead, preparation of G1M9-glycan from natural source has been achieved by hydrazinolysis/acetylation of glycopeptides, followed by labeling of the reducing end by fluorescent tags.[14,15] In this report, we describe the preparation of Asn-linked monoglucosylated high-mannose-type oligosaccharide (Fig. 1) from egg yolk IgY.

Egg yolk IgY is known to be exceptionally abundant in monoglucosylated high-mannose-type oligosaccharides,[14] which are rare in mature glycoproteins. Preparation of the G1M9-Asn was commenced by enrichment of IgY by sodium sulfate treatment.[14] For subsequent proteolysis of IgY, a previously reported method was adopted with some modifications. Namely, the IgY-rich fraction was treated by actinase E (from *Streptomyces griseus*), a commercially available protease, which is favorable for our purpose as it is not contaminated by α-glucosidase or α-mannosidase activities. After sequential purification by reverse phase and size exclusion columns, fractions containing glycoamino acids were collected and lyophilized. To facilitate purification, the α-amino group of the asparagine was converted to 9-fluorenylmethyloxycarbamate

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http://dx.doi.org/10.1016/j.carres.2015.04.011
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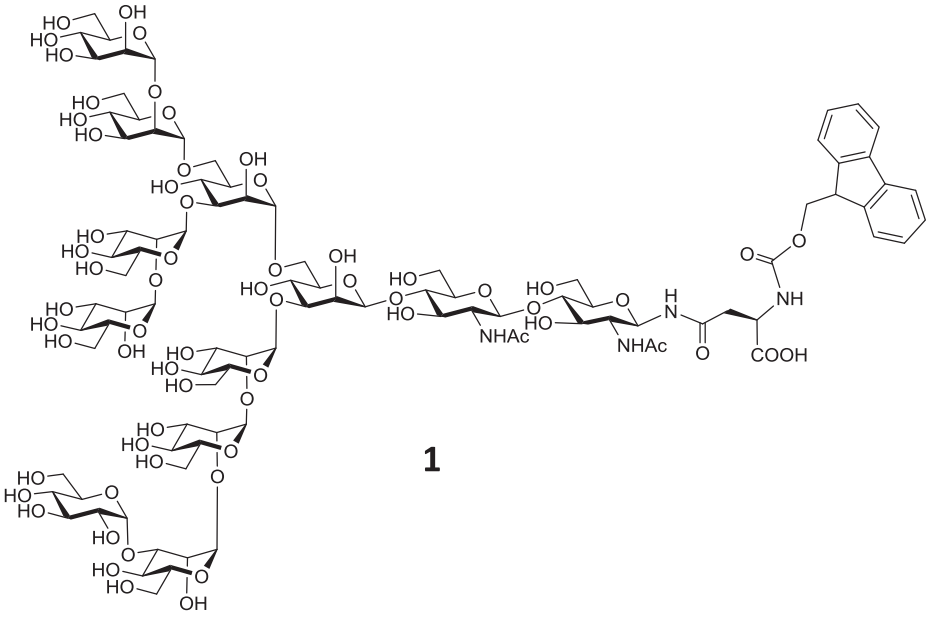

Fig. 1. Structure of G1M9-Asn-Fmoc **1**.

(Fmoc), which enables compound detection by UV-absorbance.[12] Thus, the mixture containing G1M9-Asn-Fmoc (**1**) was briefly purified by reversed phase column chromatography and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Fractions containing G1M9-Asn-Fmoc were concentrated and purified by HPLC equipped with an amide column.

