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α-L-Fucosyltransferases from Radish Primary Roots

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A novel α-L-fucosyltransferase capable of transferring L-fucose (L-Fuc) from GDP-L-Fuc to the *O*-2 of α-L-arabinofuranosyl residue (GDP-L-Fuc:α-L-arabinofuranoside 2-α-L-fucosyltransferase) has been found in the microsomal fraction of primary roots from 6-d-old radish (*Raphanus sativus* L.) seedlings. Enzyme activity was measured fluorometrically at 25°C using a pyridylaminated trisaccharide, L-arabinofuranosyl*f*α(1→3)D-galactopyranosylβ(1→6)D-galactose (AraGalGal-PA) as the acceptor. This enzyme found in the microsomal fraction is maximally active at pH 6.8 and requires 0.1% (w/v) Zwittergent 3–16 and 5 mM Mn^{2+}. Chemical and enzymatic analyses of fucosylated AraGalGal-PA confirmed the attachment of L-Fuc to the L-arabinofuranosyl (L-Ara*f*) residue at *O*-2 by α-glycosidic linkage. Radiolabeling was used to assay L-Fuc transfer to L-Ara*f*-containing galacto-oligomers and tamarind xyloglucan. The enzyme specific for the L-Ara*f* residue undergoes development- and organ-specific expression in root tissue, whereas the L-Fuc transfer to tamarind xyloglucan can be detected in microsomal fractions from various organs in developing radish plants. Enzyme assays of membranes fractionated from microsomal fractions revealed that two distinct α-L-fucosyltransferases with different acceptor specificity are associated with Golgi membranes from primary roots, whereas hypocotyl Golgi membranes completely lack the enzyme specific for the L-Ara*f* residue.

Organ-specific L-Fuc-containing AGPs were isolated from mature leaves and primary roots of 6-d-old seedlings of the radish plant (*Raphanus sativus* L.) and shown to be potent blood group O(H)-like substances that inhibit hemagglutination of human O(H) erythrocytes by eel anti-H agglutinin. (Nakamura et al., 1984; Tsumuraya et al., 1988). Structural analyses of these AGPs demonstrated that this serological activity is due to α-L-Fuc residues attached at *O*-2 to α-L-Ara*f* residues, which constitute the side chains of these AGPs (Tsumuraya et al., 1984, 1988). These findings suggest that a specific α-L-FTase(s) capable of catalyzing L-Fuc transfer to *O*-2 of the α-L-Ara*f* residue in the side chains of the AGPs may play a role in the serological activity.

In this paper, we report the occurrence of a novel α-L-FTase that catalyzes L-Fuc transfer from GDP-L-Fuc to *O*-2 of the α-L-Ara*f* residue in the microsomal fraction made from primary roots of 6-d-old radish seedlings. We used the fluorometric method with AraGalGal-PA as the acceptor. Using fluorometric and radiolabeling assays, α-L-FTases in radish primary root and hypocotyl microsomes were examined for their pH optima, metal ion and detergent requirements, acceptor specificities, and kinetic parameters. Furthermore, we investigated the variation of α-L-FTase activity using AraGalGal-PA and tamarind XG as acceptors during development of organs and their subcellular localization in primary roots and hypocotyls. Several lines of evidence are presented to support the hypothesis that there are two different α-L-FTases present in primary root Golgi membranes, which are distinguishable by their acceptor specificities.

MATERIALS AND METHODS

Materials

Seeds of radish (*Raphanus sativus* L. var *hortensis* cv Aokubi) were purchased from Tokita Seed and Plant (Saitama, Japan) and were surface sterilized successively with 70% ethanol (1 min), 0.1 M $HgCl_2$ (10 min), and calcium hypochlorite (10 min), respectively. After each step, seeds were washed with sterilized water for 10 min, allowed to imbibe on nylon grids in plastic trays filled with tap water, and allowed to germinate at 25°C in the darkness for 2 d. Seedlings were then grown under continuous illumination for 4 d and harvested. Sterilized seeds also were germinated and grown on nylon net in sterilized water containing 0.01 mg mL^{-1} penicillin G (P-L Biochemicals, Milwaukee, WI) in autoclaved culture vessels (Agripots; Kirin Brewing, Tokyo, Japan) under conditions as above.

Klebsiella pneumoniae ATCC 12658 was purchased from American Type Culture Collection (Rockville, MD).

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Abbreviations: AGP, arabinogalactan-protein; α-L-Ara*f*, α-L-arabinofuranoside; α-L-Arafase, α-L-arabinofuranosidase; α-L-Ara*f*-2-α-L-FTase, GDP-L-Fuc:α-L-arabinofuranoside 2-α-L-fucosyltransferase; AraGalGal, L-arabinofuranosyl*f*α(1→3)D-galactopyranosylβ(1→6)D-galactopyranose; AraGalGal-PA, a pyridylamino derivative of AraGalGal; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; α-L-FTase, α-L-fucosyltransferase; FucAraGalGal-PA, a pyridylamino derivative of L-fucopyranosylα(1→2)L-arabinofuranosyl*f*α(1→3)D-galactopyranosylβ(1→6)D-galactopyranose; β-Galase, β-galactosidase; β-D-Gal (XG)-2-α-L-FTase, GDP-L-Fuc:β-D-galactoside (xyloglucan) 2-α-L-fucosyltransferase; GPC, gel permeation chromatography; LSIMS, liquid secondary ion MS; 4-Me-GlcUA, 4-*O*-methyl-GlcUA; 4-*O*-Me-GlcUA(Ara)GalGal, 4-*O*-methyl-D-GlcUAβ(1→6)[L-Ara*f*α(1→3)]D-Galβ(1→6)D-Gal; PA-, pyridylamino-; PC, paper chromatography; PDase, phosphodiesterase; PNP, *p*-nitrophenyl; RP-HPLC, reverse-phase HPLC; XG, xyloglucan.

