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Free Oligosaccharides with Lewis x Structure Expressed in the Segmentation Period of Zebrafish Embryo

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We previously reported that zebrafishα1-3fucosyltrasferase 1 (zFT1) was expressed in embryos at the segmentation period, and was capable of synthesizing the Lewis x epitope [Galβ1-4(Fucα1-3)GlcNAc] [Kageyama *et.al*, *J. Biochem*., 125, 838–845 (1999)]. In the current study, we attempted to detect the enzyme products of zFT1 in zebrafish embryos. Oligosaccharides were prepared from the zebrafish embryos at 12, 18 and 48 h after fertilization and labelled with a fluorophore, 2-aminopyridine, for highly sensitive detections. Pyridylamino (PA)-oligosaccharides that were α1-3/4fucosidase sensitive and time-dependently expressed at 18 h after fertilization were identified as candidates for the *in vivo* products synthesized by zFT1. Structures of these oligosaccharides were determined by a combination of exoglycosidase digestions and two-dimensional HPLC sugar mapping to be the biantennary complex-type structures with two Lewis x epitopes: $(Gal\beta1\text{-}4)_{0,1,2}$-{Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-6[Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-3]}Manβ1-4GlcNAc, and $(Gal\beta1\text{-}4)_{0,1}$-{Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-6[Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-3]} Manβ1-4GlcNAcβ1-4GlcNAc. The presence of Lewis x structure of these oligosaccharides together with their expression time suggests that they are products of zFT1. Remarkably, most of these oligosaccharides were free form. Furthermore, we detected an endo-β-*N*-acetylglucosaminidase activity in the 18 h embryo. These results suggest that the oligosaccharides synthesized by zFT1 are present in the embryo at the segmentation period in free form, owing to the liberation from glycoproteins with endo-β-*N*-acetylglucosaminidase(s) and/or glycoamidase(s).

Key words: Lewis x, zebrafish, embryogenesis, oligosaccharide, pyridylamination.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]; DEAE, diethylaminoethyl; EDTA, ethylene diaminetetra acetic acid; Fuc, L-fucose; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; GN1 type oligosaccharide, N-linked oligosaccharide with a single GlcNAc at the reducing-end; GN2 type oligosaccharide, *N*-linked oligosaccharide with GlcNAcβ1-4GlcNAc at the reducing-end; HPLC, high-performance liquid chromatography; LTA, *Lotus tetagonolobus* agglutinin; Man, D-mannose; MALDI-TOF-MS, matrix assisted laser desorption ionization time of flight mass spectrometry; PA, pyridylamino; zFT1, zebrafish α-1,3-fucosyltransferase 1; zFT2, zebrafish α-1,3-fucosyltransferase 2.

During the process of embryogenesis, several oligosaccharides are expressed stage-specifically on the cell surface (*1*–*7*). One of these oligosaccharides, the stage-specific embryonic antigen-1 (SSEA-1), has been identified as the oligosaccharide with a Lewis x epitope [Galβ1-4(Fucα1-3) GlcNAc]. It has been suggested that the oligosaccharides with Lewis x epitope participated in events such as tight adhesion of blastomeres at compaction (*5*, *7*). However, the precise structure of these oligosaccharides has not been elucidated since previous studies used antibodies or lectins to detect the Lewis x-containing oligosaccharides and revealed only their partial structures.

Expression of the Lewis x epitope is regulated by α1-3fucosyltransferases. Kageyama *et al*. (*8*) previously isolated two α1-3fucosyltransferase genes, zebrafish α-1,3-fucosyltransferase 1 (*zFT1*) and zebrafish α-1,3-fucosyltransferase 2 (*zFT2*), from zebrafish. Both of the *zFT1* and *zFT2* gene products could transfer fucose (Fuc) from GDP-Fuc to the *N*-acetyllactosamine residue (Galβ1-4GlcNAc-) to synthesize the Lewis x epitope. The *zFT1* gene was expressed specifically in embryos at around 18 h after fertilization, corresponding to the segmentation period and then disappeared in adult zebrafish (*8*). The *zFT2* gene was expressed specifically in embryos at around 72 h after fertilization, corresponding to the juvenile and then disappeared in adult zebrafish (*8*). Therefore, fucosyloligosaccharides synthesized by *zFT1* and *zFT2* gene products should be expressed in 18 h and 72 h zebrafish embryos, respectively. In this study, we isolated the Lewis x-containing oligosaccharides from the zebrafish embryo at 18 h after fertilization, and showed that these oligosaccharides had

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N-linked structure, but were free form of a single *N*-acetylglucosamine residue (GN1 type) liberated with endo-β-*N*-acetylglucosaminidase whose activity was detected. We also isolated *N*-linked oligosaccharides whose reducing-end portion were di-*N*-acetylchitobiose sequence (GN2 type). The expression stage of both GN2 type and GN1 type free form Lewis x-containing *N*-linked oligosaccharides correlated with that of *zFT1*, suggesting that these oligosaccharides were the products of a *zFT1* gene product.

EXPERIMENTAL PROCEDURE

Materials—Zebrafish (*Danio rerio*) were obtained from Japan Animal Inc. (Osaka, Japan). Pyridylamino (PA)-isomaltooligosaccharides were purchased from Takara Biomedicals (Kyoto, Japan). Shodex Asahipak NH2P-50 columns (6.0 × 100, 4.6 × 250 and 2.0 × 150 mm) were purchased from Showa Denko (Tokyo, Japan), a Cosmosil 5C18P column (1.5 × 250 mm) from Nacalai Tesque (Kyoto, Japan), a TSK-GEL DEAE-5PW column (7.5 × 75 mm) and a TSK-GEL Sugar AX-I column (4.6 × 150 mm) from Tosoh (Tokyo, Japan), and Dowex 50W-X8 (50–100 mesh) from Dow Chemical (Richmond, VA, USA). The β-*N*-acetylhexosaminidase (jack bean) was obtained from Seikagaku Kogyo (Tokyo, Japan), β1-4galactosidase (*Streptococcus pneumoniae*) (*9*, *10*) from Boehringer Mannheim (Germany), α1-3/4fucosidase (*Streptomyces* sp. 142) from Takara Biomedicals, α-mannosidase (jack bean) and α-galactosidase (coffee bean) from Sigma (St Louis, MO, USA), neuraminidase (*Arthrobacter ureafaciens*) from Nacalai Tesque and endoglycosidase F2 (*Chrysebacterium meningosepticum*, recombinant, *Escherichia coli*) from Calbiochem (Germany). Lotus lectin agarose (LTA) was obtained from Seikagaku Corp. (Tokyo, Japan). Sep-Pak C18 Cartridge was obtained from Waters Corp. (Milford, MA, USA). Insulin chain B (oxidized, from bovine insulin) and 2,5-dihydroxybenzoic acid were obtained from Sigma, and neurotensin from Peptide Institute Inc. (Osaka, Japan). Cobra venom from *Naja kaouthia* was a kind donation from the Japan Snake Center (Gunma, Japan).

Preparation of Standard PA-Sugar Chains—PA-Fuc, PA-Gal, PA-GalNAc, PA-Glc, PA-GlcNAc, PA-Xyl and PA-isomaltooligosaccharides were prepared as described previously (*11*). M1′-PA[1], M2B′-PA, M3B′-PA and Bi′-PA were prepared as reported previously (*12*). The agaBi′-PA was obtained by digesting Bi′-PA with β1-4galactosidase. M3B-PA, M5A-PA, M6B-PA, M9A-PA, agaBi-PA, Bi-PA, BiF-PA, Tri-PA, Hyb-PA and DiSiaBi-PA were prepared as reported previously (*13*, *14*). DiFBi-PA and DiFBiF-PA were prepared from cobra venom factor from *Naja kaouthia* as reported previously (*15*, *16*) after the digestion with α-galactosidase. 3Bi-PA was prepared from ancrod (a protease purified from the venom of the Malayan pit viper) (*17*) kindly donated by Dr R. Geyer (Giessen University, Germany) and 3Bi′-PA was prepared by digesting 3Bi-PA with endoglycosidase F2 followed by pyridylamination. Abbreviations of these PA-sugar chains are shown in Table 1.

[1] The structures and abbreviations of the oligosaccharides are listed in Table 1.

Care of Zebrafish—Zebrafish and their embryos were cared for as described previously (*18*). The day–night cycle was controlled with an automatic timer (14 h light/10 h dark), and the temperature was maintained at 28.5°C. At the onset of the light cycle, zebrafish generally initiated breeding behaviour resulting in oviposition and fertilization of eggs. Therefore, the time that the light was turned on was designated as 0 h. Shortly, after fertilization, eggs were removed and incubated at 28.5°C in an embryo medium (*18*).

