Communication

350-kDa Royal Jelly Glycoprotein (Apisin), Which Stimulates Proliferation of Human Monocytes, Bears the β1-3Galactosylated N-Glycan: Analysis of the N-Glycosylation Site

Mariko Kimura,1 Yoshinobu Kimura,2† Kazunori Tsumura,2 Kiyoshi Okihara,3 Hiroyuki Sugimoto,3 Hideo Yamada,3 and Masami Yonekura4

1Faculty of Food Culture, Department of Food System, Kurashiki Sakuyo University, Nagao-Tamashima, Kurashiki 710-0292, Japan
2Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Tsushima-Naka 1-1-1, Okayama 700-8530, Japan
3Yamada Apiculture Center Inc., Ichiba 194, Kagamino-cho, Tomada-gun, Okayama 708-0393, Japan
4Department of Bioresources Science, School of Agriculture, Ibaraki University, Ami-machi, Inashiki-gun, Ibaraki 300-0393, Japan

Received June 6, 2003; Accepted June 28, 2003

While doing a structural analysis of minor component N-glycans linked to 350-kDa royal jelly glycoprotein (RJGP), which stimulates the proliferation of human monocytes, we found that a Galβ1-3GlcNAcβ1-4Man unit occurs on the insect glycoprotein. The structure of the fluorescence-labeled N-glycan was analyzed by sugar component analysis, IS-MS, and 1H-NMR. The structural analysis showed that the 350-kDa RJGP bears Galβ1-3GlcNAcβ1-4(GlcNAcβ1-2)Manα1-3(Manα1-3Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc, suggesting this insect glycoprotein is one of the substrates for both β1-3 galactosyl and β1-4 N-acetylgalcosaminyl transferases. To our knowledge, this is the first report that succeeded in identifying an insect glycoprotein bearing the β1-3 galactosylated N-glycan.

Key words: royal jelly; insect glycoprotein; N-glycosylation; site analysis; Apis mellifera

Recently, we have found some hybrid and complex-type N-glycans bearing the Galβ1-3GlcNAcβ1-4Man unit among total N-glycans prepared from the royal jelly glycoproteins mixtures, although the identification of glycoprotein(s) bearing the β1-3 galactosylated N-glycan(s) has not been done. In our previous paper,1 we showed that 350-kDa royal jelly glycoprotein (350-kDa RJGP or apisin), which stimulates the proliferation of human monocytes,2 bears a typical high-mannose type oligosaccharide (Manα3,6GlcNAc2) as major components of N-glycans. During structural analysis of minor components of N-glycans linked to this royal jelly glycoprotein, we found an N-glycan bearing a galactose residue. Since the occurrence of a galactosyl residue in N-glycans of naturally occurring insect glycoproteins has hardly been reported so far,1,4 we describe in this report the structural analysis of the galactosylated N-glycan and a glycopeptide bearing the N-glycans from the 350-kDa RJGP.

The N-glycans were liberated from the 350-kDa RJGP by hydrazinolysis (100°C for 12 hr, 100 mg protein in 5 ml of hydrazine anhydrous) and the resulting hydrazinolysate was N-acetylated with saturated sodium bicarbonate and acetic anhydride as described in our previous paper.5 The liberated N-glycans were tagged with 2-aminopyridine by the method of Kondo et al.6 After gel-filtration by a Sephadex G-10 column (2.8×45 cm) to exclude the excess reagents, the resulting pyridylaminated (PA-) derivatives were purified by RP-HPLC using a Cosmosil 5C18-AR column (4.6×250 mm) as described in our previous paper.5 As shown in Fig. 1, two major PA-sugar chain fractions (A and B) were obtained. Since we have already reported that fraction A contains three high-mannose type sugar chains (Man9GlcNAc2-PA (M9A), Man8GlcNAc2-PA

† To whom correspondence should be addressed. Fax: +81-86-251-8388; E-mail: yosh8mar@cc.okayama-u.ac.jp

Abbreviations: RJGP, royal jelly glycoprotein; RP-HPLC, reverse-phase HPLC; SF-HPLC, size-fractionation HPLC; PA, pyridylaminoligo; IS-MS, ionspray ionization mass spectrometry; MS/MS, tandem mass; Hex, hexose; HexNAc, N-acetylatedhexosamine; Man, d-mannose; Gal, d-galactose; GlcNAc, N-acetyld-galactosamine; M5A, Man1-6(Man1-3)Man1-6(Man1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; M7A, Man1-2Man1-6(Man1-3)Man1-6(Man1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; M8A, Man1-2Man1-6(Man1-3)Man1-6(Man1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; M9A, Man1-2Man1-6(Man1-2Man1-3)Man1-6(Man1-2Man1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA
Fig. 1. HPLC of PA-Sugar Chains from 350-kDa Royal Jelly Glycoprotein.