Chemicals and Enzymes

5′-GMP(2Na), 5′-GDP(2Na), Triton X-102, PNP-α-L-Araf, PNP-α-L-arabinopyranoside, PNP-β-L-arabinopyranoside, PNP-β-D-galactoside, PNP-α-L-fucoside, bis-PNP-P, PNP-β-D-glucuronide, acacia gum, larch wood arabinogalactan, and *Escherichia coli* β-Galase (EC 3.2.1.23) (grade VIII) were from Sigma. GDP-Man and Driselase were from Kyowa Hakkou (Tokyo, Japan). GDP-L-Fuc and *Fusarium oxysporum* α-L-fucosidase (EC 3.2.1.51; Yano et al., 1985) were from Seikagaku Corp. (Tokyo, Japan). GDP-[U-^{14}C]L-Fuc (260 mCi mmol^{-1}) was from DuPont NEN. D-Glucono-(1→5)-lactone, 2-aminopyridine, and Phosphor C-Test were from Wako Pure Chemical Industries (Osaka, Japan). D-Galacto-(1→4)-lactone and Nonidet P-40 were from Nakalei Tesque (Kyoto, Japan). Chaps was from Dojindo Laboratories (Kumamoto, Japan). Zwittergent 3–16 and Cellulysin were from Calbiochem. L-Arabino-(1→4)-lactone was from Koch-Light Laboratories (Colnbrook, Berkshire, UK). Other chemicals used were of reagent grade.

β-1,6-Galactobiose was prepared as described by Sekimata et al. (1989). AGPs from radish leaves, seeds, and roots were prepared according to the method of Tsumuraya et al. (1984, 1987, 1988). XG was purified from tamarind powder (Glyloid 3S, a gift from Dainippon Seiyaku, Tokyo, Japan) by the method of Kooiman (1961). Sugar beet arabinan was prepared by the method of Tagawa and Kaji (1969). XG oligosaccharides (Glc_4Xyl_3Gal [7 mg] and $Glc_4Xyl_3Gal_2$ [11 mg]) were isolated from an endo β-1,4-glucanase (EC 3.2.1.4; Cellulysin) digest of tamarind XG (50 mg) by GPC on a Bio-Gel P-2 (<400 mesh) column and by TLC (Fanutti et al., 1991; Maclachlan et al., 1992). GDP-Man was prepared by the method of Tochikura et al. (1969). GDP-L-Fuc was prepared from GDP-Man by the enzymatic method of Yamamoto et al. (1984) using the extract of *K. pneumoniae* ATCC 12658. Quantities of GDP-L-Fuc were calculated using the molar extinction of GDP at 253 nm (= 13.7×10^{-3}).

Irpex lacteus exo-β-1,3-galactanase was purified from Driselase (Tsumuraya et al., 1990). An α-L-Arafase was purified from the culture fluids of *Rhodotorula flava* (Uesaka et al., 1978).

Preparation of AraGalGal-PA

The Smith degradation product (3.72 g) of acacia gum was digested with *I. lacteus* exo-β-1,3-galactanase (73.5 units) in 50 mM acetate buffer, pH 4.6 (total volume, 210 mL), at 37°C for 24 h. After DEAE-cellulose (Brown, Berlin, NH; HCO_3^-) column chromatography, AraGalGal in the neutral sugar fraction was purified by GPC on Bio-Gel P-2, digestion with *E. coli* β-Galase (60 units) at 37°C for 12 h, and PC using Whatman 3MM paper with 1-butanol:pyridine:water (6:4:3, v/v/v) as the solvent system. AraGalGal (158 mg) was obtained, and its structure was confirmed by methylation analysis. AraGalGal was pyridylaminated according to the method of Hase et al. (1984) and subjected to GPC on a 1.7- × 70-cm Sephadex G-15 (Pharmacia Biotech) column, followed by GPC on a 1.5- × 90-cm Toyopearl HW-40F (Tosoh, Tokyo, Japan) column. The columns were equilibrated and eluted with 10 mM ammonium acetate, pH 6.0. Pure AraGalGal-PA (70 μmol) was obtained from the pooled fraction by RP-HPLC on Cosmosil 5C_{18}-P and determined to be composed of Ara and Gal in a molar ratio of 1.03 and 1.00 by GLC. Similarly, β-1,6-galactobiose was pyridylaminated and characterized as above.

Preparation of L-Araf-Containing Galacto-Oligomers

Radish root AGP (120 mg) was digested with *I. lacteus* exo-β-(1→3)-galactanase (3.6 units) at 37°C for 20 h and subjected to GPC on a 2.4- × 42-cm Sephadex G-100 column pre-equilibrated with 1% (v/v) acetic acid. The resultant galacto-oligomers were separated into neutral and acidic fractions by chromatography on a 2.1- × 15-cm DEAE-cellulose (HCO_3^-) column. The neutral sugars were eluted with water (300 mL) and then the acidic sugars were eluted from the column with 50 mM $NaHCO_3$ (total volume, 200 mL). Each sugar fraction was fractionated further separately on a 2- × 105-cm Bio-Gel P-2 column pre-equilibrated with 1% acetic acid. Fractions (1.8 mL) were collected at a flow rate of 0.12 mL min^{-1} and monitored for sugar by the phenol sulfuric acid method. The degree of polymerization of the sugar in the pooled fraction was estimated by GPC on a Bio-Gel P-2 column that had been calibrated using the elution volumes of pullulan P-50 (void volume), D-Gal (total volume), and β-1,6-linked galacto-oligomers with degrees of polymerization of 2, 3, and 4 as the standards.

Preparation of 4-*O*-Me-GlcUA(Ara)GalGal

Radish root AGP (161 mg; sugar content as D-Gal, 96.36 mg) was digested with *I. lacteus* exo-β-1,3-galactanase (4.8 units) in 50 mM acetate buffer, pH 4.6 (total volume, 5 mL), at 37°C for 24 h. 4-*O*-Me-GlcUA(Ara)GalGal in the acidic sugar fraction was purified by GPC on a 2- × 105-cm Bio-Gel P-2 column and PC using Whatman 3MM papers with 1-butanol:acetic acid:water (5:2:3, v/v/v) as the solvent system. The purified sugar (7.67 mg as D-Gal) was found to be equivalent to 11.1 mg when total sugar content was estimated using a mixture containing D-GlcUA, L-Ara, and D-Gal in a molar ratio of 1:1:2 as the standard by the phenol sulfuric acid method. This value was used to calculate the concentration of each sugar.