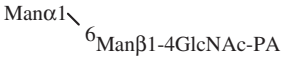
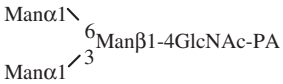
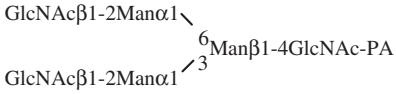
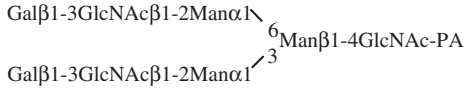
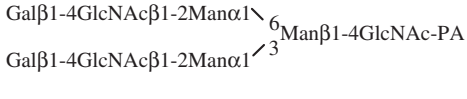
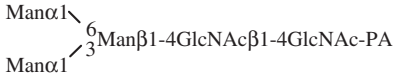
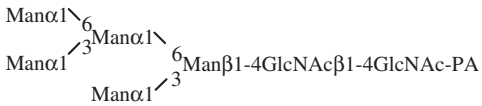
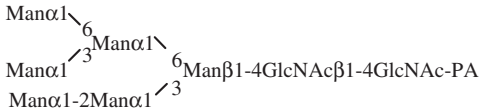
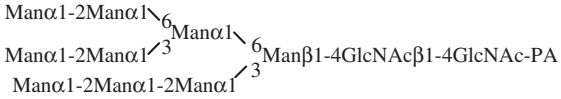
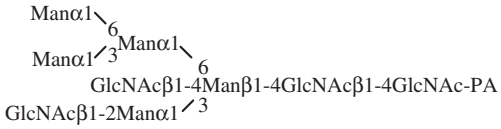
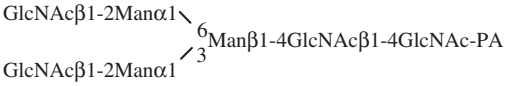
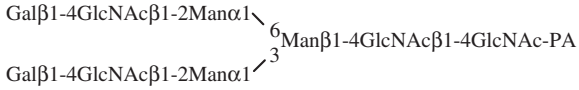
Preparation of PA-oligosaccharides from Zebrafish Embryos—After removal of chorions and yolks, the embryos were homogenized in acetone at −20°C for 2 h, and centrifuged at 16,000 × g for 15 min at 4°C. The resulting precipitates were lyophilized. Oligosaccharides were released from the lyophilized precipitates by heating with 200 μl of anhydrous hydrazine at 100°C for 10 h (for *N*-linked sugar chains) or at 60°C for 50 h (for *N*- and *O*-linked sugar chains) (*19*, *20*). The released oligosaccharides were *N*-acetylated and pyridylaminated. Excess reagents were removed by phenol–chloroform extraction and by cation-exchanger, Dowex 50W-X8 (NH_4^+ form), as reported previously (*21*). Due to many co-existing peaks, we could not find an appropriate internal standard for quantification. We quantified the recovery of PA-oligosaccharides by comparing the peak areas between the different runs. Under a careful experimental regime, the repeatability study of the difference in peak areas between the different runs was <20%.

Preparation of Membrane and Soluble Fraction from Embryos—Seven hundred zebrafish embryos at 18 h after fertilization were homogenized with a pellet mixer in 10 mM sodium phosphate buffer, pH 7.0 and the homogenates were sonicated with an ultrasonic processor (Misonix Inc.) for 1 min. The homogenates were centrifuged at 105,000 × *g* for 1 h at 4°C. The precipitates (membrane fraction) and the supernatant (soluble fraction) were collected.

High-Performance Liquid Chromatography of PA-Sugar Chains—Anion-exchange HPLC was performed at 25°C on a TSK-GEL DEAE-5PW column at a flow rate of 1.0 ml/min using Eluent A and Eluent B. Eluent A was aqueous ammonia, pH 9.0. Eluent B was 1 M ammonium acetate solution, pH 9.0. The column was equilibrated with Eluent A. After injecting a sample, neutral sugar chains were eluted with Eluent A for 10 min, and anionic sugar chains were eluted with a linear gradient to Eluent A : Eluent B (6 : 4, v/v) for 30 min, and then to Eluent A : Eluent B (0:10, v/v) for 20 min. Elution was monitored by measuring the fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

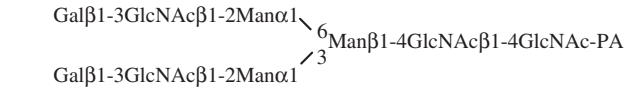
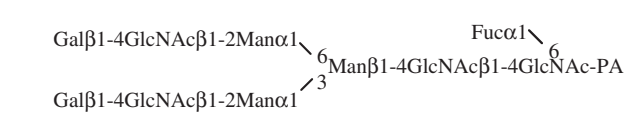
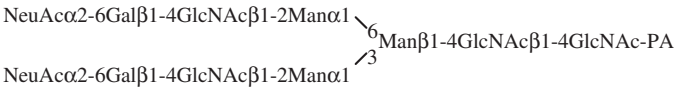
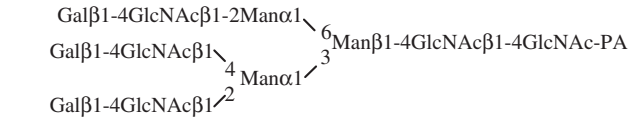
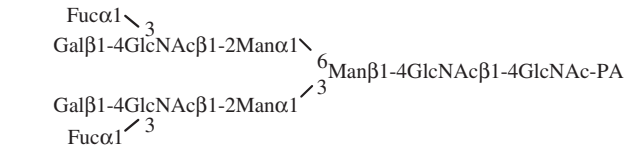
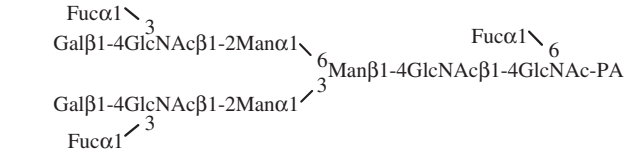
Reversed-phase HPLC was performed at 25°C on a Cosmosil 5C18P column at a flow rate of 150 μl/min. In elution Condition 1; the column was equilibrated with 100 mM ammonium acetate buffer, pH 6.0, containing 0.046% 1-butanol. After injecting a sample, the concentration of 1-butanol was increased linearly to 0.52% over 51 min, and then to 1.0% over 12 min. Elution was monitored by measuring the fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm). In elution Condition 2, the column was equilibrated

Table 1. **Abbreviation and elution positions of standard PA-oligosaccharides.**

Abbreviation	Structure	Size-fractionation[a]	Reversed-phase
M1′-PA	Manβ1-4GlcNAc-PA	1.8	1.21[b]
M2B′-PA		3.0	1.00[b]
M3B′-PA		4.1	1.17[b]
agaBi′-PA		5.8	1.33[b]
3Bi′-PA		7.5	1.26[b]
Bi′-PA		7.5	1.42[b]
M3B-PA		4.5	1.74[b]
M5A-PA		6.4	1.72[b]
M6B-PA		7.4	1.78[b]
M9A-PA		10.2	1.47[b]
Hyb-PA		7.9	1.85[b]
agaBi-PA		6.0	1.95[b]
Bi-PA		7.6	2.00[b] 1.00[c]

(continued)

Table 1. **Continued.**

Abbreviation	Structure	Size-Fractionation[a]	Reversed-phase
3Bi-PA		7.6	0.76[c]
BiF-PA		8.2	2.31[b] 1.25[c]
DiSiaBi-PA		18.6	1.81[b]
Tri-PA		9.2	2.20[b]
DiFBi-PA		9.4	0.59[c]
DiFBiF-PA		9.8	1.94[b] 0.80[c]

[a]Glucose units. [b]The elution time of Galβ1-3GalNAc-PA was taken as 1.0 when analysed on Condition 1. [c]The elution time of Bi-PA was taken as 1.0 when analysed on Condition 2.

with 20 mM ammonium acetate buffer, pH 4.0, containing 0.075% 1-butanol. After injecting a sample, the concentration of 1-butanol was increased linearly to 0.4% over 90 min. Elution was monitored by measuring the fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm).

