



Preparation of a polyclonal antibody that recognizes a unique galactose β 1-4fucose disaccharide epitope



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ABSTRACT

Galactose β 1-4fucose (Gal β 1-4Fuc) is a unique disaccharide unit that has been found only in the N-glycans of protostomia. We demonstrated that this unit has a role as an endogenous ligand for *Caenorhabditis elegans* galectins. This unit is also recognized by fungal and mammalian galectins possibly as a non-self glycomarker. In order to clarify its biological function, we made a polyclonal antibody using (Gal β 1-4Fuc)_n-BSA as the antigen, which was prepared by crosslinking Gal β 1-4Fuc-O-(CH₂)₂-SH and BSA. The binding specificity of the antibody was analyzed by frontal affinity chromatography, and it was confirmed that it recognizes naturally occurring N-glycans containing the Gal β 1-4Fuc unit linked to the reducing-end GlcNAc via α 1-6 linkage. By western blotting analysis, the antibody was also found to bind to (Gal β 1-4Fuc)_n-BSA but not to BSA or asialofetuin, which has N-glycan chains containing Gal β 1-4GlcNAc. Western blotting experiments also revealed presence of stained proteins in crude extracts of *C. elegans*, the parasitic nematode *Ascaris suum*, and the allergenic mite *Dermatophagoides pteronyssinus*, while those from *Drosophila melanogaster*, *Mus musculus*, and the allergenic mites *Dermatophagoides farinae* and *Tyrophagus putrescentiae* were negative. This antibody should be a very useful tool for research on the distribution of the Gal β 1-4Fuc disaccharide unit in glycans in a wide range of organisms.

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1. Introduction

The structural diversity of glycans across species, and even within individual organisms, is enormous.^{1,2} This characteristic is one reason for the importance of glycans in interspecies interactions such as host-pathogen interactions.^{3–7} Many glycomarkers on glycans of pathogens, which are recognized by host glycan-binding proteins, e.g., Toll-like receptors, C-type lectins, galectins, etc., have been described. The disaccharide unit galactose β 1-4fucose (Gal β 1-4Fuc),⁶ which is distributed in a variety of invertebrates, appears to be one of such glycomarkers.

Gal β 1-4Fuc is a unique disaccharide unit found only in N-glycans of protostomia,^{2,8} and some of its roles have been demonstrated in the free-living nematode *Caenorhabditis elegans*, which is

widely used as a multicellular model organism and also as a model of parasitic nematodes.⁹ We and others previously demonstrated that Gal β 1-4Fuc is an endogenous recognition unit of the *C. elegans* galectin LEC-6.^{10–13} Others and we also demonstrated that interaction between LEC-6 and Gal β 1-4Fuc, which is located in the N-glycan(s) of glycoprotein F57F4.4, promotes growth in *C. elegans*.^{12,14} On the other hand, an endogenous glycan ligand for another *C. elegans* galectin LEC-8 was reported to be devoid of this unit.¹⁵ However, affinity for Gal β 1-4Fuc was confirmed in the majority of other *C. elegans* galectins and also in annexins, which were reported to bind both phospholipids and glycosaminoglycans,^{16–18} by experiments using a chemically synthesized Gal β 1-4Fuc derivative.^{19–23} Therefore, the Gal β 1-4Fuc unit appears to have evolved as a major endogenous recognition unit of galectins and annexins in *C. elegans*.

The *Coprinosia cinerea* galectin 2, CGL2, was reported to recognize the Gal β 1-4Fuc disaccharide of *C. elegans* glycans, and its possible contribution to the nematotoxic activity of fungi through

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this interaction has been suggested.²⁴ Galectin appears to function as a part of the fungal defense system against nematodes. Human and mouse galectins were found to bind to chemically synthesized Gal β 1-4Fuc, although their binding to endogenous glycans of *C. elegans* has not been examined.²⁵ It is possible that recognition of the Gal β 1-4Fuc glycomarker of protostomia by mammalian galectins is also part of host defense systems as in the case of fungal galectin CGL2.²⁴ Therefore, knowledge on the distribution of the Gal β 1-4Fuc epitope is important for understanding not only the molecular evolution of glycans but also mechanisms of host defense.

