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Expression, Purification and Characterization of Human α-L-Fucosidase

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α-L-Fucosidases (EC 3.2.1.51) are exo-glycosidases. On the basis of the multi-alignment of amino acid sequence, α-L-fucosidases were classified into two families of glycoside hydrolases, GH-29 and GH-95. They are responsible for the removal of L-fucosyl residues from the non-reducing end of glycoconjugates. Deficiency of α-L-fucosidase results in Fucosidosis due to the accumulation of fucose-containing glycolipids, glycoproteins and oligosaccharides in various tissues. Recent studies discovered that the fucosylation levels are increased on the membrane surfaces of many carcinomas, indicating the biological function of α-L-fucosidases may relate to this abnormal cell physiology. Although the gene of human α-L-fucosidase (*h-fuc*) was cloned, the recombinant enzyme has rarely been overexpressed as a soluble and active from. We report herein that, with carefully control on the growing condition, an active human α-L-fucosidases (h-Fuc) was successfully expressed in *Escherichia coli* for the first time. After a series steps of ion-exchange and gel-filtration chromatographic purification, the recombinant h-Fuc with 95% homogeneity was obtained. The molecular weight of the enzyme was analyzed by SDS-PAGE (~50 kDa) and confirmed by ESI mass (50895 Da). The recombinant h-Fuc was stable up to 55 °C with incubation at pH 6.8 for 2 h; the optimum temperature for h-Fuc is approximately 55 °C. The enzyme was stable at pH 2.5-7.0 for 2 h; the enzyme activity decreased greatly for pH greater than 8.0 or less than 2.0. The K_m and k_{cat} values of the recombinant h-Fuc (at pH 6.8) were determined to be 0.28 mM and 17.1 s^{-1}, respectively. The study of pH-dependent activity showed that the recombinant enzyme exhibited optimum activity at two regions near at pH 4.5 and pH 6.5. These features of the recombinant h-Fuc are comparable to the native enzyme purified directly from human liver. Studies on the transfucosylation and common intermediate of the enzymatic reaction by NMR support that h-Fuc functions as a retaining enzyme catalyzing the hydrolysis of substrate via a two-step, double displacement mechanism.

Keywords: Glycoside hydrolase; Human α-L-fucosidase; Stability; Transglycosilationd; Catalytic mechanism.

INTRODUCTION

Oligosaccharides bound to proteins and lipids are present on cell surface and involve in cell-cell interaction. Fucose-containing carbohydrates play important roles in biological events involving in the critical physiological activities as oncogenesis,[1,2] blood coagulation cascade and clot dissolution,[3-5] and host-microbial interactions.[6,7] α-L-Fucosidases (EC 3.2.1.51) are responsible for the removal of L-fucosyl residues from the non-reducing end of glycoconjugates. A severe deficiency of α-L-fucosidase causes fucosidosis that can produce mental and motor retardation.[8] Several pathological conditions including inflammation,[4,9] cancer[10-12] and cystic fibrosis[13,14] are also related to an atypical activity of α-L-fucosidase.

α-L-Fucosidases have been isolated from many sources including microorganisms,[15-20] plants,[21] mollusks,[22-28] and mammals.[29-32] Since α-L-fucosidases can selectively cleave and remove specific fucosyl residues from the targets, they can serve as a powerful tool to elucidate the structure-function relationships of these fucose-containing glycoconjugates. These enzymes have been divided into two groups: one group can hydrolyze various types of fucosidic linkages as well as synthetic substrates (EC 3.2.1.51) while the others are active on the α-(1→2) (EC 3.2.1.63), α-(1→3,4)

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Abbreviations: GH, glycoside hydrolase; pNPF, p-nitrophenyl-α-L-fucopyranoside; CNPF, 2-chloro-4-nitrophenyl-α-L-fucopyranoside; MeF, methyl-α-L-fucopyranoside; pCPF, p-cyanophenyl-α-L-fucopyranoside; pNPbA, p-nitrophenyl-β-D-arabinopyranoside; pNPaA, p-nitrophenyl-α-L-arabinopyranoside; p-NPAF, p-nitrophenyl-α-L-Arabinofuranoside; pNPGal, p-nitrophenyl-β-D-Galactopyranoside; pNPGlc, p-nitrophenyl-β-D-Glucopyranoside; pNPGlcNAc, p-nitrophenyl-β-D-N-acetylglucosaminide.