Analytical Methods

Colorimetric analysis of total sugar was done with D-Gal as the standard (Dubois et al., 1956). The phloroglucinol-HCl method was used for the determination of pentose with L-Ara as the standard (Dische and Borenfreund, 1957). Uronic acid was determined using D-GlcUA as the standard according to the method of Galambos (1967). The thioglycolic acid method of Gibbons (1955) was used for the determination of L-Fuc. Protein determination was done by a dye-binding method of Bradford (1976) using BSA as the standard. Alditol acetates were analyzed in a GC-6A gas chromatograph (Shimadzu, Kyoto, Japan) following the procedure of Albersheim et al. (1967). Methylation analysis of oligosaccharides was performed as re-

ported previously (Tsumuraya et al., 1984). The quantities of methylated sugars were corrected by the method of Sweet et al. (1975). GC-MS analysis was conducted in a MAT TSQ70 mass spectrometer (Finnigan, Bremen, Germany) equipped with a 4300 gas chromatograph (Varian Harbor City, CA) fitted with a column (0.25 mm × 30 m) of DB-5 fused silica capillary (J & W Scientific, Folsom, CA), with the temperature increasing from 180 to 270°C (4°C min^{-1}). LSIMS was performed using the same mass spectrometer. The sample was dissolved in 0.5 μL of glycerol: thioglycerol (1:1, v/v) as matrix. Primary beam Cs^+ was accelerated using a potential of 20 kV.

Preparation of Microsomal Fraction

A procedure of Farkas and Maclachlan (1988) was modified to prepare the microsomal fraction. All operations were done at 4°C or below. For instance, primary roots (20–30 g total fresh weights; average fresh weights 25 mg; average length 10 cm) from 6-d-old radish seedlings were excised and cut into small pieces with a razor blade and homogenized with 2 times their weight of 0.1 M Hepes-KOH buffer, pH 6.8, containing 1 mM EDTA, 1 mM DTT, and 0.4 M Suc (buffer A) in an ice-chilled mortar. The homogenate was filtered through four layers of nylon mesh, and the filtrate was centrifuged at 6,000*g* for 10 min. The supernatant was recentrifuged at 100,000*g* for 1 h. The resulting pellets were rinsed with cold water four times and suspended in buffer A (2 mL). Thus, a microsomal fraction containing 0.8 to 1.2 mg of protein was obtained per 2 g of primary roots and used for the enzyme assays immediately after preparation. We found that the microsomes could be stored without measurable loss of activity at −80°C for 2 to 3 months but repeated freezing and thawing resulted in about 20% inactivation of the enzyme.

Hypocotyl microsomes were prepared from whole hypocotyls of 6-d-old radish seedlings by the method described above.

Enzyme Assays

The fluorometric assay was carried out in a mixture (total volume, 30 μL) composed of 40 mM Hepes-KOH buffer, pH 6.8, 1 mM AraGalGal-PA, 2 mM GDP-L-Fuc, 0.4 mM DTT, 160 mM Suc, 5 mM $MnCl_2$, 0.1% (w/v) Zwittergent 3–16, and microsomal fraction (protein content, 100–200 μg) at 25°C for 20 min. After the reaction was terminated with 0.1 M acetic acid (300 μL), the mixture was filtered through a Millipore membrane (pore size, 0.45 μm), and aliquots (5 μL) of the filtrate were applied to RP-HPLC on a 4.6- × 150-mm Cosmosil $5C_{18}$-P column (Nakalei Tesque) using a Shimadzu LC-10A system. PA-sugars were eluted isocratically with 100 mM ammonium acetate, pH 4.5, containing 0.025% (v/v) 1-butanol at a flow rate of 1.5 mL min^{-1}, and fluorescence in the effluents was monitored at 320 nm (excitation) and 400 nm (emission). PA-sugars are clearly discernible because of their retention times: β-1,6-linked galactobiose-PA, 3.4 min; AraGalGal-PA, 5.0 min; and Fuc-AraGalGal-PA, 12.9 min. The amounts of PA-sugars were calculated based on a calibration curve prepared using 2-aminopyridine as the standard. One sample of PA-sugars could be analyzed within 20 min. The increasing peak areas of fucosylated AraGalGal-PA (>10 pmol) were recorded in time intervals, and 1 unit of the enzyme is capable of transferring 1 pmol L-Fuc min^{-1} mg^{-1} protein. Control assays with no added GDP-L-Fuc were run in parallel under the same conditions.

L-Fuc transfer to neutral and acidic L-Ara*f*-containing galacto-oligosaccharides was assayed in a reaction mixture (total volume, 30 μL) containing 15 nmol of GDP-L-Fuc, 76 pmol of GDP-[^{14}C]L-Fuc (0.02 μCi), and an acceptor under the same conditions as above. The reaction was stopped by addition of cold water (1 mL), and labeled neutral sugars were desalted by passage through a Dowex (Dow Chemical, Midland, MI) 1 × 2 column (Cl^-, 1 mL) in a Pasteur pipette. Aliquots (1 mL) of the water eluates (total volume, 3 mL) were collected directly in three scintillation vials (Ponyl Vial, Packard) and mixed with the scintillation cocktail (3 mL, ACS II, Amersham). The radioactivity (dpm) of the labeled product was measured with a Tri-Carb 1600 TR liquid scintillation counter (Packard). The labeled acidic sugars on a DEAE-cellulose column (1 mL) in a Pasteur pipette were desorbed with 20 mM $NaHCO_3$ after washing with cold water. The column eluents (three, 1 mL each) were collected, and the radioactivity (dpm) of the labeled product was measured as above.

Incorporation of L-Fuc to L-Ara*f*-containing polysaccharides was measured by radiolabeling. The reaction was initiated by addition of the donor and stopped with 0.3 M acetic acid (70 μL). The labeled product was separated by PC on Whatman 3MM paper (1.5 × 23 cm) using 95% ethanol:1 M NH_4OH (2:1, v/v) as the solvent system. After ascending development for 6 to 8 h at room temperature, a 2-cm-wide paper strip containing transfer product that was immobile on the base line was cut off and inserted in a scintillation vial containing water (2 mL) and a scintillation cocktail (3 mL), and the radioactivity (dpm) of fucosylated product was counted as above.