The presence of the galactosyltransferase GALT-1, that is, responsible for the synthesis of the Gal β 1-4Fuc unit in *C. elegans* was previously confirmed.²⁶ According to a database search, potential homologs of GALT-1 have been found in vertebrates (*Xenopus* and *Danio*), invertebrates (*Caenorhabditis*, *Drosophila*, *Anopheles*, and *Ixodes*), plants, and protozoa (*Cryptosporidium*), but not in mammalian species.²⁶ However, the presence of potential GALT-1 homologs does not necessarily result in the presence of the Gal β 1-4Fuc unit, since an avian homolog of GALT-1 was reported to transfer galactose to galactose instead of fucose.²⁷ Confirmation of such a structure requires rather stringent biochemical approaches, for example, by isolation of glycans and analysis by mass spectrometry. We therefore sought a specific anti-glycan antibody against Gal β 1-4Fuc that would facilitate biological characterization of the Gal β 1-4Fuc unit.

2. Results and discussion

2.1. Preparation of (Gal β 1-4Fuc)_n-BSA, a neoglycoconjugate containing multiple Gal β 1-4Fuc epitopes, and rabbit polyclonal anti-Gal β 1-4Fuc antibody

In order to prepare an antibody against Gal β 1-4Fuc, we first produced a neoglycoconjugate modified with multiple Gal β 1-4Fuc groups, namely, (Gal β 1-4Fuc)_n-bovine serum albumin (BSA). Chemically synthesized Gal β 1-4Fuc-O-(CH₂)₂-SH (Fig. 1A)²⁰ was crosslinked with Lys residues of BSA using the bi-functional reagent 3-maleimidopropionic acid N-hydroxysuccinimide ester (BMPS), which covalently crosslinks between thiol and amino groups. After the reaction, the product was analyzed by SDS-PAGE. The presence of smeared bands with higher molecular weights than that of BSA (Fig. 1B) indicated that the Gal β 1-4Fuc group was introduced to BSA.

After removal of the unreacted Gal β 1-4Fuc derivative and BMPS by ultrafiltration, the product was further purified by affinity chromatography using an immobilized LEC-6 column. After extensive washing, bound material was eluted with lactose, which competes with the immobilized Gal β 1-4Fuc (Fig. 1C). The presence of (Gal β 1-4Fuc)_n-BSA was confirmed in the lactose-eluted fractions (Fr. 10 and 11). These fractions were pooled and, after removal of lactose by ultrafiltration, used as antigen.

The immunoglobulin fraction was isolated from the sera of rabbits immunized with (Gal β 1-4Fuc)_n-BSA by Protein A Sepharose affinity chromatography (Keari Co., Osaka, Japan). Anti-Gal β 1-4Fuc antibody was further purified by affinity chromatography by using an immobilized Gal β 1-4Fuc column.²²

2.2. Characterization of the binding specificity of the anti-Gal β 1-4Fuc antibody by frontal affinity chromatography analysis

Specificity of the antibody was studied by frontal affinity chromatography, which enables quantitative assessment of molecular interactions in a flow-based system, especially useful for the analysis of weak interactions such as those between glycans and

lectins.^{28–30} In this study, to a column of the immobilized antibody, a solution of a fluorescence labeled oligosaccharides (PA-sugars) containing Gal-Fuc or Gal-GlcNAc disaccharide unit was continuously applied. The delay in the elution front of the PA-sugar is proportional to the affinity for the immobilized antibody. In order to clarify the specificity of the antibody, we used the following PA-sugars whose (estimated) structures are shown in Fig. 2: Chemically synthesized PA-sugars,¹⁹ Gal β 1-4Fuc-Man-ol-PA and also its linkage isomer Gal β 1-3Fuc-Man-ol-PA, and PA-derivatives of *C. elegans* endogenous sugars containing Gal β 1-4Fuc unit attached to the 6-OH of the reducing-end GlcNAc residue (D1, D3, D4, E1, E3, and E4)^{10,11}. We also used D4 treated with galactosidase as a control. Other PA-sugars of vertebrate origin are commercial products.

