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Difucosylation of chitooligosaccharides by eukaryote and prokaryote α1,6-fucosyltransferases

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ABSTRACT

Background: The synthesis of eukaryotic *N*-glycans and the rhizobia Nod factor both involve α1,6-fucosylation. These fucosylations are catalyzed by eukaryotic α1,6-fucosyltransferase, FUT8, and rhizobial enzyme, NodZ. The two enzymes have similar enzymatic properties and structures but display different acceptor specificities: FUT8 and NodZ prefer *N*-glycan and chitooligosaccharide, respectively. This study was conducted to examine the fucosylation of chitooligosaccharides by FUT8 and NodZ and to characterize the resulting difucosylated chitooligosaccharides in terms of their resistance to hydrolysis by glycosidases.
Methods: The issue of whether FUT8 or NodZ catalyzes the further fucosylation of chitooligosaccharides that had first been monofucosylated by the other. The oligosaccharide products from the successive reactions were analyzed by normal-phase high performance liquid chromatography, mass spectrometry and nuclear magnetic resonance. The effect of difucosylation on sensitivity to glycosidase digestion was also investigated.
Results: Both FUT8 and NodZ are able to further fucosylate the monofucosylated chitooligosaccharides. Structural analyses of the resulting oligosaccharides showed that the reducing terminal GlcNAc residue and the third GlcNAc residue from the non-reducing end are fucosylated via α1,6-linkages. The difucosylation protected the oligosaccharides from extensive degradation to GlcNAc by hexosamidase and lysozyme, and also even from defucosylation by fucosidase.
Conclusions: The sequential actions of FUT8 and NodZ on common substrates effectively produce site-specific-difucosylated chitooligosaccharides. This modification confers protection to the oligosaccharides against various glycosidases.
General significance: The action of a combination of eukaryotic and bacterial α1,6-fucosyltransferases on chitooligosaccharides results in the formation of difucosylated products, which serves to stabilize chitooligosaccharides against the action of glycosidases.

1. Introduction

Many biomolecules, such as glycoproteins, glycolipids and oligosaccharides, are modified by the fucosylation of their oligosaccharide moiety or a polypeptide chain. In some glycoconjugates, it is known that such a modification plays a role in the regulation of their biological functions [1,2]. In eukaryotic cells, α1,6-fucosylation occurs at the innermost GlcNAc residue in asparagine-linked oligosaccharides (*N*-glycans) [3]. This fucose structure, referred to as a core fucose, is widely distributed in nature, ranging from insects to higher animals [1,2], and is formed by the action of a eukaryotic α1,6-fucosyltransferase, designated as FUT8 in the case of mammalian enzymes [4–6].

In prokaryotes, on the other hand, α1,6-fucosylation seems to be limited to Nod factor synthesis of *Rhizobium* [7]. Nod factor, which is required for the nodulation of legume roots for nitrogen-fixing, is biosynthesized from an oligosaccharide, as the result of several modifications such as methylation, sulfation, acetylation, acylation and fucosylation, all of which are catalyzed by a series of enzymes encoded by the Nod gene cluster [8]. α1,6-Fucosylation is one of the major modifications and is catalyzed by NodZ, a prokaryotic

Abbreviations: GDP, guanine nucleotide diphosphate; GN1, GlcNAc or *N*-acetylglucosamine; GN2, *N*,*N*′-diacetyl chitobiose; GN3, *N*,*N*′,*N*″-triacetyl chitotriose; GN4, *N*,*N*′,*N*″,*N*‴-tetraacetyl chitotetraose; GN5, *N*,*N*′,*N*″,*N*‴,*N*⁗-pentaacetyl chitopentaose; GN6, *N*,*N*′,*N*″,*N*‴,*N*⁗′,*N*⁗′′-hexaacetyl chitohexaose; GNF, NodZ-monofucosylated chitooligosaccharide; GNF′, FUT8-monofucosylated chitooligosaccharide; GNFF′, difucosylated chitooligosaccharide; Fuc, fucose; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; HSQC, hetero-nuclear single quantum coherence; TOCSY, total correlation spectroscopy; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight

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α1,6-fucosyltransferase. NodZ transfers a fucose residue to the reducing terminal GlcNAc residue of the oligosaccharide [9,10].