Fucosylation of XG was assayed in a reaction mixture (30 μL) containing 0.5 mM GDP-L-Fuc containing GDP-L-[^{14}C]L-Fuc (0.02 μCi), tamarind XG (1 mg mL^{-1}), 40 mM Hepes-KOH buffer, pH 6.5 or 6.8, 0.4 mM DTT, 160 mM Suc, 5 mM $MnCl_2$. After PC, the radioactivity of labeled XG was measured. Control assays without acceptors were done to correct for the radioactivity due to L-Fuc transfer to endogenous acceptor as well as for nonenzymatic degradation of GDP-[^{14}C]L-Fuc.

Activities of PDase, α-L-Arafase (EC 3.2.1.55), β-Galase, α-L-fucosidase, and GUS were assayed in a mixture (30 μL) composed of 40 mM Hepes-KOH buffer, pH 6.8, the corresponding *p*-nitrophenol derivative (1 mM), 0.4 mM DTT, 160 mM Suc, 5 mM $MnCl_2$, 0.1% (w/v) Zwittergent 3–16, and microsomal fraction (protein content, 100 μg) at 25°C. After a suitable time, the reaction was stopped by addition of 0.2 M Na_2CO_3 (970 μL), and the mixture was centrifuged at 15,000 rpm for 5 min. A_{420} in the supernatant was measured, and 1 unit of these enzymes was defined as the amount of enzyme capable of liberating 1 pmol *p*-nitrophenol min^{-1} mg^{-1} protein.

Latent IDPase activity was assayed by the methods of Kuribayashi et al. (1992) and Mitsui et al. (1990). ATPase activity was assayed according to the method of Kuribayashi et al. (1992) and Mandala and Taiz (1985). The amounts of Pi released were determined using the Phosphor C-Test, and 1 unit of activity was defined as the amount of enzyme capable of releasing 1 nmol Pi min^{-1} mL^{-1}. Nitrate- and vanadate-sensitive ATPases were assayed in the presence or absence of 50 mM $NaNO_3$ and 200 μM Na_3VO_4, respectively. Cyt *c* oxidase activity was measured using equine heart Cyt *c* according to the method of Hodges and Leonard (1974). Antimycin A-insensitive NADH-Cyt *c* reductase activity was assayed by the method of Bowles and Kauss (1976).

Isolation of Transfer Product

A reaction mixture (total volume, 3 mL) consisting of 40 mM Hepes-KOH buffer, pH 6.8, 3 μM AraGalGal-PA, 3 μM GDP-L-Fuc, 0.4 mM DTT, 160 mM Suc, 5 mM $MnCl_2$, and 0.1% (w/v) Zwittergent 3–16 was incubated with the primary root microsomal fraction (protein content, 10 mg) at 25°C for 48 h, boiled for 3 min, and filtered through a 0.45-μm membrane (Millipore filter). The transfer product (FucAraGalGal-PA) was purified by GPC on Toyopearl HW-40F, followed by RP-HPLC on Cosmosil $5C_{18}$-P, and lyophilized (yield, 1.14 μmol).

Suc Density Gradient Centrifugation

Primary root and hypocotyl microsomal fractions were diluted with 0.1 M Hepes-KOH buffer, pH 6.8, containing 1 mM EDTA and 1 mM DTT to give a density equivalent to approximately 13.5% (w/w) Suc, layered over a linear 15 to 50% (w/w) Suc gradient in buffer A, and centrifuged to equilibrium at 100,000*g* using an RPS 25–2 rotor with a 55P-72 ultracentrifuge (Hitachi, Tokyo, Japan) for 15 h. Fractions (1 mL) were collected from the bottom of the tubes and monitored for α-L-FTase and marker enzyme activities. The refractive indexes of fractions were measured with a model 1T refractometer (Atago, Tokyo, Japan).

The pellet from each membrane fraction was suspended in 2% (w/v) glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.0, and fixed overnight at 4°C. After centrifugation and rinsing with 50 mM potassium phosphate buffer, pH 7.0, for 10 min, the fixed membranes were postfixed, embedded, and stained, as described previously (Kikuchi et al., 1993). Thin sections were viewed with an Hitachi 7000H electron microscope at an accelerating voltage of 100 kV.

Serological Methods

Organs (>1 g fresh weight) were homogenized with 5 times their weights of PBS (0.145 M phosphate buffer, pH 7.2, containing 0.13 M NaCl) in an ice-chilled mortar. The homogenates were heated at 100°C for 30 min, cooled, and centrifuged at 12,000*g* for 10 min. The supernatants were used for the serological test.

The blood group H activity was assayed by the hemagglutination inhibition technique using serial 2-fold dilutions (25 μL) of H-active materials in 0.85% NaCl (saline) plus a constant amount (25 μL) of prediluted (titer 1:4) eel anti-H agglutinin. After the reaction mixtures were allowed to stand for 2 h at 4°C, the hemagglutination inhibition activity was determined by adding 2% human O erythrocytes in saline solution (25 μL). The activity was expressed as the minimum concentration (μg mL^{-1}) of the material causing observable inhibition of hemagglutination or as the reciprocal, expressed as 2^n, of the lowest dilution (*n*) to inhibit hemagglutination under these conditions; standard L-Fuc required about 200 μg mL^{-1} as the minimum concentration causing hemagglutination inhibition (Nakamura et al., 1984).

RESULTS

α-L-FTase Assays

L-Fuc transfer to AraGalGal-PA in the standard assay with primary root microsomes was traced by recording the increases in fluorescence peaks of the transfer product, which is clearly separated from the acceptor on RP-HPLC. The data obtained indicate that the reaction proceeds linearly up to 3 h. Thus, the routine enzyme assay was conducted at reaction times between 20 and 60 min. The enzyme activity was found to increase proportionally depending on the amounts of protein from 20 to 150 μg, when assayed using primary root microsomes containing 20 to 230 μg of protein after 20 min of incubation at 25°C. A pH-activity curve was obtained using cacodylate, pH 4.5 to 7.1, and Hepes-KOH, pH 6.2 to 7.8, at 20 mM, indicating a pH optimum of 6.8 for L-Fuc transfer to either AraGalGal-PA or tamarind XG with primary root microsomes, whereas hypocotyl microsomal β-D-Gal (XG)-2-α-L-FTase is maximally active at pH 6.5. The same data for the α-L-FTase activities were obtained with a microsomal fraction from primary roots from 6-d-old radish seedlings grown in sterilized Agripots to exclude the possibility of contamination by microorganisms.