Fig. 2 shows observed elution patterns. Although frontal affinity chromatography is an efficient tool capable of providing accurate binding constants, we reserve calculation of binding constants and restrict to comparison of the elution profiles, because we used a polyclonal antibody, which is a mixture of heterogeneous proteins having widely ranging different binding constants for the disaccharide epitope, and thus, representation in the term of binding constant does not seem relevant enough. The extent of retardation of Gal β 1-4Fuc-Man-ol-PA was the largest (compare the elution profile of Gal β 1-4Fuc-Man-ol-PA (solid line) and that of PA-rhamnose (dotted line; control)), while no retardation was observed for its linkage isomer Gal β 1-3Fuc-Man-ol-PA (the elution profile of Gal β 1-3Fuc-Man-ol-PA (solid line) is similar to and not retarded from that of PA-rhamnose). These results indicate that the antibody has an affinity for Gal β 1-4Fuc but not for Gal β 1-3Fuc. Significant retardation was observed for endogenous N-glycans separated from *C. elegans*, D1, D3, D4, E1, E3, and E4, containing Gal β 1-4Fuc disaccharide unit attached to the 6-OH of the reducing-end GlcNAc residue.^{10,11} Removal of the galactose residue of the disaccharide unit of D4 (D4–galactose) resulted in loss of affinity, indicating that the antibody recognizes exclusively the Gal β 1-4Fuc disaccharide unit attached to the 6-OH of innermost GlcNAc residue. The penultimate GlcNAc residue of D1, D3, and D4 are modified with a Hex-Fuc disaccharide, which is presumed to be Gal α 1-2Fuc.⁸ This disaccharide moiety, however, does not affect the interaction, because D4–galactose, which contains this disaccharide unit, was not bound by the antibody. This disaccharide unit was also shown to have no effect on the interaction with the antibody, because no difference was observed between the elution profiles of D4 and E4, though D4 contains this disaccharide unit. In the cases of D1 and E1, there is another Hex-Fuc disaccharide attached to C₃ of the reducing-end GlcNAc. Although it is presumed to be Gal β 1-4Fuc,³¹ it does not seem to interact with the antibody because the elution profiles of D1 and D3, and E1 and E3 are similar. Access to the disaccharide unit attached to C₃ of the reducing end GlcNAc by proteins seems to be prevented because of the conformation of the oligosaccharides, because Hanneman et al.³¹ and we¹⁰ found that its Gal residue is resistant to galactosidase treatment in contrast to that of the Gal-Fuc unit attached to C₆, which is easily removed. All oligosaccharides examined, derived from vertebrates, containing Gal-GlcNAc unit did not interact with the immobilized antibody. Based on these results, it is concluded that the antibody specifically recognizes the Gal β 1-4Fuc disaccharide moiety linked to C₆ of the reducing-end GlcNAc of N-glycan.

2.3. Western blotting analysis using the anti-Gal β 1-4Fuc antibody

Western blotting analysis was performed in order to further estimate the specificity of the purified antibody. (Gal β 1-4Fuc)_n-BSA, BSA, asialofetuin, and an extract from mixed-stage *C. elegans* strain N2 were subjected to SDS-PAGE and probed with the antibody (Fig. 3A). (Gal β 1-4Fuc)_n-BSA and the *C. elegans* extract were

