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Communication

Evidence for New β1-3 Galactosyltransferase Activity Involved in Biosynthesis of Unusual *N*-Glycan Harboring T-Antigen in *Apis mellifera*

Yoshinobu KIMURA,[1,†] Sho SAKAMURA,[1] Takayuki USHIJIMA,[1] Yoichiro HAMA,[2] Hiroyuki KAJIURA,[3] Kazuhito FUJIYAMA,[3] Kiyoshi OKIHARA,[4] Ken HASHIMOTO,[4] Hiroyuki SUGIMOTO,[4] and Hideo YAMADA[4]

[1]*Department of Biofunctional Chemistry, Division of Bioscience, Graduate School of Natural Science and Technology, Okayama University, Tsushima naka 1-1-1, Okayama 700-8530, Japan*
[2]*Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Honjyo 1, Saga 840-8502, Japan*
[3]*The International Center for Biotechnology, Osaka University, Yamadaoka 2-1, Suita 565-0871, Japan*
[4]*Yamada Apiculture Center, Inc., Ichiba 194, Kagamino-cho, Tomada-gun, Okayama 708-0393, Japan*

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In a previous study (Y. Kimura *et al.*, *Biosci. Biotechnol. Biochem.*, 70, 2583–2587, 2006), we found that new complex type *N*-glycans harboring Thomsen-Friedenreich antigen (Galβ1-3GalNAc) unit occur on royal jelly glycoproteins, suggesting the involvement of a new β1-3galactosyltransferase in the synthesis of the unusual complex type *N*-glycans. So far, such β1-3galactosyltransferase activity, which can transfer galactosyl residues with the β1-3 linkage to β1-4 GalNAc residues in *N*-glycan, has not been found among any eucaryotic cells. But using $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA as acceptor *N*-glycan, we detected the β1-3 galactosyltransferase activity in membrane fraction prepared from honeybee cephalic portions. This result indicates that honeybee expresses a unique β1-3 galactosyltransferase involved in biosynthesis of the unusual *N*-glycan containing a tumor related antigen in the hypopharyngeal gland.

Key words: royal jelly glycoprotein; *N*-glycan; Thomsen-Friedenreich antigen (Galβ1-3GalNAc); β1-3 galactosyltransferase; *Apis mellifera*

In a previous study, we found a new structure of *N*-glycans containing the Thomsen-Friedenreich antigen (T-antigen) unit, one of the tumor-related glycans, among *N*-glycans from royal jelly glycoproteins.[1)] Until this finding it was considered that the T-antigen structure occurs only in *O*-glycans,[2,3)] and thus this finding suggested for the first time that a new *N*-glycan processing mechanism works to construct the new tumor antigen-containing *N*-glycans[1)] in the hypopharyngeal gland of honeybee. Although many Core 1 β1-3galactosyltransferases (T-synthase) that transfer the galactose residue by β1-3 linkage to α-*N*-acetylgalactosamine (GalNAc) linked to serine or threonine residues have been characterized and the corresponding genes have been identified also in mammals,[4,5)] nematodes,[6)] and insects,[7)] no β1-3galactosyltransferase (β1-3GalT) involved in the biosynthesis of the T-antigen unit in *N*-glycan moiety has been found thus far. In this study, therefore, we analyzed the β1-3GaT activity in extract of membrane fraction (containing microsomal membrane) prepared from honeybee head portion using GalNAc-containing *N*-glycan ($GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA) as a substrate oligosaccharide.

Since royal jelly glycoproteins are synthesized in the hypopharyngeal gland, only honeybee heads were used as starting material in this study. Honeybee cephalic portion (0.5 g) was homogenized in 2 ml of 10 mM MOPS buffer (pH 7.3) containing 0.25 M sucrose, 20 mM PMSF, and 20 mM antipain (buffer A), and the resulting extract was centrifuged at 1,000 *g* for 10 min. The supernatant was then centrifuged at 105,000 *g* for 60 min to prepare a microsomal membrane fraction. The resulting precipitate was suspended in 250 µl of buffer A

† To whom correspondence should be addressed. Fax: +81-86-251-8296; E-mail: yosh8mar@cc.okayama-u.ac.jp

Abbreviations: PA-, pyridylamino-; SF-HPLC, size-fractionation HPLC; ESI-MS, electrospray ionization mass spectrometry; Hex, hexose; HexNAc, *N*-acetyl-D-hexosamine; Man, D-mannose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; GN2M3, $GlcNAc_2Man_3GlcNAc_2$-PA (GlcNAcβ1-Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA); E2, $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA; E4, $Gal_1GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA; E5, $Gal_2GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6(Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA); T-antigen, Thomsen-Friedenreich antigen (Galβ1-3GalNAc); β1-3GalT, β1-3 galactosyltransferase; PMSF, phenylmethanesulfonyl fluoride; MOPS, 3-(*N*-Morpholino)-propanesulfonic acid