The residual activities of primary root α-L-Ara*f*-2-α-L-FTase after 1 h of incubation at various temperatures (15, 20, 25, 30, 37, and 45°C) indicate that the enzyme has a maximal activity at 25°C and is inactivated rapidly above 30°C. Of divalent metal ions tested at 5 mM, Mn^{2+} and Mg^{2+} are effective in causing 2.3- and 1.6-fold increases in enzyme activity, respectively, whereas the enzyme was inactivated completely by Hg^{2+} and Cu^{2+} and inhibited 80 and 70% by Zn^{2+} and Fe^{3+}, respectively. BSA was omitted from the reaction mixture because it has a small stimulating effect within a concentration range of 0.1 to 10 μg mL^{-1}.

Phosphodiesterase (782 pmol min^{-1} mg^{-1} protein) and β-Galase (291 pmol min^{-1} mg^{-1} protein) activities were detected in radish primary root microsomal fraction at pH 6.8. Sodium fluoride (20 mM) and D-galactono-(1→4)-lactone (50 mM) were effective in reducing PDase and β-Galase activities to less than 50%, respectively. However, these inhibitors had no stimulatory effect on the L-Fuc

transfer reaction and thus were omitted from the assay system. Activities of α-L-Arafase, α-L-fucosidase, and GUS were negligible (<30 pmol min^{-1} mg^{-1} protein) even after incubation for 60 min.

Effects of Detergents on α-L-FTase Activities

Figure 1A shows the effects of various detergents (final concentration, 0.1%, w/v) on α-L-FTase activity in primary root microsomes. Obviously, the detergents added to the reaction mixture enhanced L-Fuc transfer to AraGalGal-PA, except for SDS, with which about 50% of the enzyme was inactivated. In particular, Zwittergent 3–16, Nonidet P-40, Triton X-102, and Triton X-100 were effective in causing approximately 2-fold increases in enzyme activity within concentrations of 0.1 to 0.5% (w/v). Zwittergent 3–16 was used for the standard enzyme assay.

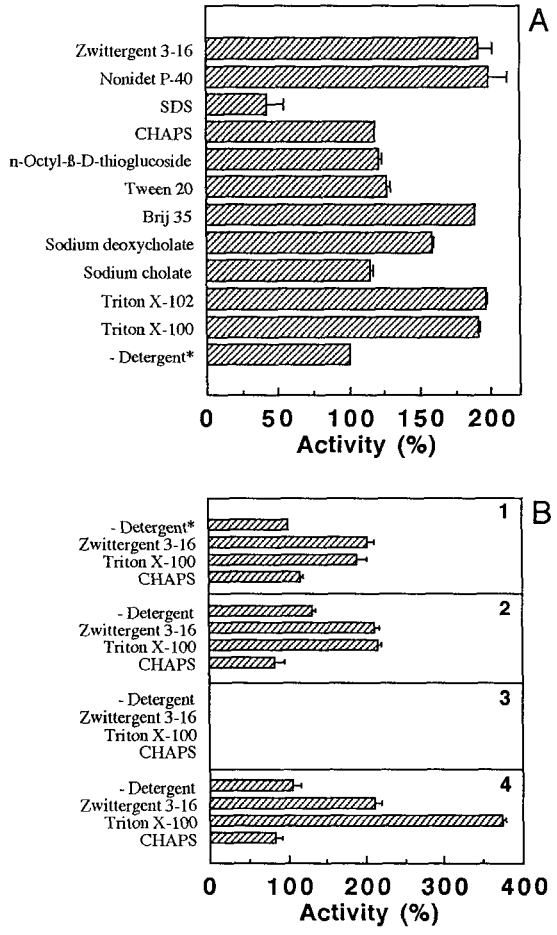

Figure 1. Effects of detergents on α-L-FTase activities. A, Effects of various detergents on α-L-FTase activity (AraGalGal-PA as the acceptor) in radish primary root microsomes. B, Comparison of α-L-FTase activities in the microsomal fractions from radish primary roots and hypocotyls in the presence of three detergents. Panels 1 and 3, α-L-FTase activities (AraGalGal-PA as the acceptor) in primary root and hypocotyl microsomes; panels 2 and 4, α-L-FTase activities (tamarind XG as the acceptor) in primary root and hypocotyl microsomes. α-L-FTase activities were assayed in a standard reaction mixture containing 0.1% (w/v) detergent and expressed as relative activities (percentages), taking those obtained without added detergent as unity (100%, − Detergent*). Error bars represent the range of triplicate determinations.

Figure 1B shows a comparison of the effects of three detergents on α-L-FTase activities in primary root and hypocotyl microsomal fractions from 6-d-old radish seedlings. Chaps had no activating effect on primary root microsomal α-L-FTases involved in the fucosylation of AraGalGal-PA and tamarind XG. In contrast, Triton X-100 and Zwittergent 3–16 caused 1.5- to 2-fold increases in both enzyme activities. Furthermore, it became evident that both Zwittergent 3–16 and Triton X-100 were able to increase L-Fuc transfer to tamarind XG 2- and 3.4-fold in hypocotyl microsomes, respectively, whereas as with Chaps, no measurable fucosylation AraGalGal-PA could be detected with these detergents.