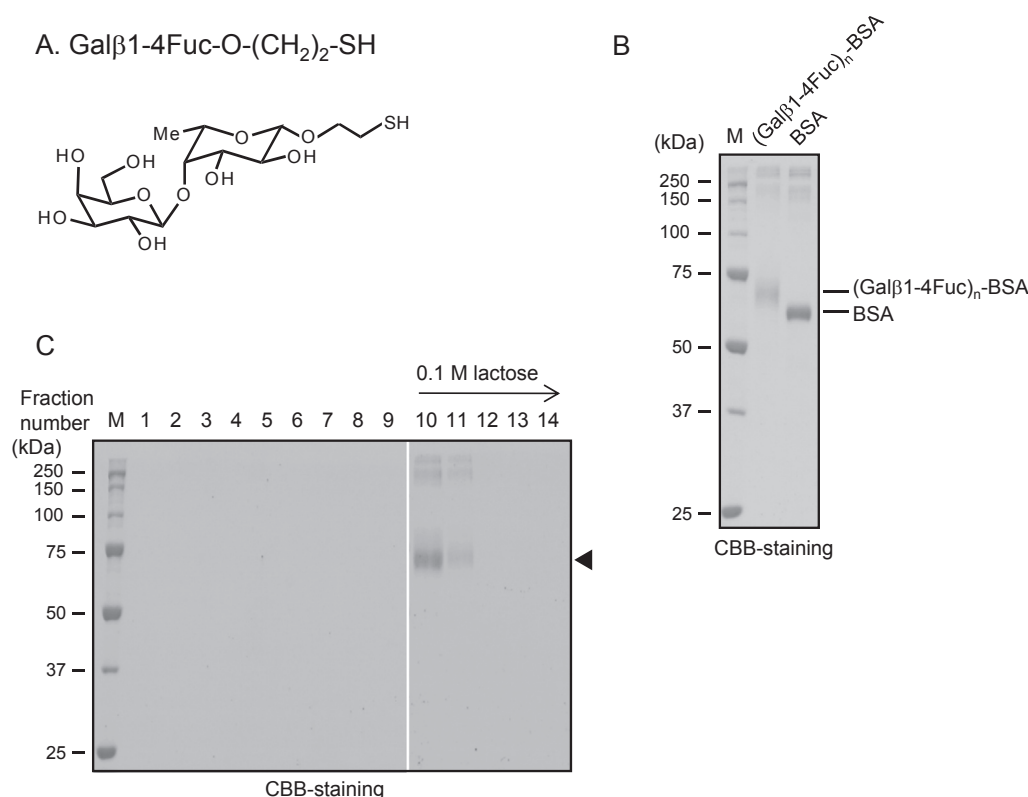


Fig. 1. Preparation of (Galβ1-4Fuc)_n-BSA, a neoglycoconjugate containing multiple Galβ1-4Fuc epitopes (A) Structure of Galβ1-4Fuc-O-(CH₂)₂-SH, a chemically synthesized Galβ1-4Fuc derivative. Its conformation is based on ¹H NMR spectra. (B) For the synthesis of (Galβ1-4Fuc)_n-BSA, Galβ1-4Fuc-O-(CH₂)₂-SH was covalently crosslinked to BSA using BMPs. The products were then resolved in SDS-PAGE and proteins were stained with Coomassie brilliant blue (CBB). (C) Synthesized (Galβ1-4Fuc)_n-BSA was purified by immobilized LEC-6 column chromatography. After extensive washing, bound material was eluted with 0.1 M lactose. Each fraction was resolved in SDS-PAGE, and proteins were stained with CBB. The numbers on the left of the panel are the molecular masses of standard proteins. Arrowhead indicates the position of (Galβ1-4Fuc)_n-BSA. Arrow indicates lactose-eluted fractions.

found to react strongly with the antibody. In contrast, no signal was observed in the cases of the unmodified BSA and asialofetuin, which contain Galβ1-4GlcNAc, a disaccharide unit commonly found in glycans of vertebrates. These results indicate that the antibody is specific for Galβ1-4Fuc.

Because the anti-Galβ1-4Fuc antibody successfully recognized endogenous Galβ1-4Fuc disaccharide units, we tested its ability to recognize Galβ1-4Fuc in extracts derived from various species.

First, crude extracts of *C. elegans* at different developmental stages were analyzed by western blotting (Fig. 3B), because some previous reports suggested possible roles for the interaction between the Galβ1-4Fuc unit of endogenous glycans and LEC-6 in the growth of *C. elegans*.^{12,14} However, no significant differences were observed in the pattern of bands stained by the antibody among different developmental stages. Although this preliminary experiment did not support our previous findings, more detailed analysis such as at the level of individual proteins, and localization at the cellular and tissue levels, etc., are necessary.

Second, crude extracts from various developmental stages of *Drosophila melanogaster* and from the brain and thymus of *Mus musculus*, the mites *Dermatophagoides farinae*, *Tyrophagus putrescentiae*, and *Dermatophagoides pteronyssinus*, and the parasitic nematode *Ascaris suum* were analyzed for their reactivity with the antibody (Fig. 3C). Only for proteins in the *D. pteronyssinus* and *A. suum* extracts gave positive signals. The result with *A. suum* is consistent with a previous report that showed the presence of the Galβ1-4Fuc unit.⁸ Given that mammalian galectins can also interact with Galβ1-4Fuc,²⁵ this structure might have a role in the parasitism of worms. Among the three species of mites that are known to cause allergic reactions in humans, the Galβ1-4Fuc unit was