(EC 3.2.1.111), and α-(1→6) linkages (EC 3.2.1.127) of the L-fucosidic bond of glycoconjugates. Based on the similarity of amino acid sequence, α-L-fucosidases are classified into two glycoside hydrolase families, namely GH-29 and GH-95. More information about these families are available on a permanently updated web site (http://www.cazy.org/fam/acc_GH.html).[33]

Human α-L-fucosidases have been purified from various cells, fluids and tissues; their kinetic, electrophoretic and immunological properties were reported.[34] In general, h-Fuc is a multimeric protein derived from two closely related polypeptide subunits with mass ~50 kDa-60 kDa, and differs in carbohydrate composition and possibly other post-translational modifications.[35-37] Human α-L-fucosidase from blood plasma is a sialoglycoprotein that contains ~7-8% carbohydrate by mass. Although the exact functions of the carbohydrate component are unclear, the stability and kinetic properties of the enzyme, the protein folding, intracellular transport, secretion and generation of multiple molecular forms are suggested to be influenced by the level of glycosylation.[38-40] cDNA clones containing the coding sequence of *h-fuc* were isolated and identified.[41-43] The open reading frame encodes human liver-tissue α-L-fucosidase, containing 461 amino acids with the first 22 amino acids as the signal peptide.[41] The expression of the recombinant enzyme was reported only as a fusion protein forming an inactive inclusion body.[44] We report herein the first successful study that human α-L-fucosidase was expressed as an active form in *E. coli*. The purification and characterization of this important recombinant enzyme was presented and its catalytic mechanism was also discussed.

EXPERIMENTAL SECTION

Materials and Chemicals

Vector pCMV.SPORT6 containing a human tissue α-L-fucosidase gene (*h-fuc*) (NCBI accession number M29877) (Stragene, La Jolla, CA, USA), pET22b(+) vector system (Invitrogen, Carlsbad, CA, USA), *Vent* DNA polymerase (New England Biolabs, Ipswich, MA, USA) for PCR reactions, restriction endonucleases and T4 DNA ligase (Roche Applied Science, Basel, Switzerland), *Escherichia coli* strain JM109 and BL21(DE3) (ECOS, Taipei, Taiwan) served as the host for recombinant plasmids. Oligonucleotides were synthesized by Integrated DNA Technologies (Mission Biotechnology, Taipei, Taiwan), protein marker standards of small molecular mass for electrophoresis (GE Healthcare, Piscataway, NJ, USA). Buffers, p-Nitrophenyl-α-L-fucospyranoside (pNPF), and other chemicals for substrate synthesis were purchased from Aldrich-Sigma Chemical Co. (St. Louis, MO, USA). Columns and gels for protein purification were obtained from GE Healthcare.

Construction of expression vector

Vector pCMV.SPORT6 containing the human α-L-fucosidase gene served as a DNA template for PCR amplification. The upstream (5'-CTGCAGCACATATGGT GCGTCGGGCCCAGCCTC-3') and downstream (5'-GGTACCAAGCTTTCAC TTCACTCCTGTCAGCT-3') primers, containing *Nde*I and *Kpn*I restriction sites as underscored, respectively, were employed to amplify the *h-fuc* gene coding the mature protein. The sequence for the first 22 amino acids (the signal peptide) was omitted from the DNA amplification. DNA was amplified in 25 cycles (1 min at 95 °C, 35 sec at 55 °C, and 2 min at 69 °C) with a final extension of 5 min at 69 °C. The amplified DNA fragment (~1.4 Kb), containing the *h-fuc* gene for mature protein, was purified by using the Viogen kit (Viogen, Mountain View, CA, USA) and digested with *Nde*I and *Kpn*I. The resulting DNA fragment was purified and further inserted into pET22b(+). The inserted h-fuc was fully sequenced to confirm the gene correctness before protein expression.

Protein expression and purification

E. coli BL21 (DE3) cells bearing pET22 b(+)_*h-fuc* plasmid were cultivated overnight in LB medium (1 L, with 10 g trypton, 7.5 g yeast extract and 5.5 g NaCl per liter, pH 6.0, 37 °C). This culture (5 mL) was freshly inoculated into the same medium (1 L) supplemented with ampicillin (final concentration 0.1 mg/mL). When cells were grown to the mid-log phase, recombinant protein was induced on addition of isopropyl thio-β-D-galactoside (IPTG, 2 mM). After 20 h induction, cells were harvested and resuspended in 20 mL sodium acetate buffer (20 mM, pH 5.5) for sonication. The crude proteins in the supernatant were precipitated by the addition of ammonium sulfate to 85% saturation. The precipitant was then resuspended in 10 mL of sodium acetate buffer (20 mM, pH 5.5). The solution was then desalted with a HiTrap desalting column. The filtrate (15 mL) was loaded onto a cation-exchanged column (HiTrap SP, 2.6 × 40 cm) that was pre-equilibrated with sodium acetate buffer (20 mM, pH 5.5). Chromatograhpic purification was performed by eluting (3.0 mL/min) the proteins with acetate buffer and a linear gradient concentration of NaCl