Effects of Various Compounds

L-Fucosyl transfer to AraGalGal-PA was assayed in the standard reaction mixtures to which various compounds were added. GMP and GDP inhibit enzyme activity completely at 5 and 50 mM, respectively. Other nucleotides such as ADP, AMP, UDP, and UMP exhibited little inhibitory effect even at 50 mM. The enzyme was completely inactivated by 10 mM EDTA, and PMSF caused 40% inhibition at 1 mM. EDTA-inactivated microsomes no longer regained activity when 20 mM Mn^{2+} was added afterward. D-Xyl, L-Fuc, L-Fuc-1-P, D-galactono-(1→4)-lactone, D-glucono-(1→5)-lactone, and L-arabino-(1→5)-lactone had no effect on the enzyme at 10 mM.

Acceptor Specificity and Kinetics

Relative activities for the acceptors were AraGalGal-PA, 100; AraGalGal, 85.5; and 4-O-Me-GlcUA(Ara)GalGal, 114.6. Neutral and acidic L-Ara*f*-containing galacto-oligomers (degree of polymerization 3–8) were found to act as L-Fuc acceptors with efficiencies comparable to that of AraGalGal-PA. D-Ara, L-Ara, PNP-α-L-arabinofuranoside, and PNP-α- and -β-L-arabinopyranoside were totally inactive as acceptors. L-Fuc incorporation to the following polysaccharides was negligible: acacia gum and its Smith degradation products, radish leaf AGP (fucosylated), larch wood arabinogalactan, and sugar beet arabinan. Nonfucosylated radish root and seed AGPs (2.2 and 3.5% incorporation) were slightly labeled after 20 h of incubation with primary root microsomes. Exogenous tamarind XG served as a good acceptor: 66.3% of L-Fuc was incorporated after 20 min of incubation with primary root microsomes. The microsomal α-L-FTase catalyzing fucosylation of XG requires 5 mM Mn^{2+} and 0.1% (w/v) Zwittergent 3–16 or Triton X-100 for maximal activity. XG oligosaccharides (Glc_4Xyl_3Gal and $Glc_4Xyl_3Gal_2$) at 5 mg mL^{-1} were not acceptors (1 and 2.6%, respectively, compared to AraGalGal-PA after 20 min of incubation) but caused about 20% inhibition of β-D-Gal (XG)-2-α-L-FTase activity in primary root microsomes.

Apparent K_m and V_{max} values for GDP-L-Fuc and acceptors were calculated according to the Hanes-Woolf plot

(Howard et al., 1987) of the saturation data obtained by the fluorometric or radiolabeling method after 20 min of reaction using a microsomal fraction containing 100 μg of protein. In Table I are summarized the kinetic parameters of L-Fuc transfer to L-Ara*f*-containing tri- and tetrasaccharides and tamarind XG. The K_m values for L-Ara*f*-containing acceptors are of a similar order of magnitude but tend to decrease with increasing molecular size. The concentrations of tamarind XG that resulted in half-maximal initial velocity ($V_{max}/2$) were calculated to be 0.68 and 0.65 mg mL^{-1} with primary root and hypocotyl microsomes, respectively.

Structure of the Transfer Product

The transfer product was unsusceptible to *R. flava* α-L-Arafase, but the peak completely disappeared upon incubation with *F. oxysporum* α-L-fucosidase, yielding a PA-sugar eluting at the same position as the acceptor. This provides solid evidence for an α-anomeric configuration of the L-fucosidic linkage. Subsequent action of α-L-Arafase on the defucosylated product gave rise to a peak that corresponded to that of β-1,6-linked galactobiose-PA.

GLC and GC-MS analyses of methylated sugars derived from the permethylated transfer product confirmed the formation of 2,3,4-tri-*O*-methyl L-Fuc, 3,5-di-*O*-methyl L-Ara, and 2,4,6-tri-*O*-methyl D-Gal in a molar ratio of 0.94:1.00:0.89, indicating that the L-Fuc residue was linked to the L-Ara*f* residue at *O*-2. On LSIMS analysis this product afforded $(M + H)^+$ and low intense $(M + Na)^+$ ions at *m/z* 699 and 721, respectively, consistent with the predicted values calculated for the PA-sugar (M_r = 698). A series of fragment ions at *m/z* 553, 419, and 259 confirmed that the sugar sequence, Fuc→Ara→Gal→Gal, is valid. From these data, the product was identified as FucAraGalGal-PA.

The transfer product inhibited hemagglutination of human O erythrocytes by eel anti-H agglutinin (titer 1:2) at 170 μg mL^{-1} (based on the amount of L-Fuc), the value being comparable to those of L-Fuc (375 μg mL^{-1}) and PNP-α-L-Fuc (61.6 μg mL^{-1}).

Table I. *Kinetic parameters of α-L-FTase in L-Fuc transfer to L-Ara*f*-containing acceptors and tamarind XG*

Initial rates were estimated after 20 min of reaction under standard assay conditions.

Donor or Acceptor	K_m	V_{max}
Donors[a]	mM	pmol min^{-1} mg^{-1} protein
GDP-L-Fuc[b,d] (AraGalGal-PA)	0.17	1130
GDP-L-Fuc[b] (tamarind XG)	0.043	212
GDP-L-Fuc[c] (tamarind XG)	0.052	157
Acceptors		
AraGalGal-PA[b,d]	3.70	855
AraGalGal[b]	16.3	1380
4-*O*-Me-GlcUA(Ara)GalGal[b]	2.47	458

[a] Acceptor used for the reaction is shown in the parentheses. [b] Primary root microsomes. [c] Hypocotyl microsomes. [d] Determined by fluorometric method.