detected only in *D. pteronyssinus*, and the glycans of *D. pteronyssinus* was suggested to be responsible for its allergenic properties.^{32,33} However, presence of the Galβ1-4Fuc unit was not confirmed in the other two mites. Relation between the presence of the Galβ1-4Fuc unit and allergenic properties of parasitic animals remains to be investigated. The results for *Mus musculus* are not surprising because the potential GALT-1 homolog is absent in this species. On the other hand, the result on *Drosophila melanogaster* is somewhat unexpected because this species is known to express a potential GALT-1 homolog,²⁶ although the presence of Galβ1-4Fuc has not been confirmed by structural analyses.^{34–36} It remains to be clarified whether the level of GALT-1 expression/activity in *D. melanogaster* is too low or the potential homolog has a different role as in the case of avian homolog.²⁷

In the present study, we produced an antibody capable of detecting the endogenous Galβ1-4Fuc disaccharide unit. This new tool should be very useful for elucidation of the biological roles of this disaccharide in protostomia, especially from the viewpoints of symbiotic and antagonistic relationships between human and other organisms.

3. Experimental

3.1. Preparation of (Galβ1-4Fuc)_n-BSA, a neoglycoconjugate containing multiple Galβ1-4Fuc epitopes

For the preparation of a neoglycoconjugate containing the Galβ1-4Fuc epitope, a chemically synthesized Galβ1-4Fuc derivative, Galβ1-4Fuc-O-(CH₂)₂-SH (Fig. 1A)²⁰ and bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO, USA) were crosslinked