Size-fractionation HPLC was carried out at 25°C on Shodex Asahipak NH2P-50 columns (6.0 × 100 mm or 4.6 × 250 mm) at a flow rate of 0.6 ml/min, and an NH2P-50 column (2.0 × 150 mm) at a flow rate of 75 μl/min. For NH2P-50 columns (4.6 × 250 and 2.0 × 150 mm), Eluent C and D were used. Eluent C was acetonitrile: water: acetic acid (900: 100: 3, v/v/v) titrated to pH 7.0 with 12.5% aqueous ammonia; Eluent D acetonitrile: water: acetic acid (200:800:3, v/v/v) titrated to pH 7.0 with 12.5% aqueous ammonia. The columns were equilibrated with Eluent C: Eluent D (95: 5, v/v). After injecting a sample, a linear gradient was run first to Eluent C: Eluent D (86: 14, v/v) for 3 min, then to Eluent C: Eluent D (73: 27, v/v) for 17 min, then to Eluent C: Eluent D (52: 48, v/v) for 59 min and finally to Eluent C: Eluent D (25: 75, v/v) for 6 min. Elution was monitored by measuring the fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm). For the NH2P-50 column (6.0 × 100 mm), Eluent E and F were used. Eluent E was acetonitrile: water: acetic acid (800: 200: 3, v/v/v) titrated to pH 7.0 with 12.5% aqueous ammonia; Eluent F acetonitrile: water: acetic acid (200: 800: 3, v/v/v) titrated to pH 7.0 with 12.5% aqueous ammonia. The column was equilibrated with Eluent E. After injecting a sample, a linear gradient was run to Eluent E: Eluent F (1: 1, v/v) over 60 min. The molecular sizes of PA-sugar chains were expressed as glucose units using standard PA-isomaltooligosaccharides as a scale. Elution was monitored by measuring fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

Reducing-end monosaccharide analysis was performed on a TSK-GEL Sugar AX-I column at a flow rate of 0.3 ml/min at 74°C. The elution buffer was 0.8 M boric acid, which had been adjusted to pH 9.0 with potassium hydroxide, containing 10% acetonitrile. Elution was monitored by measuring the fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

Glycosidase Digestion of PA-Sugar Chains—PA-Oligosaccharides were digested with the following enzymes; 100 mU of neuraminidase in 50 μl of 0.1 M ammonium acetate buffer, pH 6.0, at 37°C for 16 h; 10 mU of β-*N*-acetylhexosaminidase in 50 μl of 50 mM ammonium acetate buffer, pH 6.0, at 37°C for 16 h; 4 mU of α1-3/4fucosidase in 20 μl of 30 mM potassium phosphate buffer, pH 6.0, at 37°C for 16 h; 100 mU of

α-mannosidase in 50 μl of 50 mM sodium citrate buffer, pH 4.5, at 37°C for 5 h or 16 h; 250 mU of α-galactosidase in 50 μl of 300 mM sodium phosphate, pH 6.5, containing 50 μg of D-galacturonic acid-γ-lactone at 25°C for 16 h and 15 mU of β1-4galactosidase in 25 μl of 0.1 M sodium citrate-phosphate buffer, pH 6.0, at 37°C for 16 h. The substrate specificity of β1-4galactosidase was confirmed by using 3Bi-PA and Bi-PA as substrates. The 3Bi-PA was not hydrolysed, whereas Bi-PA was hydrolysed under the conditions used in the present study. All enzymatic reactions were terminated by heating at 100°C for 3 min.

Acid Hydrolysis of PA-Oligosaccharides—Acid hydrolysis of PA-oligosaccharide was performed as described previously (*22*). The PA-monosaccharides obtained were analysed on a TSK-GEL Sugar AX-I column as described earlier. Reducing-end monosaccharide analysis was performed after acid hydrolysis of a PA-oligosaccharide as reported on a TSK-GEL Sugar AX-I column as indicated earlier (*22*).

Partial acid hydrolysis to cleave α-fucose residues was performed as described previously (*23*). Briefly, a PA-sugar chain (10 pmol) was incubated with 20 μl of 1 M trifluoroacetic acid at 70°C for 15 min, and the solution was lyophilized. A sample of the residue was analysed on a NH2P column (2.0 × 150 mm).

Two-Dimensional Sugar Mapping—The structures of the PA-oligosaccharides were assessed by two-dimensional sugar mapping. The elution positions of more than 100 standard PA-*N*-linked glycans have already been previously reported, and the introduction of a reversed-phase scale made it possible to predict the elution positions even if standard PA-*N*-linked sugar chains were not available (*23*). PA-oligosaccharides were separated by reversed-phase HPLC and size-fractionation HPLC, and the elution position of each oligosaccharide was compared with those of standard PA-oligosaccharides on the two-dimensional sugar map. Each PA-oligosaccharide was then digested with exoglycosidases, and the structures of the products were analysed on the two-dimensional sugar map as reported previously (*13*).

Detection of the Enzymatic Activities of Endo-β-N-acetylglucosaminidase—The extraction buffer (16 μl, 50 mM sodium phosphate, pH 7.0, containing 1 mg/ml of BSA) was added to 100 zebrafish embryos at 18 h after fertilization. The resulting suspension was homogenized with a pellet mixer and centrifuged at 105,000 × *g* for 60 min at 4°C. The supernatant was used as a soluble enzyme fraction. The resultant precipitates were then solubilized in 16 μl of 10 mM Tris–HCl, pH 7.0, containing 1% 3-[(3-cholamidopropyl)dimethylammonio] (CHAPS). The supernatant obtained by centrifugation at 105,000 × *g* for 1 h at 4°C was used as a membrane enzyme fraction. As substrates, 200 pmol of M6B-PA and Bi-PA were used. To 8 μl of a soluble enzyme fraction obtained from 50 embryos were added 2 μl of 10 M substrate and 5 μl of 300 mM sodium citrate buffer, pH 6.0, containing 0.1% BSA. Incubation was performed at 37°C for 20 h, and the reaction was terminated by heating at 100°C for 3 min. After centrifugation, the reaction mixtures were analysed by reversed-phase HPLC (elution condition 1), and the hydrolysis rates were calculated from the amount of PA-GlcNAc produced. For the analysis of the optimum pH and substrate specificity of endo-β-*N*-acetylglucosaminidase, a soluble enzyme fraction (prepared from 20 embryos for M6B-PA as an accepter and 50 embryos for Bi-PA as acceptors) obtained as above was taken as the enzyme solution. Sodium citrate buffers (100 mM, pH 4.0–7.0) containing 0.1% BSA, and 100 mM Tris–HCl buffers (pH 7.0–8.0) containing 0.1% BSA were used for the analysis of optimal pH. The enzyme reaction was carried out as described earlier by incubating at 37°C for 4 h and was terminated by heating at 100°C for 3 min. The reaction mixtures were analysed by reversed-phase HPLC (elution condition 1).

MALDI-TOF MS Analysis—A PA-oligosaccharide was co-crystallized in a matrix of 2,5-dihydroxybenzoic acid and analysed with a Voyager-DE-RP BioSpectrometry Workstation (Perspective Biosystems, Framingham, MA, USA) equipped with delayed extraction operated in the reflector mode. Peptide standards (neurotensin and insulin chain B) were used to achieve a two-point external calibration for mass assignment of ions.

Purification of α-Fucose Containing PA-Oligosaccharides by LTA-Affinity Chromatography—PA-Oligosaccharides prepared from 1,000 zebrafish embryos as described earlier were dissolved in 100 μl of 10 mM Tris–HCl buffer, pH 7.2, containing 150 mM NaCl and 0.1% Triton X-100. An LTA-affinity column (3.0 × 70 mm) was equilibrated with the same buffer at a flow rate of 100 μl/min. After the sample solution was placed on the column, the column was washed with the buffer and α-fucose containing PA-oligosaccharides were then eluted with the buffer containing 0.5 M Fuc. This fraction was freeze-dried, dissolved in 100 μl of 0.1% acetic acid, and the solution was applied to a Sep-Pak C18 Cartridge equilibrated with 0.1% acetic acid. This cartridge was washed with 2 ml of 0.1% acetic acid. α-fucose containing PA-oligosaccharides were eluted with 2 ml of 0.1% acetic acid containing 20% methanol. The solution was freeze-dried and diluted in 50 μl of water.

RESULTS

Detection of zFT1 Products in Zebrafish Embryos—We tried to detect in zebrafish embryo oligosaccharides that were synthesized by the action of α1-3fucosyltransferase (zFT1) and were presumed to be expressed stage specifically at 18 h after fertilization (*8*). Since this enzyme can synthesize fucosylated sugar chains, we also tried to detect sugar chains with a Fucα1-3 residue expressed at 18 h after fertilization. To detect and isolate these sugar chains, we compared the HPLC elution profiles of PA-oligosaccharides prepared by hydrazinolysis-*N*-acetylation of the lyophilized embryos followed by pyridylamination. The PA-oligosaccharide fractions obtained from 2,000 zebrafish embryos at 12, 18 and 48 h after fertilization were separately purified by DEAE-HPLC (Fig. 1). Each pass-through fraction (fraction N) containing PA-neutral oligosaccharides was further separated by size-fractionation HPLC (Fig. 2), and fractions

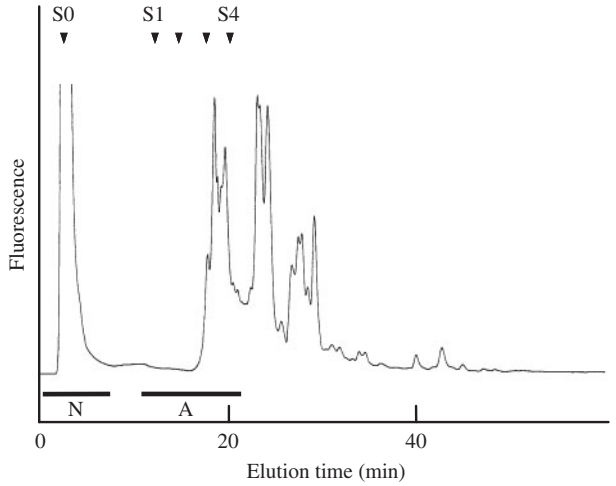


Fig. 1. **DEAE-HPLC of PA-*N*-glycans obtained from zebrafish embryos at 18 h after fertilization.** HPLC was performed on a TSK-GEL DEAE-5PW column. Arrowheads S0–S4 indicates the elution positions of authentic asialo, monosialo, disialo, trisialo and tetrasialo PA-*N*-glycans, respectively, obtained from α_1-acid glycoprotein. Fractions were pooled as indicated by the bars; N, the neutral fraction; A, the acidic fraction.

