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

Yoshinobu Kimura,1,2* Sho Sakamura,1 Takayuki Ushijima,1 Yoichiro Hama,2 Hiroyuki Kajura,1 Kazuhiro Fujiyama,3 Kiyoshi Okihara,4 Ken Hashimoto,4 Hiroyuki Sugimoto,4 and Hideo Yamada4

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

Received February 9, 2007; Accepted February 23, 2007; Online Publication, April 7, 2007

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

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 β1-4 GalNAc residues in N-glycan, has not been found among any eucaryotic cells. But using GalNAc2GlcNAc2Man3-GlcNAc2-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.

**Evidence for New β1-3 Galactosyltransferase Activity Involved in Biosynthesis of Unusual N-Glycan Harboring T-Antigen in *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.14) 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-glycans15) 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 (GalNAc2GlcNAc2Man3-GlcNAc2-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 30,000 g for 10 min. The resulting precipitate was suspended in 250 μl of buffer A
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 (GalNAc2GlcNAc2Man5GlcNAc2-PA) was prepared by the β1-3 galactosidase digestion of Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA (ES5) or royal jelly glycoproteins, as described in our previous report.1, b, GN2M3 (GlcNAcMan5GlcNAc2-PA).8) The resulting PA-sugar chains were separated by reverse-phase (RP) HPLC using a Shiseido Asahipak NH2P-50 column (10 × 250 mm, Showa Denko, Tokyo), as described in a previous report.10) As shown in Fig. 1-I, PA-sugar chain E2 was converted to GlcNAc2Man5GlcNAc2-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, Man3GlcNAc2-PA, with diplococcal β-N-acetylgalactosaminidase (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: 2,4-di-O-methyl-Man (peak-a), 2,3,4,6-tetra-O-methyl-Man (peak-b), 3,4,6-tri-O-methyl-HexNAc (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 be Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA (ES5)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.
Crude extract (15 μl) prepared from honeybee cephalic portion was incubated with 680 pmol of GalNAc₂GlcNAc₂Man₃GlcNAc₂-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 MnCl₂, 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 Gal₁GalNAc₂GlcNAc₂Man₂GlcNAc₂-PA (E₄) (m/z 983.0 [M + 2H]⁺ and m/z 993.5 for [M + 2Na]⁺), suggesting that a galactose residue was transferred to GalNAc₂GlcNAc₂Man₃GlcNAc₂-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 β₁-3 galactosidase, as described in our previous paper.¹ The elution position of the β₁-3 galactosidase digest of the product corresponded to that of GalNAc₂GlcNAc₂Man₂GlcNAc₂-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. 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.

**Fig. 3. Detection of Honeybee β₁-3GalT Activity.**
I, SF-HPLC of E2 (GalNAc₂GlcNAc₂Man₂GlcNAc₂-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, GalNAc₂GlcNAc₂Man₂GlcNAc₂-PA (E₂); b, Gal₁GalNAc₂GlcNAc₂Man₂GlcNAc₂-PA (E₄). II, SF-HPLC of β₁-3 galactosidase digest of the product obtained in I-3. 1, Authentic PA-sugar chains; a, GalNAc₂GlcNAc₂Man₂GlcNAc₂-PA (E₂); b, Gal₁GalNAc₂GlcNAc₂Man₂GlcNAc₂-PA (E₄); c, Gal₂GalNAc₂GlcNAc₂Man₂GlcNAc₂-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 (Gal₂GalNAc₂GlcNAc₂Man₃GlcNAc₂-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 Gal₁GalNAc₂GlcNAc₂Man₃GlcNAc₂ or GalNAc₂GlcNAc₂Man₃GlcNAc₂ should be done in the future.

Based on genetic information on Drosophila β1-3 galactosyltransferase,11 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 GalNAc₂GlcNAc₂Man₃GlcNAc₂-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 (Cosme for vertebrate O-glycan β1-3 GalT activity)111 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 (pNP-β-GalNAc) as another substrate will be described in a subsequent paper.

Acknowledgments

We thank the IS-MS laboratory of Okayama University for MS analysis. This work was supported in part by grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Basic Research C, no. 17580300).

References