FUT8 and NodZ have similar structures and enzymatic properties, as indicated by our crystallographic analysis of FUT8 and other studies related to NodZ [11–13]. Both enzymes are structurally classified into the GT-B group of glycosyltransferases [14,15]. The catalytic domains of these enzymes are very homologous except for two additional domains in FUT8, an N-terminal coiled-coil structure and a C-terminal SH3 domain [11,12]. Consistent with being structurally similar, both enzymes transfer a fucose residue to the reducing terminal GlcNAc residue or its equivalent in spite of distinct specificities toward the acceptor substrates.

NodZ utilizes a chitooligosaccharide as the substrate for Nod factor synthesis. It is known that chitohexaose is one of the most favorable substrates for the enzymatic activity of NodZ, while NodZ is able to significantly react with an *N*-glycan, albeit less actively than FUT8, producing a core fucose structure [8]. On the other hand, our previous study demonstrated that FUT8 shows a broad substrate tolerance and, in fact, is able to fucosylate chitooligosaccharides at a comparable level to NodZ [16]. It has also been unexpectedly found that FUT8 and NodZ transfer a fucose to different GlcNAc residues in common chitooligosaccharides: the reducing terminal residue for NodZ and the third residue from the non-reducing end for FUT8 (Fig. 1).

In this study, on the basis of findings obtained to date, we examined the issue of whether difucosylated chitooligosaccharides can be synthesized by the combined use of FUT8 and NodZ. This kind of investigation may contribute to further development of neoglycoconjugates that can be used for various industrial and medical applications. To accomplish such a position-specific difucosylating reaction, it was necessary to investigate how active a monofucosylated chitooligosaccharide formed by either of the two enzymes is as a substrate for the other. Structural analyses involving normal phase-HPLC, mass spectrometry and NMR study were carried out to confirm whether the oligosaccharide was successfully and specifically difucosylated as expected. In addition, sensitivities to various glycosidases were compared between non-fucosylated and difucosylated chitooligosaccharides in order to examine a possible effect(s) of such a unique fucosylation on the properties of the oligosaccharide.

2. Material and methods

2.1. Chemicals

GDP-β-L-fucose was purchased from Wako Pure Chemicals (Osaka, Japan) and YAMASA Corporation (Chiba, Japan). β-*N*-acetylglucosaminidase from Jack bean and α-fucosidase from bovine kidney were purchased from Sigma (MO, USA). Chitooligosaccharides and lysozyme from hen egg white were purchased from Seikagaku Biobusiness Corporation (Tokyo, Japan). For expression of recombinant protein, pET28 vector was purchased from Merck (Darmstadt, Germany). Other common chemicals were obtained from Wako Pure Chemicals, Nacalai Tesque (Kyoto, Japan) and Sigma.

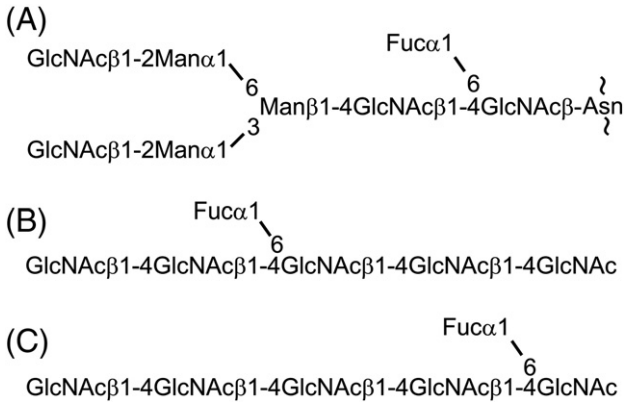

Fig. 1. Products of **α**1,6-fucose transfer reactions by FUT8 and NodZ. (A) FUT8 catalyzes the transfer of a fucose residue to the innermost GlcNAc of an *N*-glycan. (B) FUT8 also fucosylates a chitooligosaccharide at the third GlcNAc residue from the non-reducing end. (C) NodZ catalyzes the transfer of a fucose residue to the reducing terminal GlcNAc residue of a chitooligosaccharide.