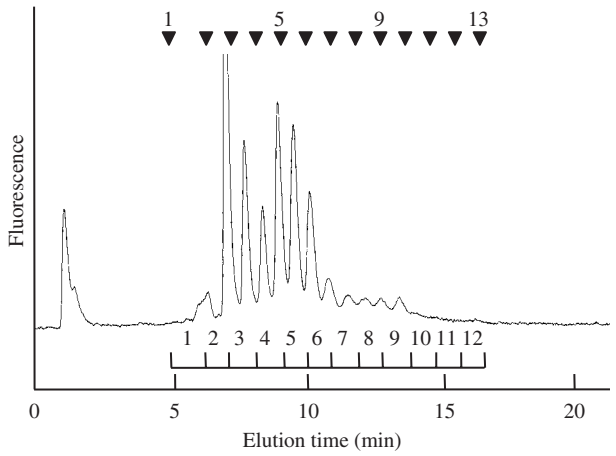


Fig. 2. **Size-fractionation HPLC of fraction N from zebrafish embryos.** HPLC was performed on a Shodex Asahipak NH2P-50 column (4.6 × 50 mm). Arrowheads 1–13 indicate the elution positions (glucose unit) of standard PA-isomalto oligosaccharides. Fractions were collected as indicated by numerals. The peaks appearing at around 2 min were due to contaminating materials.

1–12 were collected according to the elution positions of standard PA-isomaltooligosaccharides. Each fraction was then separated by reversed-phase HPLC. To find an oligosaccharide(s) expressed mainly at 18 h after fertilization, the HPLC chromatograms obtained from embryos at the different fertilization times were compared. Several such peaks were observed (data not shown). To detect fucosyloligosaccharides among these peaks, the fractions were digested with α1-3/4fucosidase, and the products were analysed by reversed-phase HPLC. Fraction N9A in fraction 9 (Fig. 3B) increased at 18 h after fertilization and was largely reduced after digestion with α1-3/4fucosidase (Fig 3B-1 and B-2). At 48 h after fertilization, although a large peak was also observed, only a part of this peak was digested with α1-3/4fucosidase. These results indicate that this peak contained fucosyloligosaccharide(s) expressed stage-specifically in zebrafish embryos at 18 h after fertilization. Since a larger quantity of this fraction was needed for the structural analysis, fraction N in Fig. 1 was directly separated by reversed-phase HPLC and fraction N1 at the elution position of N9A was collected (Fig. 4). Fraction N1 was further separated by size-fractionation HPLC and seven peaks eluting between 8.0 G.U. and 11.0 G.U. were assessed for the presence of fucosylated sugar chains (Fig. 5, bars) as N9A appeared in fraction 9 which covers between 9 GU and 10 GU (Fig. 2). Fractions N1a and N1b were digested with α1-3/4fucosidase (data not shown). In contrast, five other fractions were not hydrolysed with α1-3/4fucosidase. However, only fraction N1c was digested with α1-3/4fucosidase after digestion with β1-4galactosidase (data not shown). Therefore, fractions N1a, N1b and N1c were considered to contain fucose-containing oligosaccharides, and their corresponding structures were determined as described subsequently.

Structure Analysis of Fraction N1a—N1a was hydrolysed with acid, and the products were *N*-acetylated. The PA-monosaccharide originating from the reducing end was analysed on a TSK-GEL Sugar AX-I column as described in Experimental Procedures (Fig. 6). A major peak was detected at the elution position of PA-GlcNAc, indicating that the reducing-end residue of N1a was PA-GlcNAc. No peak was detected at the elution position of PA-di-*N*-acetylchitobiose (Fig. 6, arrow 1), indicating that the structure of fraction N1a did not have the GlcNAcβ1-4GlcNAc-PA structure since our in-house data suggested that about 54% of GlcNAcβ1-4GlcNAc-PA should remain after the acid hydrolysis of *N*-linked glycans under the acid hydrolysis conditions used (unpublished data). When fraction N1a with a molecular size of 9.3 glucose unit was digested with α1-3/4 fucosidase, the molecular size was reduced by 1.8 glucose units, indicating that two fucose residues were removed and a peak appeared at the elution position of Bi′-PA by two-dimensional sugar mapping (Table 2). Further digestion of the product with β1-4galactosidase led to the hydrolysis of two Gal residues, and the appearance of a peak at the position of agaBi′-PA. The product was further digested with β-*N*-acetylhexosaminidase, resulting in a peak at the position of M3B′-PA. This product was digested with α-mannosidase initially to M2B′-PA as an intermediate product and finally to a product eluting at the position of M1′-PA (Table 2). Fraction N1a was considered to have two Lewis x structures on its non-reducing end based on the results that fraction N1a digested with α1-3/4fucosidase was hydrolysed with β1-4galactosidase that can hydrolyse a Galβ1-4 linkage but not a Galβ1-3 linkage under the conditions used, and that fraction N1a digested with α1-3/4fucosidase eluted at Bi′-PA but not at 3Bi′-PA (Table 2). Taken together, these results indicated that the structure of fraction N1a was Galβ1-4(Fucα1-3)GlcNAcβ1-2 Manα1-6[Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-3]Manβ1-4