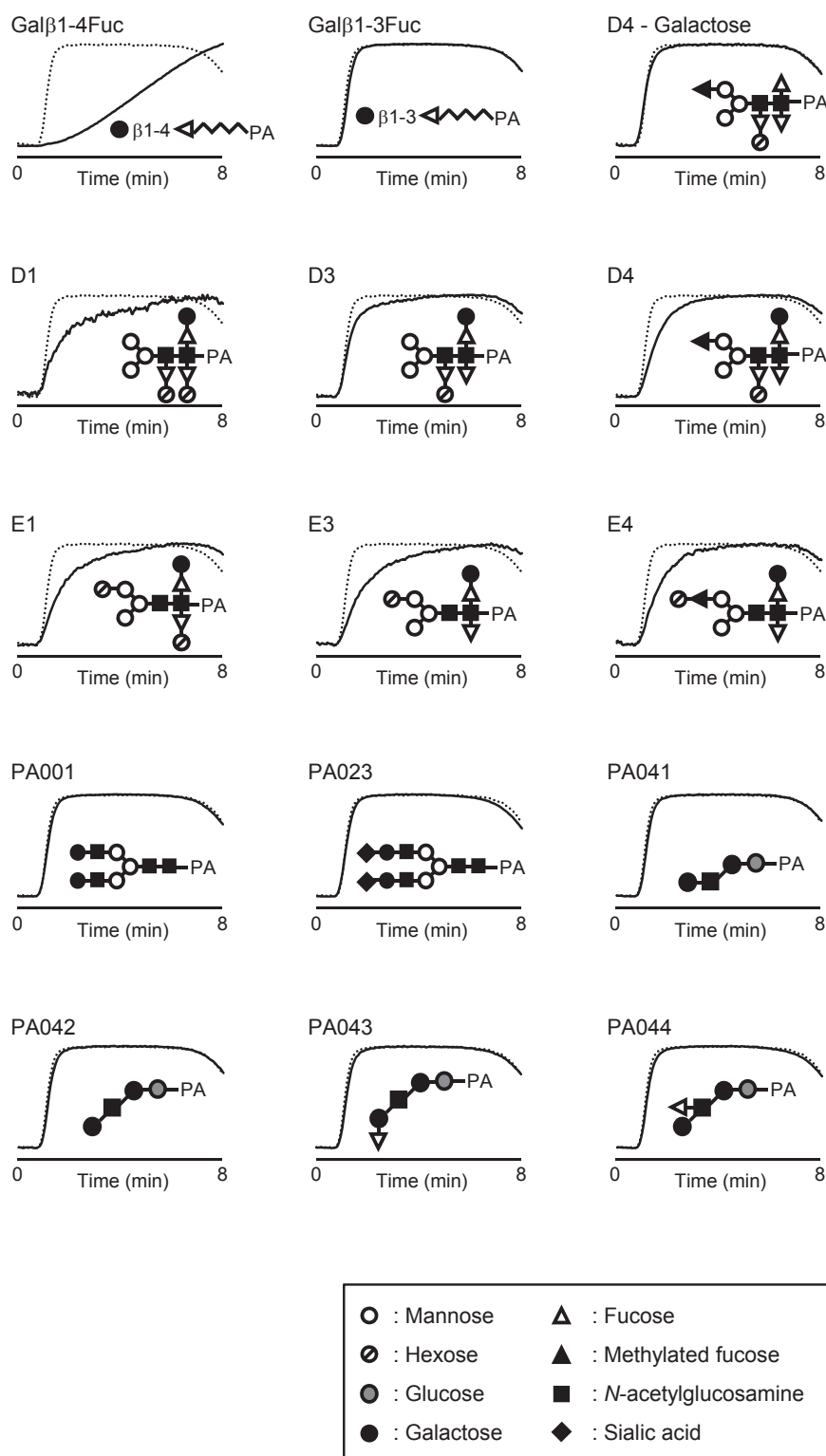


Fig. 2. Frontal affinity chromatography analysis using an immobilized *anti*-Galβ1-4Fuc antibody adsorbent. Elution profiles of PA-sugars from an immobilized *anti*-Galβ1-4Fuc antibody column are shown. Structure of each PA-sugar is depicted in each panel of elution profile. The elution profile of each PA-sugar (solid line) is superimposed on that of PA-rhamnose (broken line), which has no affinity for *anti*-Galβ1-4Fuc antibody. For Galβ1-3Fuc, PA001, PA023, PA041, PA042, PA043, and PA044, a high affinity column (4.8 mg protein/mL gel) was used. For the others, a low affinity column (1.2 mg protein/mL gel) was used.

using 3-maleimidopropionic acid N-hydroxysuccinimide ester (BMPS; Sigma–Aldrich). Galβ1-4Fuc-O-(CH₂)₂-SH (160 μL of 50 mg/mL, dissolved in ultrapure water), 1 mL of 20 mg/mL BSA dissolved in phosphate-buffered saline-ethylenediaminetetraacetic

acid (PBS-EDTA; 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, 2.68 mM KCl, pH 7.4, supplemented with 2 mM EDTA), 25 μL of 100 mg/mL BMPS dissolved in dimethylformamide, and 815 μL of PBS-EDTA were mixed and incubated for 15 h at room temperature