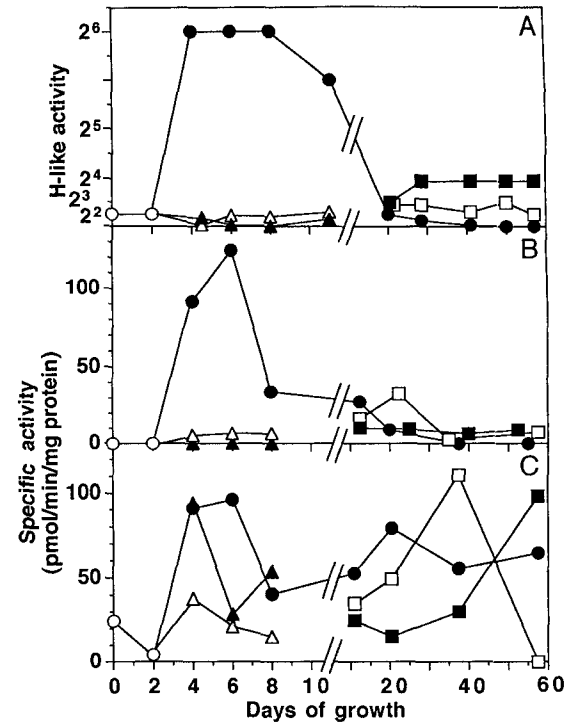

Figure 2. Variations of α-L-FTase activities and blood group H-like activity in developing organs of radish plant. A, Blood group H-like activity in the extracts from various organs. Inhibitory activity was expressed as 2^n of the lowest dilution to inhibit hemagglutination. B, α-L-FTase activity (AraGalGal-PA as the acceptor). The activity was fluorometrically measured for microsomal membranes prepared from organs. C, α-L-FTase activity (tamarind XG as the acceptor). Radiolabeling was used to assay the enzyme activity in microsomal membranes from organs. ○, Seeds and 2-d-old seedlings; ●, roots; △, cotyledons; ▲, hypocotyls; □, midribs; ■, leaves.

Variation of α-L-FTase Activities in Developing Organs

Figure 2 shows the variation of α-L-FTase activities. These were determined using AraGalGal-PA and tamarind XG as acceptors in microsomal fractions prepared from developing organs of the radish plant after germination. It is evident that α-L-Ara*f*-2-α-L-FTase activity in microsomes from growing roots increased transiently to a maximal level at the initial stage of development and declined rapidly in accord with a parallel decrease of blood group H-like activity (Fig. 2, A and B). Weak but significant α-L-FTase activity using AraGalGal-PA could be detected along with a parallel increase of serological activity in microsomes from developing leaves (Fig. 2, A and B). It is apparent that fucosylation of tamarind XG occurs in elongating regions of stems, roots, and leaves regardless of the growth stages of the plant (Fig. 2C).

Subcellular Localization

Figure 3 shows the distribution of protein, the activities of marker enzymes, and α-L-Ara*f*- and β-D-Gal (XG)-2-α-L-FTase activities in membrane fractions prepared from radish primary roots microsomes by centrifugation on a linear 15 to 50% (w/w) Suc density gradient. Both α-L-FTase

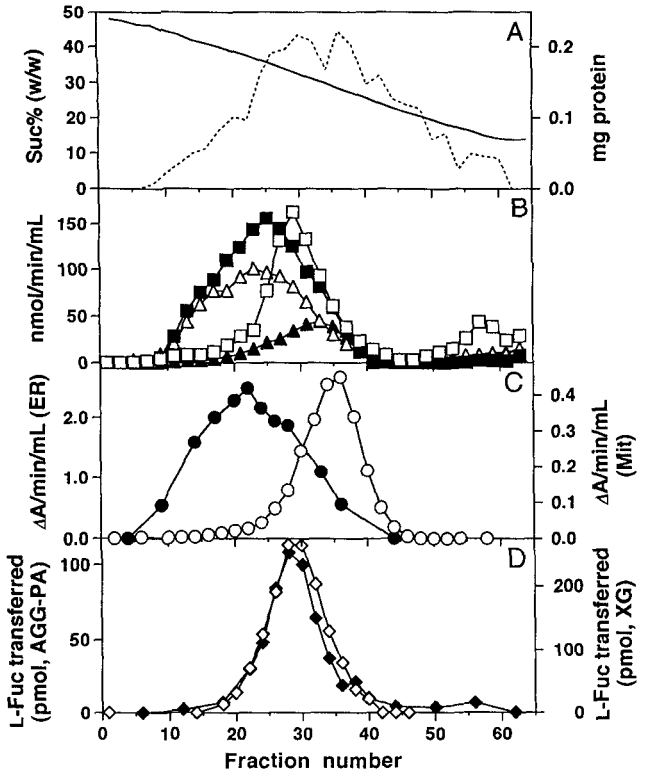

Figure 3. Distribution of α-L-FTases and marker enzymes in radish primary root membranes on Suc density gradients. α-L-Araf-2-α-L-FTase activity was assayed fluorometrically using 1 mM AraGal-Gal-PA as the acceptor. β-D-Gal (XG)-2-α-L-FTase activity was assayed by radiolabeling using 1 mg mL^{-1} tamarind XG as the acceptor. Assays for marker enzymes were conducted by the methods described in "Enzyme Assays." A, Suc gradient (——), expressed as percentage equivalent Suc, and total protein content per tube (·····). B, ■, Total ATPase activity; □, IDPase activity, a marker for Golgi membranes; △, vanadate-sensitive ATPase, a marker for plasma membranes; ▲, nitrate-sensitive ATPase, a marker for tonoplast membrane. C, ●, Cyt *c* oxidase, a marker for mitochondria (Mit); ○, antimycin-insensitive NADH-Cyt *c* reductase, a marker for ER. D, ◇, α-L-Araf-2-α-L-FTase; ◆, β-D-Gal (XG)-2-α-L-FTase.

activities co-distributed in fractions of 30 to 38% (w/w) Suc densities, and their peaks largely overlapped that of latent IDPase, a marker enzyme for Golgi membranes, which was clearly distinguishable from that of NADH-Cyt *c* reductase, a marker of ER (Fig. 3, B–D). A similar distribution of β-D-Gal (XG)-2-α-L-FTase and IDPase activities was observed with hypocotyl membrane fractions, in which α-L-FTase activity with AraGalGal-PA as the acceptor was totally lacking (Fig. 4, B–D). Electron microscopic observation showed that membrane fractions equilibrated at 30 to 38% (w/w) Suc densities with associated IDPase and α-L-FTase activities consisted mostly of smooth membranes and vesicles without ribosomes.