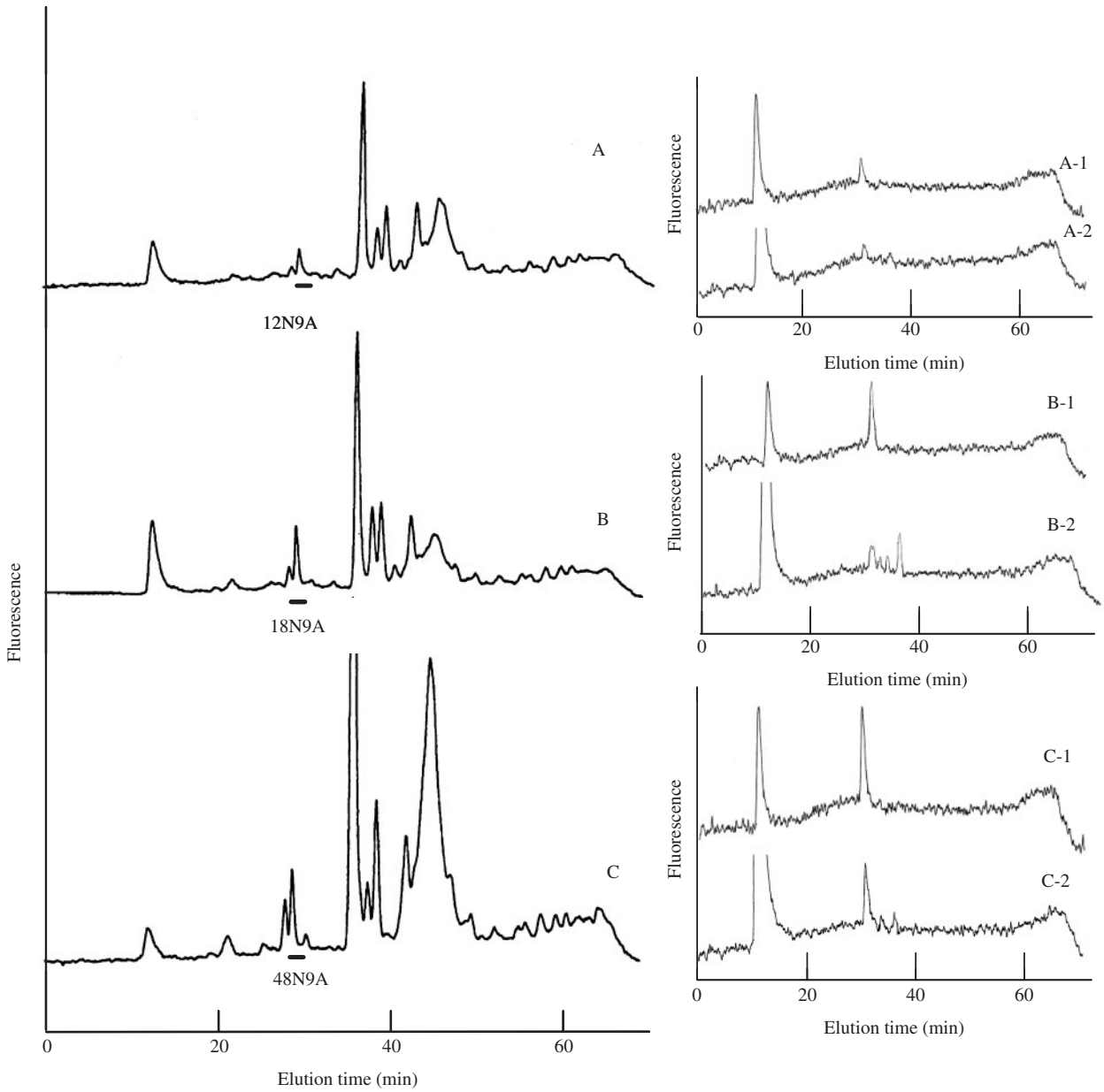


Fig. 3. **Reversed-phase HPLC of fraction 9 in Fig. 2 obtained from zebrafish embryos at 12 h, 18 h and 48 h after fertilization.** HPLC was performed on a Cosmosil 5C18P column (elution condition 1). (A) Fraction 9 obtained from zebrafish embryos at 12 h after fertilization; (B) Fraction 9 at 18 h; (C) Fraction 9 at 48 h. Fractions were pooled as indicated by the bars. (A-1) Fraction 12N9A; (A-2) the α1-3/4fucosidase digest of fraction 12N9A; (B-1) Fraction 18N9A; (B-2) the α1-3/4fucosidase digest of Fraction 18N9A; (C-1) Fraction 48N9A; (C-2) the α1-3/4fucosidase digest of fraction 48N9A.

GlcNAc-PA (Table 3). Analysis of fraction N1a by MALDI-TOF MS yielded the peak ions $[M+H]^+$ (m/z 1808.39, calculated 1808.69), $[M+Na]^+$ (m/z 1830.48, calculated 1830.68), and $[M+K]^+$ (m/z 1846.59, calculated 1846.78), in support of the earlier described results.

Structure Analysis of Fraction N1b—When fraction N1b was digested with β1-4galactosidase, a peak appeared at the elution position of Fraction N1a indicating the hydrolysis of one Gal residue (Table 2). Further, digestion of the product gave the same results as observed for N1a, and M3B'-PA was obtained (Table 2), indicating that fraction N1b contained one Gal attached fraction N1a. To analyse the linkage position of the Gal residue, the partial acid hydrolysis that cleaves mainly α-fucosyl residues was performed. If the Galβ1-4 residue was bound to a Fucα1-3 residue of fraction N1a, a peak was expected at the elution position of Bi′-PA. When fraction N1b was partially hydrolysed, no peak was detected at the elution position of Bi′-PA by size-fractionation HPLC (Fig. 7) and reversed-phase HPLC (data not shown), but a peak was found at the elution position corresponding to Gal-N1a (Fig. 7). These results indicated that the Galβ1-4 residue of fraction N1b was not bound to a Fucα1-3 residue but to

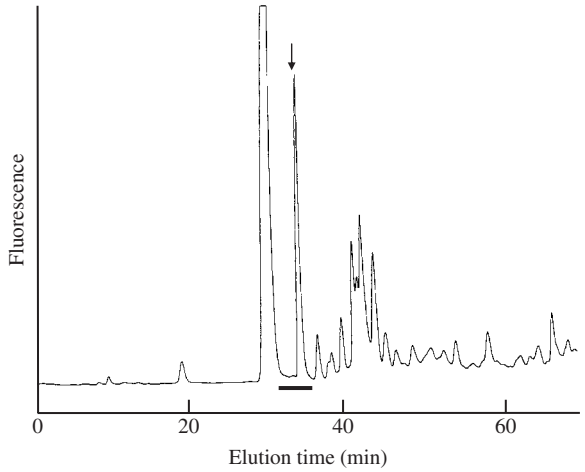


Fig. 4. **Reversed-phase HPLC of fraction N.** HPLC was performed on a Cosmosil 5C18P column (elution condition 1). The arrow indicates the elution position of N9A. Fraction N1 was pooled as indicated by the bar.

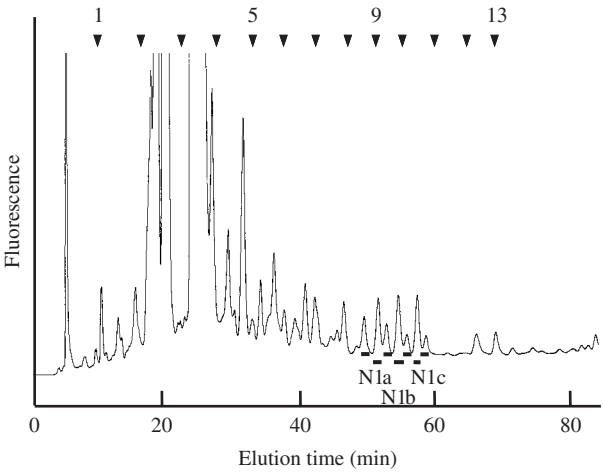


Fig. 5. **Size-fractionation HPLC of Fraction N1.** HPLC was performed on a Shodex Asahipak NH2P-50 column (4.6 × 200 mm). Arrowheads 1–13 indicate the elution positions (glucose unit) of PA-isomalto oligosaccharides. Fractions were pooled as indicated by the bars.

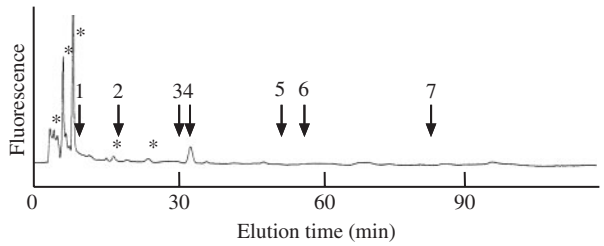


Fig. 6. **Reducing-end PA-monosaccharide analysis obtained from fraction N1a.** HPLC was performed on a TSK-GEL Sugar AX-I column. The elution positions of standard PA-sugars are indicated by the arrows; 1, PA-*N,N′*-diacetylchitobiose (GlcNAcβ1-4GlcNAc-PA); 2, PA-GalNAc; 3, PA-Xyl; 4, PA-GlcNAc; 5, PA-Glc; 6, PA-Fuc; 7, PA-Gal. Peaks indicated with asterisk were due to contaminating materials.

one of the two non-reducing terminal Galβ1-4 residues (Table 3). Analysis of fraction N1b by MALDI-TOF MS yielded the peak ions $[M+Na]^+$ (m/z 1992.93, calculated 1992.73) and $[M+K]^+$ (m/z 2008.96, calculated 2008.84), in support of the structure proposed.

Structural Analysis of Fraction N1c—Although fraction N1c was digested with α1-3/4fucosidase only after digestion with β1-4galactosidase as described earlier, indicating that fraction N1c was larger than fraction N1b by one Gal residue and that each Lewis x on the non-reducing end was capped with a β1-4galactose residue. When fraction N1c was digested sequentially with β1-4galactosidase and α1-3/4fucosidase, peaks were detected at the elution position of fraction N1a and Bi′-PA by two-dimensional HPLC (Table 2), respectively. Although the linkage position of the Galβ1-4 residues of fraction N1c was not analysed, the two Galβ1-4 residues were probably bound to the non-reducing terminal Galβ1-4 residues as found for fraction N1b (Table 3). Analysis of fraction N1c by MALDI-TOF MS yielded the peak ions $[M+Na]^+$ (m/z 2154.51, calculated 2154.78) and $[M+K]^+$ (m/z 2170.78, calculated 2170.89), in support of the above results. These results indicated that fraction N1c was digalactosylated fraction N1a and was also expressed in the segmentation period of zebrafish. From the results shown in Fig. 5, the amounts of fractions N1a, N1b and N1c were almost similar.

Detection of Oligosaccharides with the Fraction N1a Structure with GlcNAcβ1-4GlcNAc or GlcNAcβ1-4(Fucα1-6)GlcNAc at the Reducing Ends—Since the structures of fractions N1a, N1b and N1c were similar to *N*-linked oligosaccharides but the reducing end was only GlcNAc instead of GlcNAcβ1-4GlcNAc, we next analysed for the presence of oligosaccharides with the GlcNAcβ1-4GlcNAc and GlcNAcβ1-4(Fucα1-6)GlcNAc structures at the reducing end: namely, DiFBi and DiFBiF, respectively. The neutral fraction (fraction N) obtained from 1,000 embryos was separated by reversed-phase HPLC (Fig. 8). A fraction (fraction N2) eluting at the position of standard DiFBi-PA with GlcNAc-GlcNAc-PA at the reducing end was collected and further separated by size-fractionation HPLC, and fraction N2a at the elution position of DiFBi-PA was collected (data not shown). Fraction N2a showed a peak at the elution position of DiFBi-PA by the size-fractionation HPLC and reversed-phase HPLC (Table 2). The structure of fraction N2a was further confirmed after the digestion with α1-3/4fucosidase, which resulted in a peak at the elution position of Bi-PA, but not 3Bi-PA, by two-dimensional HPLC mapping (Table 2), indicating the presence of the Lewis x structure.