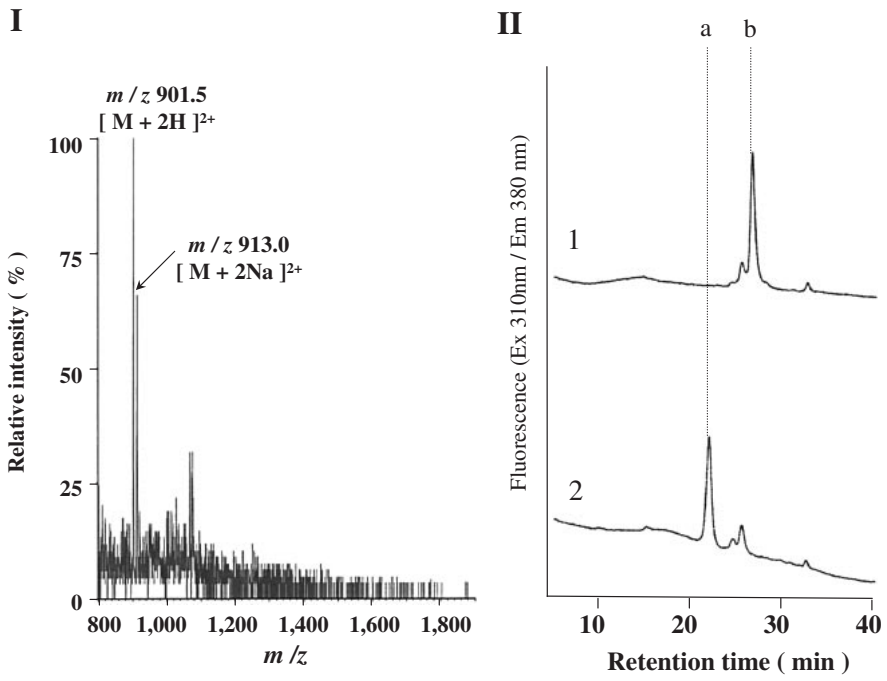

Fig. 1. Structural Analysis of PA-Sugar Chain E2 ($HexNAc_4Hex_3HexNAc_2$-PA) as Substrate for β1-3 GalT.

I, ESI-MS analysis of E2. II, β-*N*-acetylgalactosaminidase digestion of E2. 1, E2; 2, *Bacillus* β-*N*-acetylgalactosaminidase digest of 1. The letters a and b indicate the elution positions of authentic PA-sugar chains; a, $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA prepared from $Gal_2GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E5) by β-galactosidase digestion, as described in our previous report;[1] b, GN2M3 ($GlcNAc_2Man_3GlcNAc_2$-PA).[8]

containing 1% Triton X-100 and sonicated in an ice bath for 1 min. After incubation at 20 °C for 60 min, the extract was centrifuged again at 105,000 *g* for 60 min, and the resulting supernatant (about 200 µl) was used in the subsequent experiment as crude enzyme solution.