DISCUSSION

A fluorometric assay of α-L-FTase activity was performed using AraGalGal-PA as the acceptor, which represents the minimal unit of a typical side chain structure in radish AGPs (Tsumuraya et al., 1990). This method allowed us to quantify more than 10 pmol of the fucosylated product eluting at predetermined retention times on RP-HPLC. Isolation of sufficient amounts of fucosylated product facilitated its structural analysis by chemical and enzymatic methods, leading to the confirmation of α-L-fucosidic linkage and showing the attachment of L-Fuc to the α-L-Araf residue at *O*-2, which is responsible for the blood group H activity of radish primary root AGP (Tsumuraya et al., 1988). These data and those obtained by radiolabeling using AraGalGal and 4-*O*-Me-GlcUA(Ara)GalGal suggest the occurrence of a discrete α-L-FTase, which we propose to name α-L-Araf-2-α-L-FTase because of its high degree of specificity for *O*-2 L-Araf attached to β-1,6-linked D-Gal in microsomes from radish primary roots.

Detergents such as Zwittergent 3–16, Triton X-100, and Nonidet P-40 are indispensable for the activation of microsomal α-L-FTases in radish primary roots and hypocotyls, as observed for membranous α-L-FTases from other plant tissues (James and Jones, 1979; Camirand et al., 1987). Importantly, no measurable L-Fuc transfer to AraGal-Gal-PA could be detected with radish hypocotyl microsomes even in the presence of detergents. This is true for Golgi membranes that were obtained from radish hypocotyl microsomes by Suc density gradient centrifugation as well. However, it is conceivable that there may be an

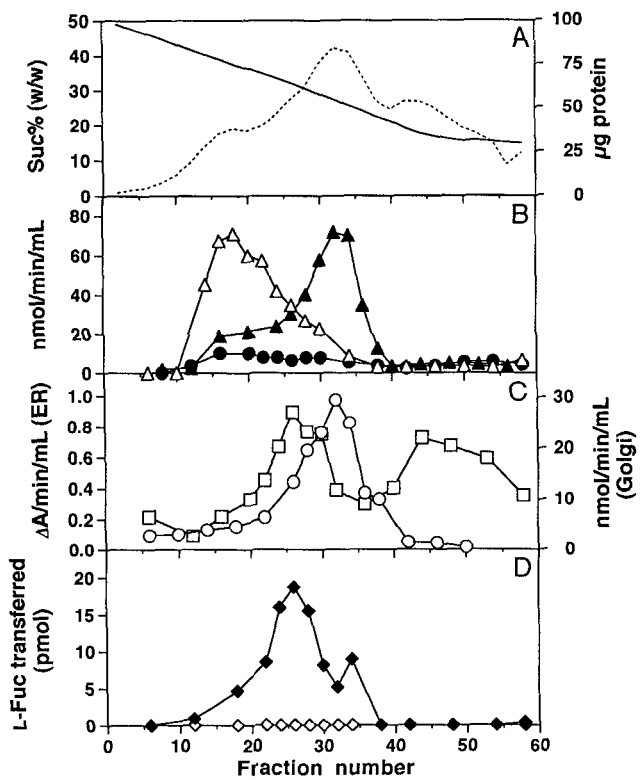

Figure 4. Distribution of α-L-FTases and marker enzymes in radish hypocotyl membranes on Suc density gradients. A, A linear Suc gradient (15–50%, w/w). B, △, Plasma membrane marker enzyme; ▲, tonoplast membrane marker enzyme; ●, mitochondria marker enzyme. C, □, Golgi membrane (Golgi) marker enzyme; ○, ER marker enzyme. D, ◆, β-D-Gal (XG)-2-α-L-FTase; ◇, α-L-Araf-2-α-L-FTase.

inhibitory substance(s) in hypocotyl microsomes, which is able to suppress specifically α-L-Araf-2-α-L-FTase activity. Hence, the enzyme activity was measured for the mixtures of primary root and hypocotyl microsomes (protein content, 100 μg each) under the standard assay conditions. No appreciable change in the L-Fuc transfer reaction was observed when microsomal fractions from the tissues were mixed in the ratios of 1:1 and 1:2, suggesting that no such inhibitor(s) is present (data not shown). These findings present evidence to support the idea that α-L-Araf-2-α-L-FTase is distinguishable from that involved in the fucosylation of tamarind XG. The stimulating effect of Mn^{2+} or Mg^{2+} on α-L-FTase activity and the complete inactivation of the enzyme with EDTA suggest dependence of the enzyme on divalent metal ions. However, Hanna et al. (1991) reported no appreciable activating effect of $MnCl_2$ on α-L-FTase solubilized from pea epicotyl microsomes in L-Fuc transfer to tamarind XG. Thus, the role of these metal ions in the activation of α-L-FTases in radish primary root microsomes is not entirely clear and further studies will be needed to solve this problem. All α-L-FTase activities in primary root and hypocotyl microsomes were found to be inhibited by GDP and GMP, similar to results reported for pea epicotyl enzyme (Hanna et al., 1991).

AraGalGal-PA, 4-*O*-Me-GlcUA(Ara)GalGal, and AraGalGal are good acceptors. The rates and kinetic parameters in the L-Fuc transfer to these sugars appear to be related to their molecular sizes. Attachment of 4-Me-GlcUA to the nonreducing terminal D-Gal residue at *O*-6 of AraGalGal resulted in an increase in the initial rate of the reaction. Furthermore, both acidic and neutral L-Araf-containing galacto-oligomers with higher degrees of polymerization were found to act as acceptors at comparable rates when their relative activities were compared on the basis of pentose content (as L-Ara) in each pooled fraction.

β-D-Gal (XG)-2-α-L-FTase has been detected in the microsomal fraction from various tissues and localized in Golgi membranes in primary roots and hypocotyls. The enzymes in microsomes from these tissues resemble each other closely in kinetic parameters and reaction with XG oligosaccharides, which are inhibitory. The properties of radish enzymes are very similar to those of the enzyme in pea hypocotyl microsomes (Maclachlan et al., 1992). It is interesting that the activating effect of CHAPS on pea enzyme (Hanna et al., 1991) contrasts with the inability of this detergent to enhance radish enzymes.

In this work, a novel α-L-FTase specific for L-Araf residues has been characterized with respect to its properties, specificity distribution in tissues, and subcellular localization. Temporary synthesis of fucosylated AGP can be ascribed to a transient expression of the enzyme in Golgi membranes in radish primary roots.

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