Fraction N3 in Fig. 8 eluting at the position of standard DiFBiF-PA with GlcNAc-Fuc(α1-6)GlcNAc-PA at the reducing end was also collected and separated by size-fractionation HPLC. Fraction N3a at the elution position of DiFBiF-PA was collected, however, after digestion of N3a with α1-3/4fucosidase, no peak appeared at the elution position of BiF-PA analysed in the same way used for fraction N2a (data not shown). These results indicated that a GlcNAcβ1-4GlcNAc type oligosaccharide (DiFBi) was present in zebrafish embryos, but a GlcNAcβ1-4(Fucα1-6)GlcNAc type

Table 2. **Two-dimensional sugar mapping combined with sequential exoglycosidase digestion of PA-Oligosaccharides.**

Fraction	Successive treatment with exoglycosidases	Size-fractionation[a]	Reversed-phase[b]	PA-oligosaccharide identified
N1a	No treatment	9.3	1.23	
	α1-3/4fucosidase	7.5	1.42	Bi′-PA
	β1-4galactosidase	5.8	1.34	agaBi′-PA
	β-*N*-acetylhexosaminidase	4.1	1.17	M3B′-PA
	α-mannosidase (5 h)	3.0	1.00	M2B′-PA
	α-mannosidase (16 h)	1.8	1.21	M1′-PA
N1b	No treatment	9.9	1.24	
	β1-4galactosidase	9.3	1.23	N1a
	α1-3/4fucosidase	7.5	1.42	Bi′-PA
	β1-4galactosidase	5.8	1.34	agaBi′-PA
	β-*N*-acetylhexosaminidase	4.1	1.17	M3B′-PA
	No treatment	9.9	1.24	
	α1-3/4fucosidase	9.1	1.33	
N1c	No treatment	10.7	1.24	
	α1-3/4fucosidase	10.7	1.24	
	β1-4galactosidase	9.4	1.23	N1a
	α1-3/4fucosidase	7.6	1.42	Bi′-PA
N2a	No treatment	9.5	0.59	DiFBi-PA
	α1-3/4fucosidase	7.6	1.00	Bi-PA
N4	No treatment	10.1	0.61	
	β1-4galactosidase	9.4	0.59	DiFBi-PA
	α1-3/4fucosidase	7.6	1.00	Bi-PA
AN1b	neuraminidase	9.9	1.24	N1b
	β1-4galactosidase	9.3	1.23	N1a

[a]Glucose unit. [b]The elution time of Galβ1-3GalNAc-PA analysed using elution condition 1 is taken as 1.0 (fraction N1a, N1b and N1c), and the elution times analysed using elution condition 2 are indicated (fraction N2a and N4).

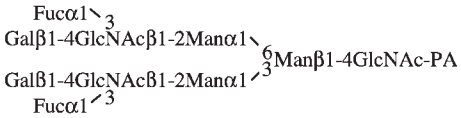
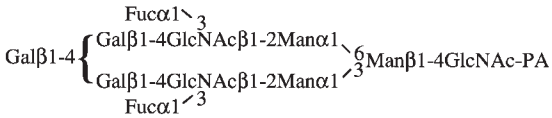
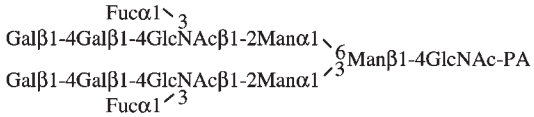
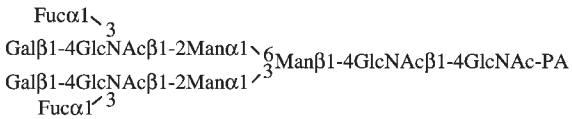
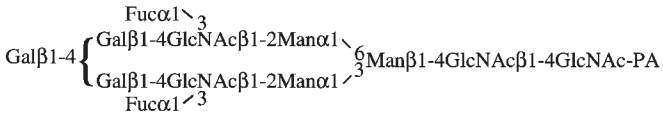
oligosaccharide (DiFBiF) was not. The proposed structure of fraction N2a is shown in Table 3.

Detection of an Oligosacccharide with the Fraction N1b Structure with GlcNAcβ1-4GlcNAc at the Reducing End—Using similar techniques as described earlier for fraction N2a, we tried to detect an oligosaccharide with the Galβ1-4DiFBi structure at the non-reducing end and GlcNAcβ1-4GlcNAc at the reducing end: namely, Galβ1-4DiFBi. PA-oligosaccharides prepared from embryos were separated by DEAE-HPLC. The neutral fraction (N) collected was separated by size-fractionation HPLC. Based on glucose units of fraction N1a, the glucose unit of an oligosaccharide of fraction N1b with a di-*N*-acetylchitobiose residue was estimated to be 10.2. A fraction containing PA-oligosaccharides with 9.95–10.35 glucose units was collected (data not shown). This fraction collected was further separated by reversed-phase HPLC. A peak (fraction N4) which appeared at the estimated elution position of Galβ1-4DiFBi-PA disappeared after digestion with α1-3/4fucosidase (Fig. 9) (Table 2). Fraction N4 was digested with β1-4galactosidase, resulting in a peak at the elution position of DiFBi-PA by two-dimensional HPLC sugar mapping. This product was further digested with α1-3/4fucosidase, leading to a peak at the elution position of Bi-PA (Table 2). These results suggest that an oligosaccharide of fraction N1b with GlcNAcβ1-4GlcNAc (fraction N4) was also expressed in zebrafish embryos at 18 h after fertilization. The proposed structure of fraction N4 is shown in Table 3.

Detection of Oligosaccharides with Sialylated Lewis x Epitope—The presence of sialyl derivatives of N1a, N1b, N1c, N2a and N4 was examined. The acidic fraction (fraction A) from the separation shown in Fig. 1 was digested with neuraminidase. The digests were separated by DEAE, reversed-phase and size-fractionation HPLC to see whether Lewis x containing fucosyloligosaccharides were included. Fraction AN1b at the elution position of PA-Sugar N1b was digested with β1-4galactosidase and a peak was detected at the elution position of fraction N1a by two-dimensional HPLC (Table 2), indicating that sialylated N1b was expressed, but sialylated fraction N1a, N1c, N2a and N4 were not detected. Since the amount of fraction AN1b was limited, further structural analysis such as the linkage position of sialic acid could not be performed.

Affiliation of Fractions N1a, N1b and N1c—Under the conditions employed for the preparation of PA-oligosaccharides as described earlier, PA-oligosaccharides were possibly obtained from free oligosaccharides in addition to *N*- and *O*-linked oligosaccharides attached to glycoproteins. To confirm whether fractions N1a, N1b and N1c were expressed as free oligosaccharides or were attached to proteins, zebrafish

Table 3. **Proposed structures for Fucα1-3 containing PA-oligosaccharides obtained from zebrafish embryos in the present study.**

Fraction	Structure
N1a	
N1b	
N1c	
N2a	
N4	

embryos (700 embryos) were separated into the membrane and the soluble fractions as described in Experimental Procedures. The membrane fraction was sequentially lyophilized, hydrazinolysed (60°C, 50 h), *N*-acetylated and pyridylaminated to obtain PA-oligosaccharides (fraction M). The soluble fraction was pyridylaminated without hydrazinolysis and the PA-oligosaccharides thus obtained (fraction S) were from free oligosaccharides. Fractions M and S were separately purified by DEAE-HPLC and neutral fractions were collected. Each neutral fraction was further separated by reversed-phase HPLC, and fractions M1 and S1 covering the elution positions of fractions N1a, N1b and N1c were collected (data not shown). Fractions M1 and S1 were further separately purified by size-fractionation HPLC (Fig. 10). Fractions at the elution positions of fractions N1a, N1b and N1c were collected, and each fraction was analysed. Analysis of fraction S1 resulted in a fraction eluting at the position of N1a, which was identified as fraction N1a by two-dimensional HPLC before and after the digestion with α1-3/4fucosidase (data not shown), and the fractions eluting at the positions of fractions N1b and N1c that were confirmed as fractions N1b and N1c, respectively, by two-dimensional HPLC before and after the digestion with β-galactosidase (data not shown). However, no peak was detected from the fraction eluting at fractions N1a, N1b and N1c from fraction M1 as analysed by two-dimensional HPLC mapping (data not shown). These results indicated that fractions N1a, N1b and N1c existed mainly as free oligosaccharides.

We also analysed whether fraction N2a was bound to proteins or existed as a free oligosaccharide by applying a method similar to that used for fractions N1a, N1b and N1c as described earlier. The results indicated that about 70% of N2a was bound to proteins and 30% thereof existed as a free oligosaccharide, when the losses owing to the procedures were taken into consideration (data not shown).