Pyridylaminated *N*-glycan substrate ($GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA) was prepared by the β1-3 galactosidase digestion of Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6(Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA (E5)[1] or royal jelly glycoproteins, as described in our previous paper. Briefly, glycopeptides were prepared from actinase digest of crude royal jelly (32 g, Yamada Apiculture Center, Okayama, Japan) by gel-filtration with a Sephadex G-25 column (3.8 × 40 cm). *N*-Glycans were released by hydrazinolysis (100 °C, 10 h, in 10 ml of hydrazine anhydrous) from lyophilized glycopeptides. Liberated oligosaccharides were *N*-acetylated, desalted, and labeled with 2-aminopyridine, as described in a previous report.[8] The resulting PA-sugar chains were separated by reverse-phase (RP) HPLC using a Shiseido Capcell pak C18 MG column (10 × 250 mm, Shiseido, Tokyo), as described in a previous report.[1] *N*-Glycans containing the HexNAc-HexNAc unit were detected in fraction E (Fig. 1-I in our previous paper),[1] and PA-sugar chains in the fraction were further separated by size fractionation (SF) HPLC using a Shodex Asahipak NH2P-50 column (10 × 250 mm, Showa Denko, Tokyo, Japan) (Fig. 1-II in our previous paper).[1] ESI-MS analysis revealed that PA-sugar chain E2 consisted of HexNAc5, Hex3, and HexNAc-PA (*m/z* 901.5 for $[M + 2H]^{2+}$ and *m/z* 913.0 for $[M + 2Na]^{2+}$), as shown in Fig. 1-I. As shown in Fig. 1-II, PA-sugar chain E2 was converted to $GlcNAc_2Man_3GlcNAc_2$-PA by recombinant *Bacillus* β-*N*-acetylgalactosaminidase,[9] indicating the occurrence of two β-GalNAc residues in E2. This product was further digested to the trimannosyl core structure, $Man_3GlcNAc_2$-PA, with diplococcal β-*N*-acetylhexosamindase (Boehringer Mannheim, Germany) (data not shown). Furthermore, the branching structure of E2 was analyzed by methylation analysis by the method described in a previous paper.[10] As shown in Fig. 2, the following permethylated alditol acetates were detected: 3,4,6-tri-O-methyl-Man (peak-a), 2,4-di-O-methyl-Man (peak-b), 3,4,6-tri-O-methyl-HexNAc (might be 3,4,6-tri-O-methyl-GalNAc, peak-c), and 3,6-di-O-methyl-GlcNAc (peak-d). Although the recovery rates of permethylated alditol acetates of HexNAc were relatively low in comparison with those of Man, methylation analysis indicated that the structure of E2 must carry the GalNAc1-4GlcNAc unit. The β-galactosidase digest of Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6(Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA (E5)[1] was eluted at the same positions as those of E2 on SF-HPLC and RP-HPLC. Considering these results of ESI-MS analysis, exoglycosidase digestion, and methylation analysis, the structure of E2 must be GalNAcβ1-4GlcNAcβ1-2Manα1-6(GalNAcβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA.

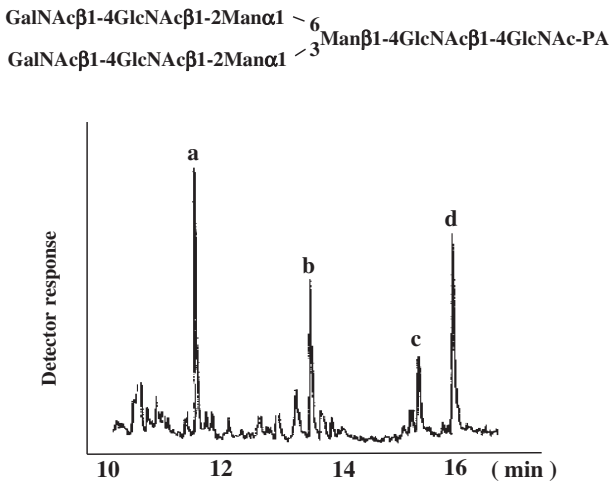

Fig. 2. Methylation Analysis of E2.

Each methylated alditol acetate derivative was analyzed by GC–MS, as described in our previous report.[9] a, 3,4,6-tri-O-methyl-Man; b, 2,4-di-O-methyl-Man; c, 3,4,6-tri-O-methyl-GalNAc; d, 3,6-di-O-methyl-GlcNAc.

Crude extract (15 μl) prepared from honeybee cephalic portion was incubated with 680 pmol of Gal$NAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E2), 20 mM UDP-Gal, 1.6 mM chitotriose (the generous gift of Dr. Takeshi Yamagami, Kyushu University) in 125 mM MOPS buffer (pH 7.3) containing 7.5 mM $MnCl_2$, 10% glycerol, and 0.5% Triton X-100 at 37 °C for 4 h or 16 h. As shown in Fig. 3-I, a new product was detected at about 28 min in a time-dependent manner, and the elution position corresponded to that of $Gal_1GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E4) ($m/z$ 983.0 $[M + 2H]^{2+}$ and m/z 993.5 for $[M + 2Na]^{2+}$), suggesting that a galactose residue was transferred to $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E2). This was further confirmed by β1-3 galactosidase (recombinant expressed in *E. coli*, Sigma, St. Louis, MO, USA) digestion. The product was pooled, as indicated by a horizontal bar in Fig. 3-I-3, and treated with β-galactosidase, as described in our previous paper.[1] The elution position of the β1-3 galactosidase digest of the product corresponded to that of Gal$NAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E2), as shown in Fig. 3-II-3, indicating that the galactosyl residue was transferred by β1-3 linkage but not by β1-4 linkage. These results clearly indicate that the extract of membrane fraction (containing microsomal membrane) prepared from honeybee cephalic portion contained β1-3 galactosyltransferase activity involved in the biosynthesis of T-antigen in the *N*-glycan moiety. To our knowledge, this is the first report indicating the occurrence of a new β1-3 galactosyltransferase that can transfer galactosyl residue by β1-3 linkage to β1-4 GalNAc residue in *N*-glycan. It is noteworthy that