2.2. Preparation of recombinant proteins

The recombinant protein of a soluble form of FUT8 was prepared using a baculovirus/insect cell expression system as described previously [17]. The recombinant NodZ protein was expressed in *Escherichia coli* (*E. coli*), as described previously [18], and was purified by Ni^{2+} chelate affinity chromatography. The cDNA for NodZ was amplified by the polymerase chain reaction from a genome of *Bradyrhizobium japonicum* USDA110, which was a generous gift from Dr. Akihiro Suzuki (Faculty of Agriculture, Saga University). The oligonucleotide primer set for amplification of the cDNA flanked by 5′ Nco I and 3′ Xho I sites are 5′-CGCGCCATGGGCGAAGTTCTACCGATGCAG-3′ as the forward primer and 5′-GCGCCCTCGAGCGAAGCCATAAGCGCTTGC-3′ as the reverse primer. The amplified fragment for NodZ was digested and inserted into a pET28 vector using the Nco I and Xho I sites. The obtained plasmid was used to transform *E. coli* BL21 (DE3). The recombinant NodZ protein was expressed in the transformed cells with MagicMedia™ *E. coli* expression medium (Life Technologies, California, USA), according the manufacturer's protocols. The cells were harvested and resuspended in 50 mM Tris–HCl buffer, 100 mM NaCl, pH 7.5. The resuspended cells were lysed by sonication using a sonifier S-150D (BRANSON, CT USA). The lysate was then centrifuged at 12,000 ×*g* for 15 min, and the supernatant was applied on Ni^{2+}-charged metal chelating Sepharose column (GE Healthcare, Tokyo, Japan) that had been pre-equilibrated with 50 mM Tris–HCl buffer, 100 mM NaCl, 50 mM imidazole, pH 7.5. After washing with 50 mM Tris–HCl buffer, 100 mM NaCl, 50 mM imidazole, pH 7.5, the recombinant protein was eluted from the column by increasing the concentration of imidazole up to 500 mM. The eluted protein was dialyzed against 50 mM Tris–HCl buffer, 100 mM NaCl, pH 7.5. If necessary, the purified protein was concentrated using an Amicon YM-10 (Millipore).

2.3. Protein determination

Protein concentrations were determined by a BCA Kit (PIERCE, IL, USA) using bovine serum albumin as a standard.

2.4. Assay for α1,6-fucosyltransferase activities

Activities of FUT8 and NodZ were assayed using chitooligosaccharides, as reported previously [16,18,19]. The recombinant enzymes were incubated at 37 °C with 1 mM of chitooligosaccharides and 1 mM GDP-β-L-fucose in 0.1 M MES–NaOH, pH 7.0. The reactions were terminated by boiling after an appropriate reaction time, and the reaction mixtures were centrifuged at 15,000 ×*g* in a microcentrifuge for 10 min. The resulting supernatants were analyzed by normal phase HPLC (2695 Separation Module, Waters, MA, USA) equipped with TSKgel Amide-80 (4.6 × 250 mm, Tosoh, Tokyo, Japan), as reported for chitinase activity assay [20,21]. The reaction products and the unreacted substrate were isocratically separated at 30 °C with 70% acetonitrile at a flow rate of 1.0 ml/min. Absorbance of the column eluent was monitored at 210 nm with a UV monitor (2487 Multi λ Absorbance Detector, Waters) [20,21].