To determine whether fractions N1a, N1b and N1c were released by enzymes during the preparation of PA-oligosaccharides, the above experiment with embryos heated at 100°C for 5 min before homogenization to inactivate enzymes was performed. Chromatograms similar to those shown in Fig. 10B were obtained (data not shown), indicating that fractions N1a, N1b and N1c were present as free oligosaccharides and were not hydrolysed during the preparation.

Stage Specific Expression of Oligosaccharides with Lewis x Epitope—Time dependent expression of fractions N1a, N1b and N2a was analysed using embryos at 12, 18 and 48 h after fertilization. PA-oligosaccharides were prepared from embryos and the amounts were quantified after HPLC separation using a method similar to that shown in Fig. 5 for fractions N1a and N1b, and that shown in Fig. 9 for fraction N2a. The amount of fractions N1c and N4 could not be calculated since the contaminating materials overlapped with fraction N1c at 48 h

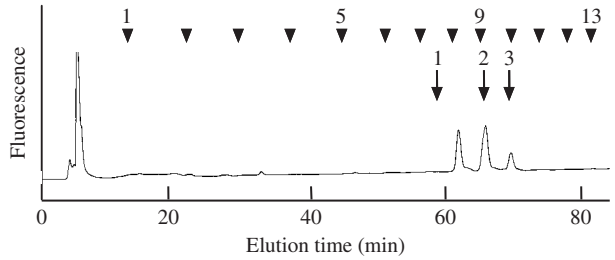


Fig. 7. **Size-fractionation HPLC of the partial acid hydrolysates of fraction N1b.** HPLC was performed on a Shodex Asahipak NH2P-50 column (2.0 × 150 mm). The elution positions of PA-oligosaccharides were indicated by the arrows; 1, Bi′-PA; 2, the α1-3/4fucosidase digest of N1b; 3, N1b. Arrowheads 1–13 indicate the elution positions (glucose unit) of PA-isomalto oligosaccharides.

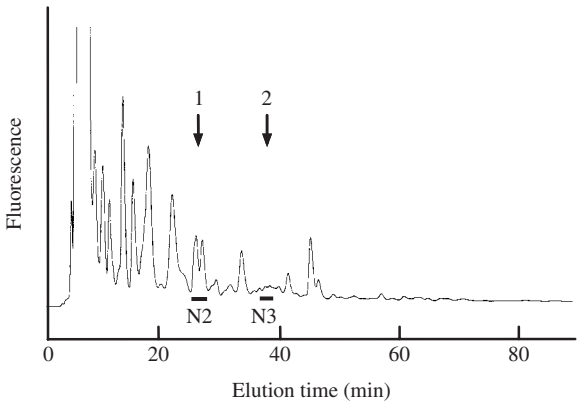


Fig. 8. **Reversed-phase HPLC of fraction N of PA-*N*-glycans obtained from zebrafish embryos at 18 h after fertilization.** HPLC was performed on a Cosmosil 5C18P column (elution condition 2). The elution positions of standard PA-oligosaccharides were indicated by the arrows; 1, DiFBi-PA; 2, DiFBiF-PA. Fractions were pooled as indicated by the bars.

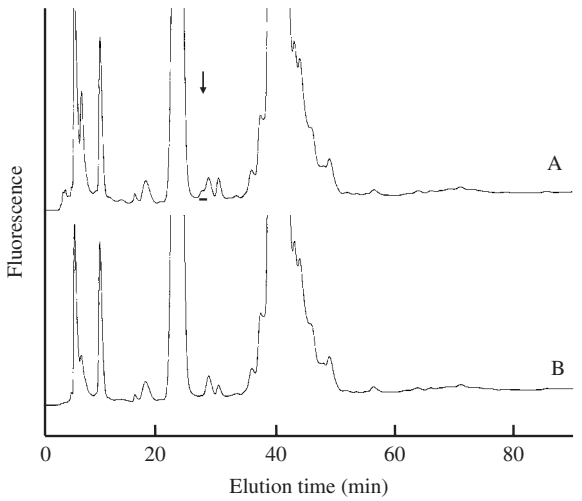


Fig. 9. **Reversed-phase HPLC of 9.95–10.35 glucose unit fraction.** HPLC was performed on a Cosmosil 5C18P column (elution condition 2). (A) 9.95–10.35 glucose unit fraction; (B) the α1-3/4fucosidase digest of the fraction. The arrow indicates the estimated elution position of Galβ1-4DiFBi-PA. Fraction N4 was pooled as indicated by the bar.

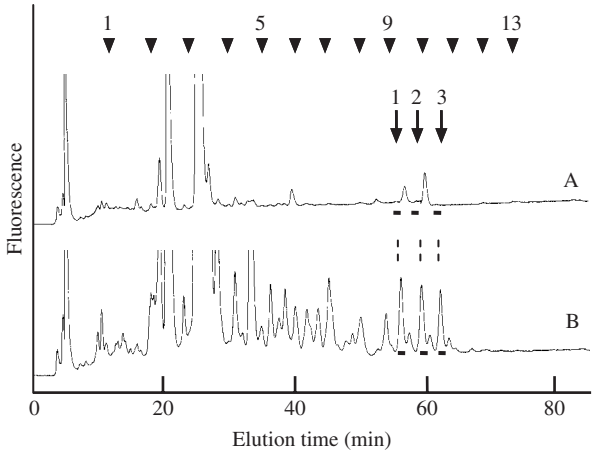


Fig. 10. **Size-fractionation HPLC of fractions M1 and S1.** HPLC was performed on a Shodex Asahipak NH2P-50 column (4.6 × 200 mm). (A) Fraction M1; (B) Fraction S1. Arrowheads 1–13 indicate the elution positions (glucose unit) of standard PA-isomalto oligosaccharides. The elution positions of PA-oligosaccharides are indicated by the arrows; 1, N1a; 2, N1b; 3, N1c.

after fertilization and fraction N4 at 12, 18 and 48 h after fertilization. Three PA-oligosaccharides were mainly expressed in the zebrafish embryos at 18 h after fertilization (Fig. 11), indicating that the oligosaccharides with the Lewis x epitope were expressed at the similar expression stage of *zFT1*.

Majority of Fucosyloligosaccharides Detected in this Study—Since contaminating peaks were observed in the chromatograms, it was possible that other fucosyloligosaccharides were included in zebrafish embryos. To examine this possibility, PA-fucosyloligosaccharides were purified with LTA-affinity chromatography specific for α-fucose containing oligosaccharides. A fraction eluted with 0.5 M fucose contained a major peak (Fig. 12) as most PA-oligosaccharides derived from *N*-linked oligosaccharides are larger than four glucose unit. The peak at the elution positions of DiFBi'-PA and DiFBi-PA was digested with α1-3/4fucosidase (Fig. 12) and a peak was observed at the positions of Bi'-PA and Bi-PA, however, no other peaks were digested with α1-3/4fucosidase (Fig. 12). This result indicated that the fucosyloligosaccharides detected in this study were the major fucosyloligosaccharides expressed in the neurula of zebrafish.

Detection of Endo-β-N-Acetylglucosaminidase Activity Expressed in Zebrafish Embryos—Since fractions N1a, N1b and N1c were free oligosaccharides and closely resemble N linked glycans, we have speculated that these oligosaccharides were first synthesized as glycoproteins, and then subsequently oligosaccharides were hydrolysed with enzyme(s). Therefore, the activity of endo-β-*N*-acetylglucosaminidase was analysed in zebrafish embryos. Two crude enzyme fractions (membrane enzyme fraction and soluble enzyme fraction) were prepared as described in EXPERIMENTAL PROCEDURES. A high mannose type PA-oligosaccharide (M6B-PA) was digested with the both enzyme fractions, but complex type PA-oligosaccharide (Bi-PA) was digested with only

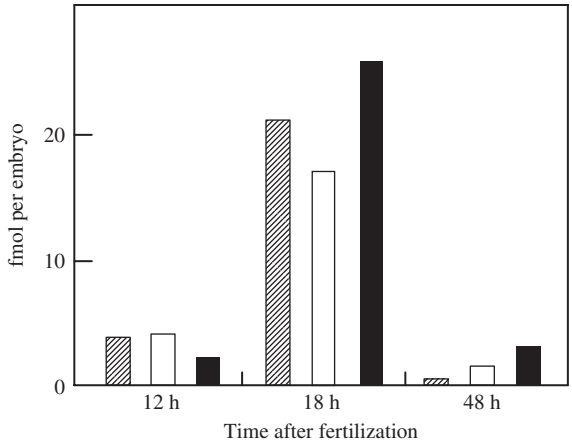


Fig. 11. **Stage specific expression of Fractions N1a, N1b and N2a**. The expression of fraction N1a, N1b and N2a were analysed using embryos of 12 h, 18 h and 48 h after fertilization. Bevel bar, expression of N1a; empty bar, N1b; closed bar, N2a. Amount of Lewis x containing oligosaccharides were calculated considering of the yields of the reactions and the losses during the purification.