[I] RP-HPLC of PA-derivatives from the 350-kDa RJGP using a Cosmosil 5C18-AR (6.0 × 250 mm). [II] Size-fractionation HPLC of the fraction B obtained in [I], using a Shodex Asahipak NH2P-5 column (4.6 × 250 mm). [III] MS/MS spectrum of PA-sugar chain B2 obtained in [II].

(M8A), and Man7GlcNAc2-PA (M7A)),20 we focused on Fraction B in Fig. 1-[I]. PA-sugar chains in the fraction were further purified by SF-HPLC using a Shodex Asahipak NH2P-50 column (Showa Denko, 4.6 × 250 mm) as described in our previous paper.5) As shown in Fig. 1-[II], two PA-sugar chains (B1 and B2) were obtained. Although we have already analyzed the structure of B1 (Man5GlcNAc2-PA (M5A)), the structure of B2 has remained to be determined. Sugar component analysis of B2 by GLC (Hitachi G-3000 with a DB-1 capillary column (30 m × 0.25 mm)), after methanolysis followed by trimethylsilylation, showed that this PA-sugar chains consists of mannose, galactose, and a GlcNAc residue, indicating that this PA-sugar chain belongs to the galactose-containing complex-type of structure.

A double-charged ion [M + 2H]2+ of B2 was observed at m/z 861.0 on ion-spray mass spectrometry (Perkin Elmer Sciex API-III),5) which agreed with the expected mass of HexNAc2Hex5HexNAc2-PA. As shown in Fig. 1-[III], the relevant signals observed by IS-MS/MS analysis of B2 could be reasonably assigned as fragment ions derived from Gal1GlcNAc2Man4GlcNAc2-PA; m/z 1516.5 (Gal1GlcNAc1Man4GlcNAc2-PA), m/z 1354.5 (GlcNAc1Man4GlcNAc2-PA), m/z 1193.0 (Gal1GlcNAc1Man2GlcNAc2-PA and/or GlcNAc1Man3GlcNAc2-PA), m/z 1151.5 (Man4GlcNAc2-PA), m/z 1031.0 (GlcNAc1Man2GlcNAc2-PA), m/z 989.0 (Man3GlcNAc2-PA), m/z 827.5 (Man2GlcNAc2-PA), m/z 665.5 (Man1GlcNAc2-PA), m/z 503.5 (GlcNAc2-PA), m/z 366.0 (Gal1GlcNAc1 and/or Man1GlcNAc1), and m/z 300.0 (GlcNAc-PA).

The structure of B2 was further analyzed by 500 MHz 1H-NMR (Fig. 2). Comparing the chemical shifts of structural reporter groups between B2 and GlcNAc1Man4GlcNAc2-PA,5) two additional signals (4.450 ppm and 4.567 ppm) were observed in the spectrum for B2. The chemical shifts observed at 4.450 ppm (with the coupling constant J1.2 (7.6 Hz), indicating the b-conformation) and 4.567 ppm agreed well with those of the b1–3 Gal residue (δ = 4.441 ppm) and b1–4 GlcNAc residue (δ = 4.570 ppm) in the N-glycan linked to bovine fetuin, respectively,7) suggesting that the galactosyl residue is bound to the galactose-containing complex-type of structure. To reveal the amino acid sequence around the glycosylation site(s) of 350-kDa RJGP, we purified some glycopeptides from a tryptic digest of the royal jelly glycoprotein and sequenced them. 350-kDa RJGP (100 mg) was carboxymethylated8) and digested with 2 mg of trypsin (TPCK-treated, Sigma) in 0.1 M Tris-HCl (pH 8.8) at 37°C for 24 hr. After gel-
Insect Glycoprotein Bearing $\beta1-3$Glactosylated $N$-Glycan

Fig. 2. 500 MHz $^1$H-NMR Spectrum.