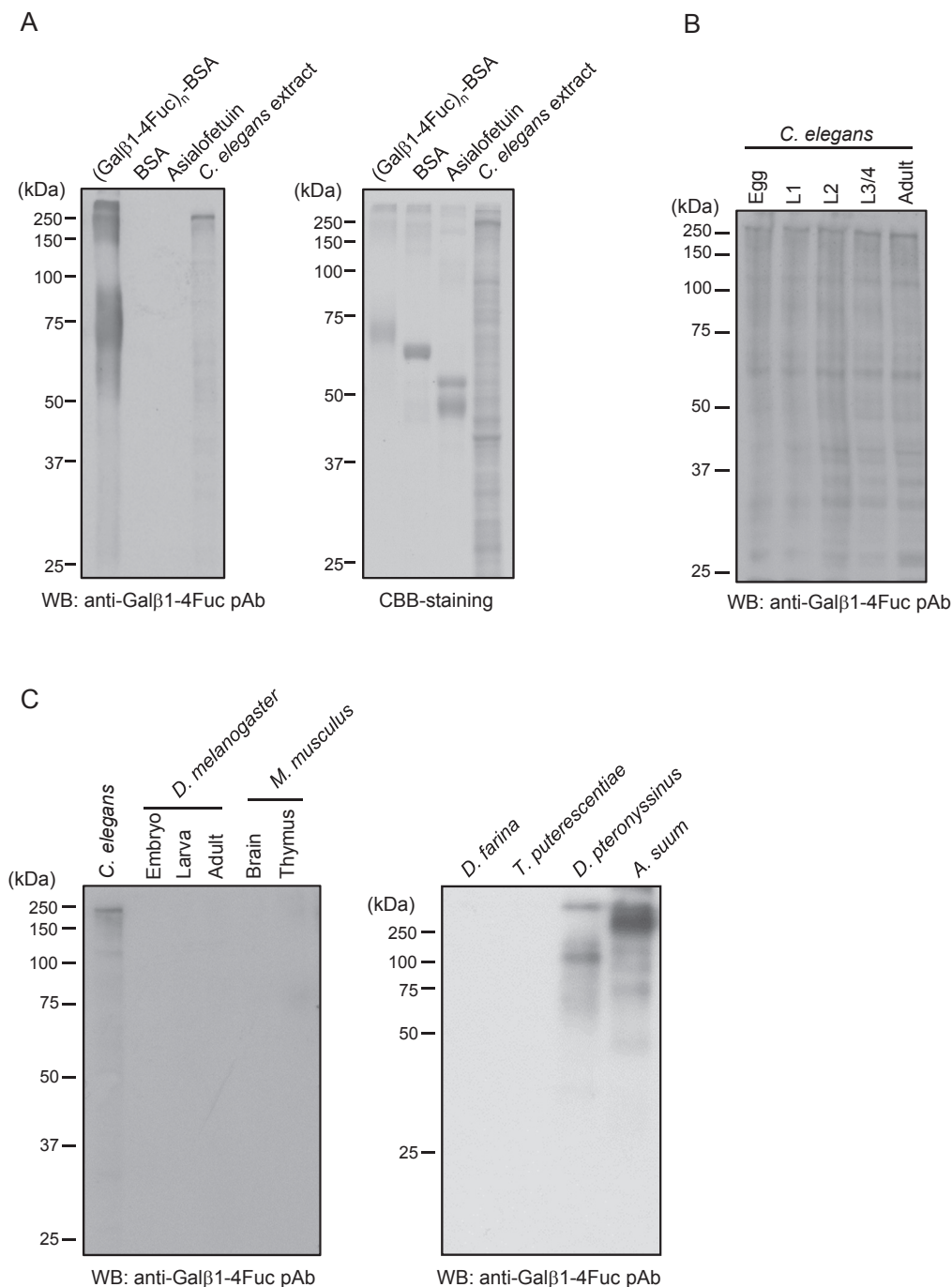


Fig. 3. Western blotting analysis using *anti*-Gal β 1-4Fuc antibody (A) Synthesized (Gal β 1-4Fuc)_n-BSA, BSA, asialofetuin, and extract from a preparation of mixed-stage *C. elegans* N2 strain were analyzed by western blotting with the *anti*-Gal β 1-4Fuc antibody. These samples were also resolved in SDS-PAGE, and proteins were stained with CBB. (B) Extracts from the indicated developmental stages of *C. elegans* N2 strain. (C) Extracts from a mixed-stage preparation of *C. elegans* N2 strain, the indicated developmental stages of *D. melanogaster*, the indicated tissues from *M. musculus* C57BL/6 strain, *D. farinae*, *T. putrescentiae*, *D. pteronyssinus*, and *A. suum* were analyzed by western blotting with the *anti*-Gal β 1-4Fuc antibody. The numbers on the left of the panel are the molecular masses of standard proteins. Equal protein loading of each panel was confirmed by CBB staining of the gel or Ponceau S staining of the membrane (data not shown).

and then were ultrafiltered using an Amicon Ultra-15 Centrifugal Filter Unit (Merck Millipore, Darmstadt, Germany) to remove unreacted Gal β 1-4Fuc-O-(CH₂)₂-SH and BMPS.

Synthesized (Gal β 1-4Fuc)_n-BSA was further purified by affinity chromatography using an immobilized LEC-6 column. Preparation of an immobilized LEC-6 column and affinity purification were performed essentially as previously described.¹⁰ In brief, (Gal β 1-4Fuc)_n-BSA was applied to the column, and after extensive washing of the column with PBS-EDTA, bound material was eluted with PBS-

EDTA containing 0.1 M lactose. Fractions 10 and 11, which contained (Gal β 1-4Fuc)_n-BSA, were pooled and ultrafiltered using an Amicon® Ultra-15 Centrifugal Filter Unit (Merck Millipore) in order to remove lactose, and used as the antigen.