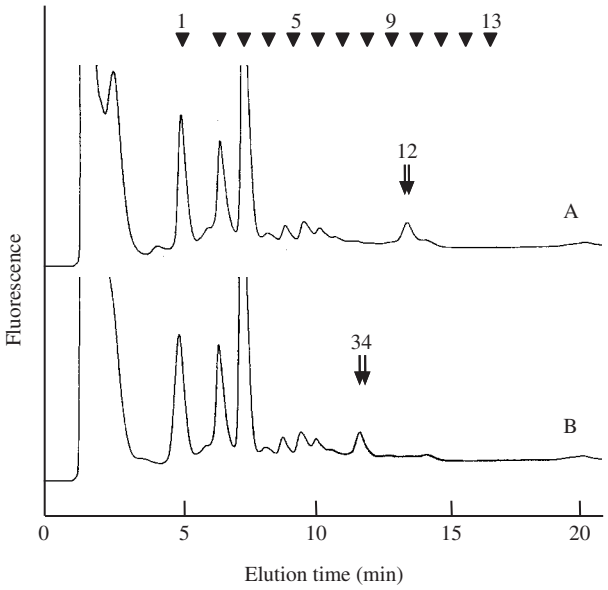


Fig. 12. **Size-fractionation HPLC of PA-fucosyloligosaccharides purified by LTA affinity chromatography.** HPLC was performed on a Shodex Asahipak NH2P-50 column (4.6 × 50 mm). (A) The LTA-bound fraction; (B) α1-3/4fudosidase digests of the LTA-bound fraction. Arrowheads 1–13 indicate the elution positions (glucose unit) of standard PA-isomalto oligosaccharides. The elution positions of PA-oligosaccharides are indicated by the arrows; 1,DiFBi'-PA; 2, DiFBi-PA; 3, Bi'-PA; 4, Bi-PA.

the soluble enzyme fraction under the conditions used. The relative initial hydrolysis rates of the soluble enzyme fraction are summarized in Table 4. PA-oligosaccharides with GlcNAcβ1-4(Fucα1-6)GlcNAc at the reducing end (BiF-PA and DiFBiF-PA) were not hydrolysed, being consistent with the observation that the free oligosaccharides with the GlcNAcβ1-4(Fucα1-6)GlcNAc structure were not detected. The soluble enzyme fraction possibly

Table 4. **Hydrolysis of PA-oligosacchrides with Soluble Enzyme Fraction from zebrafish embryos at 18 h after fertilization.**

Substrate	Relative rate of hydrolysis [a]	Substrate	Relative rate of hydrolysis[a]
M9A-PA	48	agaBi-PA	0.09
M6B-PA	100	Bi-PA	1.4
M5A-PA	44	BiF-PA	<0.01
M3B-PA	24	DiSiaBi-PA	0.91
Hyb-PA	24	DiFBiF-PA	<0.01
		Tri-PA	0.13

[a]The value obtained with M6B-PA was taken as 100.

contained more than one endo-β-*N*-acetylglucosaminidase. The optimum pH of the soluble enzyme fraction was 7.5 for both M6B-PA and Bi-PA as substrates (data not shown).

DISCUSSION

In this study, we have detected oligosaccharides that were presumably synthesized by zFT1 stage-specifically expressed in zebrafish embryos. Most surprisingly, however, was our finding of free *N*-linked type oligosaccharides with two Lewis x structures. Three oligosaccharides (fractions N1a, N1b and N1c) were found to be stage-specifically expressed in the segmentation period of zebrafish embryo at about 18 h after fertilization, suggesting that these oligosaccharides are produced by the gene products of *zFT1* based on the observation that the oligosaccharides were expressed mostly at 18 h after fertilization and that recombinant zFT1 can synthesize the Lewis x structure (*8*). These oligosaccharides were the major fucosyloligosaccharides from *N*-linked oligosaccharides found in the neurula of zebrafish (Fig. 12). Fractions N1a, N1b and N1c were partial structures of *N*-linked glycans in that their reducing-end portion was not a di-*N*-acetylchitobiose sequence (GN2 type) but a single *N*-acetylglucosamine residue (GN1 type). These oligosaccharides are therefore considered to be produced from glycoproteins by digestion with an endo-β-*N*-acetylglycosaminidase or with a combination of a peptide-*N*-glycosidase and an endo-β-*N*-acetylglycosaminidase. Complex type glycans first appeared in the embryo after 12 h after fertilization (*24*). In fact, the endo-β-*N*-acetylglycosaminidase activity was detected in embryos at 18 h after fertilization although the presence of peptide *N*-glycosidase activity was not examined in this study. Endo-β-*N*-acetylglycosaminidase activity against complex-type oligosaccharides was found although this activity was low as compared with that against high mannose type glycans. We speculate that endo-β-*N*-acetylglycosaminidase(s) in the embryo was responsible for the hydrolysis leading to free oligosaccharides. The presence of endo-β-*N*-acetylglucosaminidase has been reported in several eukaryotes (*25–28*) and prokaryotes (*29–33*). Most of these enzymes can hydrolyse high mannose type glycans, but not complex type glycans. Exceptions that are able to act on complex type oligosaccharides have been reported in human saliva (*27*) and in *Flavobacterium meningosepticum* (*32*). Recently,

the gene of a human cytosolic endo-β-*N*-acetylglucosaminidase has been cloned, and the orthologue of this gene has been found in zebrafish (*34*). The substrate specificities against BiF-PA and DiFBiF-PA indicate that the *N*-linked glycans with the GlcNAcβ1-4(Fucα1-6)GlcNAc residue at the reducing-end portion was not hydrolyzed (Table 4). This specificity agrees with the observation that oligosaccharides with GlcNAcβ1-4(Fucα1-6)GlcNAc residue at the reducing-end were not detected. In addition, the substrate specificity against agaBi-PA also indicates that the addition of a Galβ1-4residue to the GlcNAc residue is necessary for recognition by endo-β-*N*-acetylglucosaminidase(s). We did not analyse a peptide-*N*-glycosidase activity in this study, but this activity is likely also present since fractions N2a was detected as free oligosaccharides.

Further studies are needed to reveal the characteristics of these enzymes. GN1-type free oligosaccharides have been detected in several organs and cultured cells (*12*, *35–45*) and are present mostly in cytosol (*12*, *37–41*) or endoplasmic reticulum (ER) (*42–44*), and their structures are high mannose-type glycans (*37–41*). GN1-complex-type free oligosaccharides as detected in the present study have been reported in fish eggs (*45*), the urine of a fucosidosis patient (*36*) and mouse liver (*12*).

Whereas sialylated fraction N1b (Table 2) was detected, sialylated fraction N1a was not detected, indicating that sialylation occurs on the Galβ1-4Galβ1-4GlcNAc structure and not on the Lewis x structure. The Galβ1-4Galβ1-4GlcNAc structure has been detected in unfertilized fish eggs (*35*, *46*) and fertilized fish eggs (*45*). In those reports, the Galβ1-4 residue is linked to bi- or tri-antennary complex-type glycans, and most of it was detected as sialylated free oligosaccharides (*35*, *45*, *46*). Recently, oligosaccharide with sialyl Lewis x and the Galβ1-4Galβ1-4GlcNAc structure at the non-reducing end residue are also reported in zebrafish embryos (*47*). In that report, the expression level of non-sialylated Lewis x-containing oligosaccharides is lower than that of sialylated Lewis x-containing oligosacchrides and unchanged during the segmentation. The genomic study for zebrafish revealed the existence of orthologues of FUT7 and FUT9 (*48*). Among these fucosyltransferases, only FUT9 is capable of synthesizing Lewis x structure. This gene was reported to be expressed from 50% epibody (5.25 h after fertilization at the gastrula stage) to long-pec (48 h after fertilization at hatching) (*48*), which was not consistent with the expression stage of Lewis x containing oligosaccharides detected in this study. Meanwhile, the expression stage of Lewis x-containing GN1 type free oligosaccharides and GN2 type *N*-linked oligosaccharides correlated with *zFT1*, and most of them are non-sialylated, strongly suggesting that they are the enzymatic products of the gene product of *zFT1*. The detection of oligosaccharides with the Lewis x structure that are freed from proteins have not been reported to our knowledge.

In this study, a portion of fraction N2a was found as glycans attached to membrane bound proteins, therefore the other oligosaccharides likely also originated from glycoproteins. It has been previously reported that Lewis x containing glycans were expressed in the neural tube and involved directly in the neural induction of chick embryos using the antibody against Lewis x epitope (*41–51*). The current results indicate that Lewis x containing glycans were present in membrane-bound glycoproteins. However, most Lewis x containing oligosaccharides were detected as soluble free oligosaccharides. Liberation of Lewis x containing glycans during the neural formation period may suggest the importance of the free oligosaccharides that we observed in this study.

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