[I], $^1$H-NMR Spectrum of PA-Sugar Chain B2. The spectrum was measured with a Varian VXR 500 instrument at 27°C as described in earlier.5) [II], Chemical Shifts of Structural reporter groups of PA-Sugar Chain B2 and authentic $N$-glycans. a) GlcNAc$\beta1-2$Manol$-3$(Manol$-3$Manol$-6$)Man$β1-4$GlcNAc$β1-4$GlcNAc-PA, 5) b) (Gal$β1-3$GlcNAc$β1-4$)(Gal$β1-4$GlcNAc$β1-2$)Manol$-3$(Gal$β1-4$GlcNAc$β1-2$Manol$-6$)Man$β1-4$GlcNAc$β1-4$GlcNAc.7)

filtration through a Sephadex G-50 column, the positive fraction for the phenol-$H_2$SO$_4$ method9) (elution volume 320–400 ml, Fig. 3-[A]) was pooled and concentrated to dryness in vacuo. The resulting peptides were separated by RP-HPLC with a Shiseido Capcell Pak C18MG column (10×250 mm) by a linear gradient of acetonitrile from 0 to 60% in 0.1% trifluoroacetic acid solution. As shown in Fig. 3-[B], two peaks (TGP-1 and TGP-2) were positive for the phenol-$H_2$SO$_4$ method, suggesting these peaks contained glycopeptides. Sugar component analysis of these glycopeptide fractions with GLC showed that TGP-1 contained glycopeptides bearing the galactosyl residue in addition to Man and GlcNAc residues, while TGP-2 contained glycopeptide bearing mainly Man and GlcNAc residues. The purified peptides were analyzed by automatic Edman degradation using an Applied Biosystem Model 494. The amino acid sequences of the purified glycopeptides were analyzed by the pulsed-liquid blot method after blotting the peptides onto a PVDF membrane. The amino acid sequence of TGP-1 was found to be QVEIPHDVAV(X)ATTGK, although the amino acid sequence of the glycopeptide in TGP-2 could not be identified due to contamination by some other peptides. Although the eleventh amino acid could not be identified, a homology search of the amino acid sequence of TGP-1 with ExPASy (http://www.expasy.org/) found that this sequence agreed well with Q(167)VEIPHDVAVATTGK(182) of one of the major royal jelly proteins (MRJ).10) Considering the results of sequence homology search and sugar component analysis of TPG-1, it seems to be reasonable to conclude that the eleventh amino acid must be the glycosylated asparagine residue.

It has been considered that insect cells can produce the high-mannose type structure (Man$_9β1$GlcNAc$_2$),2,5,11) hybrid-type structure (GlcNAc$_1Man$_4GlcNAc$_2$),5) truncated and fucosylated structure (Man$_3Fuc$_2–0GlcNAc$_2$),12) and GalNAc-containing biantennary structure (GalNAc$_2$–1GlcNAc$_2$Fuc$_2$–0Man$_3$GlcNAc$_2$),12,13) but not the galactose-containing complex type $N$-glycans. Recently, we have found for the first time some hybrid and complex type $N$-glycans bearing the Gal$β1-3$GlcNAc$β1-4$Man unit among the total $N$-glycans prepared from the royal jelly glycoproteins (RJGPs) mixture.1) However, it has still been obscure which glycoprotein(s) of the RJGPs carry the $β1-3$ galactosylated $N$-glycan. In this report, we could identify the 350- kDa RJGP (apisin)2) as the first example of an insect glycoprotein bearing the Gal$β1-3$GlcNAc$β1-4$Man unit in $N$-glycan moiety, indicating that this royal jelly glycoprotein is one of the substrates for both $β1-3$ galactosyl and $β1-4$ $N$-acetylglucosaminyl transferases.
Fig. 3. Gel-filtration and RP-HPLC of Tryptic Digest of Carboxymethylated 350-kDa RJGP.

[A], Gel-filtration profile. The tryptic digest was applied onto the Sephadex G-50 gene column (1.8 × 180 cm) in 0.1 N NH₄OH. The peptides were monitored by the absorption at 230 nm and the carbohydrate was detected by the phenol-H₂SO₄ method.⁹)

[B], RP-HPLC profile. The glycopeptide-fraction obtained in [A] was applied to the Shiseido Capcell Pak C18MG column (10 × 250 mm) and the peptides were eluted as described in the text.

Acknowledgments

We thank to the IS-MS laboratory and The SC-NMR Laboratory of Okayama University. We also thank to Professor Hidenori Yamada (Okayama University) for the amino acid sequence analysis of the glycopeptides.

References