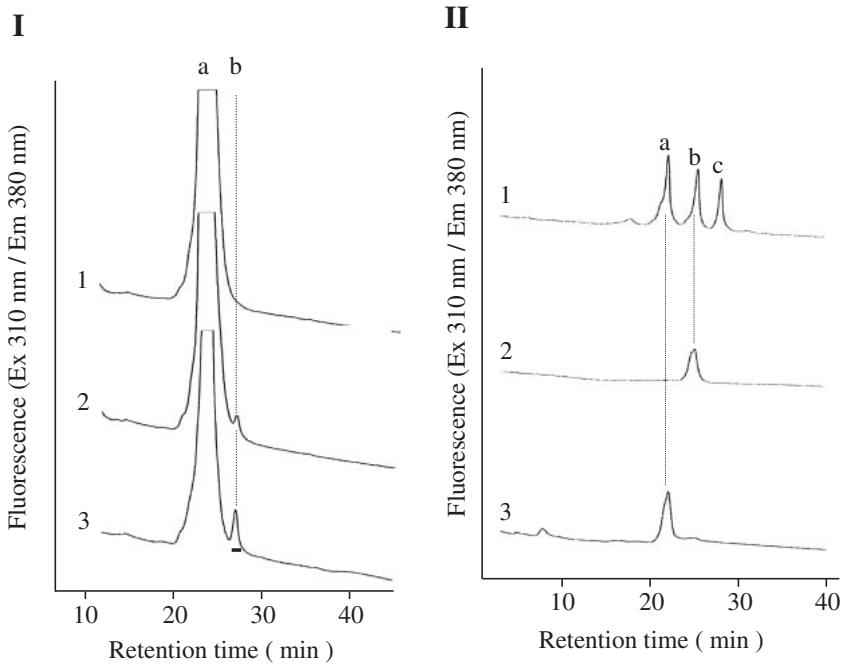

Fig. 3. Detection of Honeybee β1-3GalT Activity.

I, SF-HPLC of E2 ($GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA) treated with microsomal extract prepared from honeybee cephalic portion. 1, incubated with heat-treated microsomal extract. 2, incubated with the microsomal extract for 4 h. 3, incubated for 16 h. The product was pooled as indicated by the horizontal bar and treated with β1-3 galactosidase (Sigma). The letters a and b indicate the elution positions of authentic PA-sugar chains; a, $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E2); b, $Gal_1GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E4). II, SF-HPLC of β1-3 galactosidase digest of the product obtained in I-3. 1, Authentic PA-sugar chains; a, $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E2); b, $Gal_1GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E4); c, $Gal_2GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E5). 2, the product obtained in I-3. 3, β1-3 galactosidase digest of 2.

prolonged incubation with the extract of membrane fraction, UDP-Gal, and E2 did not produce E5 ($Gal_2GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA), suggesting that another β1-3 GalT is necessary for transfer of the second galactosyl residue, or that the first galactosyl residue transferred causes steric hinderance to the transferase activity in the case of the free form oligosaccharide substrate. To clarify this issue, transferase assays using higher concentrations of $Gal_1GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E4) and glycopeptides bearing $Gal_1GalNAc_2GlcNAc_2Man_3GlcNAc_2$ or $GalNAc_2GlcNAc_2Man_3GlcNAc_2$ should be done in the future.

Based on genetic information on *Drosophila* β1-3 galactosyltransferase,[7] we found a putative β1-3 galactosyltransferase gene in the honeybee genome database (http://racerx00.tamu.edu/bee_resources.html), and succeeded in expression of the gene in *E. coli* and purification of the recombinant protein, but we could not detect β1-3 galactosyltransferase activity of the expressed protein towards $GalNAc_2GlcNAc_2Man_3GlcNAc_2$-PA (E2) or benzyl 2-acetamido-2-deoxy-α-galactopyranoside (benzyl-α-GalNAc). Since it has been found that *O*-glycan β1-3 GalT (T-synthase) requires a species-specific molecular chaperone (Cosmc for vertebrate *O*-glycan β1-3 GalT activity)[11] for transferase activity, it might be necessary that the putative honeybee *N*-glycan β1-3 GalT gene should be expressed in insect cells to achieve enzyme activity. Expression of the putative β1-3 GalT in insect cells is in progress. It is also important to distinguish whether the *N*-glycan β1-3 GalT activity arose from new β1-3 GalT or by productive activity of Core 1 T-synthase. Hence the transferase assay of the recombinant *Apis* β1-3 galactosyltransferase-like protein using *para*-nitrophenyl 2-acetamido-2-deoxy-β-galactopyranoside (*p*NP-β-GalNAc) as another substrate will be described in a subsequent paper.

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