3.2. Frontal affinity chromatography

Frontal affinity chromatography was performed basically as described previously.³⁷ In brief, 2.0 mL of a PA-sugar in a buffer

comprising of 20 mM Na₂HPO₄, 150 mM NaCl, and 1 mM EDTA, pH7.2, at a concentration of 5 nM was applied to an immobilized anti-Galβ1-4Fuc antibody column (1.2 or 4.8 mg protein/mL gel), at a flow rate of 0.25 mL/min, at 25 °C. The elution of PA-sugars was detected by using a fluorescence detector at an excitation wavelength of 310 nm and an emission wavelength of 380 nm. The PA-sugars used are follows: Galβ1-4Fuc-Man-ol-PA and Galβ1-3Fuc-Man-ol-PA were chemically synthesized as described previously.¹⁹ D1, D3, D4, E1, E3, and E4, pyridylaminated endogenous ligand N-glycans of *C. elegans* galectin LEC-6, were isolated from *C. elegans* as reported previously.¹⁰ D4-Galactose, D4 treated with galactosidase was prepared as described previously.¹⁰ PA001 (Galβ1-4GlcNAcβ1-2Manα1-3 (Galβ1-4GlcNAcβ1-2Manα1-6) Manβ1-4GlcNAcβ1-4GlcAc-PA), PA023 (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3 (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6) Manβ1-4GlcNAcβ1-4GlcAc-PA), PA041 (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA), PA042 (Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA), PA043 (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA), PA044 (Galβ1-3 (Fucα1-4) GlcNAcβ1-3Galβ1-4Glc-PA), and PA-rhamnose were purchased from Takara Bio (Shiga, Japan).

3.3. Preparation of asialofetuin and extracts

For the preparation of asialofetuin, which is the desialylated form of fetuin and contains multiple terminal Galβ1-4GlcNAc units, bovine fetuin (Sigma–Aldrich) was treated with neuramidase (New England Biolabs, Ipswich, MA, USA) for 24 h at 37 °C, and purified using a LEC-6 column essentially as described above. The purified asialofetuin was then concentrated using an Amicon® Ultra-15 Centrifugal Filter Unit (Merck Millipore).

For the preparation of *C. elegans* extracts, the *C. elegans* N2 strain was cultured as described previously.³⁸ Mixed-stage *C. elegans* was harvested and lysed by SDS-PAGE sample buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 8% glycerol, 0.01% Pyronin Y, and 2% 2-mercaptoethanol) with sonication and boiling. For the *C. elegans* extracts of different developmental stages, mixed-stage *C. elegans* was treated with bleach for the isolation of eggs. The eggs were grown at 20 °C under normal laboratory conditions, and harvested after 24 (L1), 48 (L2/3), 72 (L4), and 96 (adult) h. The harvested eggs and worms were suspended in SDS-PAGE sample buffer and disrupted by sonication and boiling.

For the preparation of *D. melanogaster* extracts, embryo, third instar larva, and adult fly were collected and the extracts were prepared as described previously.³⁹

For the preparation of *M. musculus* brain and thymus extracts, a C57BL/6 mice was humanely sacrificed and the brain and thymus were collected in accordance with Teikyo University guide for the care and use of laboratory animals. These tissues were suspended in SDS-PAGE sample buffer and disrupted by sonication and boiling.

The extracts of *Dermatophagoides farinae*, *Tyrophagus putrescentiae*, *Dermatophagoides pteronyssinus*, and *Ascaris suum* were obtained from Cosmo Bio (Tokyo, Japan).

3.4. SDS-PAGE and western blotting

Proteins or extracts were resolved in SDS-PAGE and stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA, USA) or transferred electrophoretically onto nitrocellulose membranes (Bio-Rad). The membranes were stained with Ponceau S (Sigma–Aldrich) for the verification of equal protein loading, and then were incubated in Super Block blocking buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at room temperature. Then, the membranes were blotted with anti-Galβ1-4Fuc antibody diluted in TBS containing 0.1% Tween 20 (TBS-T) and subsequently incubated with

horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibody (GE Healthcare, Little Chalfont, UK) diluted in TBS-T, followed by detection with enhanced chemiluminescence immunoblotting detection reagents (GE Healthcare).

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