As shown in Fig. 2a, the analytical HPLC profile revealed the presence of several Fmoc-protected glycan derivatives, as detected by absorbance at 254 nm. They were separately collected and analyzed by MALDI-TOF MS. The major peak, which was eluted at 38.2 min, had the *m*/*z* value 2404.89 (Fig. 3), assignable as G1M9-Asn-Fmoc (**1**) $[M+Na]^+$, in light of the calculated molecular weight of **1** (2382.16). The peak, which eluted slightly faster than **1** (at 37.1 min) was assigned as $Hex_9GlcNAc_2$-Asn-Fmoc, since its *m*/*z* value was one hexose unit (162) smaller than **1**. In light of the previous report,[14] it is likely to be a mixture of M9-and G1M8-Asn-Fmoc, whereas further analysis was not conducted. Additional peaks eluted earlier (32–36 min) exhibited *m*/*z* values in the range of 2500–3000, which were concluded to be a mixture of glycopeptides, which were not fully digested by proteolysis.

The structure of the purified product (Fig. 2b) assigned as **1** was further confirmed by 500 MHz NMR analysis as shown in Fig. 4. By comparison with previous reports,[6,12,16,17] anomeric signals were assigned as shown in Fig. 4b. In addition, two-dimensional NMR spectra, HSQC-DEPT and COSY, are shown in Fig. 4c and d, respectively. Through the HSQC-DEPT experiment, the other anomeric signals of two Man residues, which were obscured by water peak in ^1H NMR could be clearly detected. Comparing with M9-Asn-Fmoc spectrum,[12] we assigned the proton signal with a chemical shift of 5.13 ppm as the anomeric proton of terminal glucose (Fig. 4d), a doublet with $J_{1,2}$=3.8 Hz, supporting its anomeric configuration as α. All data are fully consistent with the identification of G1M9-Asn-Fmoc **1** as the structure of the major product.

In summary, a practical procedure to isolate monoglucosylated high-mannose-type oligosaccharide derivative **1** from the biological source was established. It enables preparation of ca. 3.7 mg of G1M9-Asn-Fmoc (**1**) from 1 g of egg yolk IgY. The compound **1** is expected to be versatile as an intermediate for chemical or enzymatic syntheses of glycopeptides and glycoproteins, which carry G1M9 glycans.

1. Experimental

1.1. General procedure

Actinase E was from Kaken pharmaceutical (Tokyo, Japan). N-(9-Fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) was from Watanabe Chemical Ind. Ltd. (Hiroshima, Japan). All other reagents were from the Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemicals Co. Inc. (Tokyo, Japan), or Tokyo Chemical Industries Co. Ltd. (Tokyo, Japan). All solutions were prepared immediately before use. NMR spectra were recorded with a Bruker Advance 500 spectrometer. Normal phase HPLC purifications were carried out on JASCO PU-2087 and UV-2070 using Inertsil Amide column (5 μm, 10×250 mm, GL Science, Tokyo, Japan). Analytical HPLC was carried out on Waters e2695 and 2998 using Inertsil Amide column (3 μm, 4.6×150 mm, GL Science, Tokyo, Japan). Mass spectroscopic measurements were carried out on Kratos Analytical AXIMA-LNR MALDI TOF Mass Spectrometer using DHB (2,5-dihydroxybenzoic acid) as a matrix.

1.2. Preparation of G1M9-Asn rich fraction from egg yolk

Enrichment of IgY-rich from egg yolk was carried out using sodium sulfate as reported.[14] In brief, the hen egg yolk was separated from egg white and mixed with 5 volumes of water, stood at 4 °C for 2 h and centrifuged (10,000 rpm, 20 min). Sodium sulfate (Na_2SO_4) was added to the supernatant to give a final concentration of 19%. After stirring over night at room temperature, the mixture was centrifuged (7000 rpm, 10 min) to give IgY-rich precipitate, which was then dissolved in water (30 mL) and dialyzed against water with a cellulose tube (UC36-32-100, MWCO 14 kDa, EIDIA Co. Ltd., Tokyo, Japan) for 2 h. The fraction containing 0.9 g of the protein (obtained from 40 eggs) was digested with 50 mg of actinase E in 50 mM Tris–HCl (pH 8.0), 5 mM $CaCl_2$, and 0.05% NaN_3 at 37 °C for 1 day. After addition of 50 mg of the enzyme again, the mixture was stood for another 1 day. The liberated glycans were