(0-1000 mM, 5 mM/min) (Fig. 1a). The fractions with α-L-fucosidase activity (with NaCl at 450-550 mM) were collected, concentrated (25-fold) and further equilibrated with sodium phosphate buffer (20 mM, pH 6.2) for subsequent chromatography. A sample solution (5 mL) derived from the previous step was loaded onto an anion-exchanged column (HiTrap Q, 1.6 × 20 cm) pre-equilibrated with sodium phosphate buffer (20 mM, pH 6.2). Proteins were eluted (1.2 mL/min) with a phosphate buffer and a linear gradient of concentration of NaCl (0-1000 mM, 6.5 mM/min) (Fig. 1b). Fractions (250-350 mM NaCl) with activity were pooled (5 mL final) and concentrated (10-fold) for further separation. 1 mL of the previous sample solution was loaded onto a size exclusion column (Sephadex_G-75, 1.6 × 20 cm) and eluted (0.7 mL/min) with sodium acetate buffer (20 mM, pH 5.5) containing NaCl (10 mM). Fractions with activity were collected and concentrated 15-fold and kept at 4 °C for further studies.

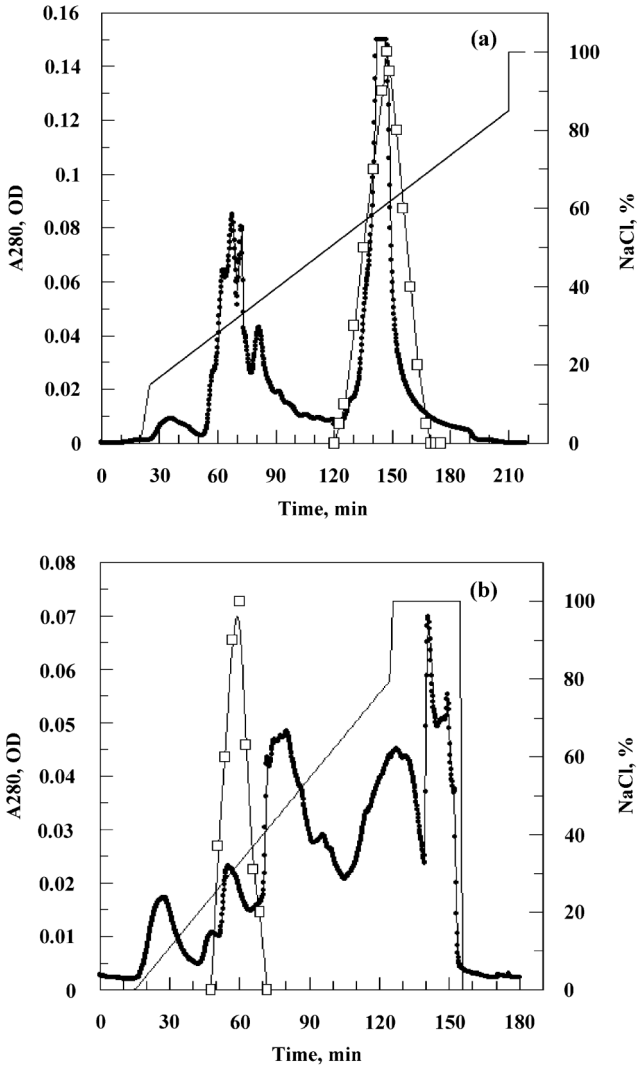

Fig. 1. The chromatograms of the purification of the recombinant h-Fuc. (a) Separation on cation-exchanged column (HiTrap SP) at acetate buffer, pH 5.5. (b) Separation on anion-exchanged column (HiTrap Q) at phosphate buffer, pH 6.2. The aborbance (-•-) was monitored at 280 nm. The fractions with h-Fuc activity (-□-) was eluted with linear gradient of 1 M NaCl (—).

Mass analysis of protein

Protein concentration was determined according to the method of Brandford (Protein Assay; Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standards. The purity and molecular mass of the enzyme was examined by SDS-PAGE.[45] Mass analyses were performed using a quadrupole time-of-flight mass spectrometer (Q-TOF, Micromass, Manchester, UK). The instrument was step to scan with a ratio of mass to charge in the range of 100-2500 units (*m/z*), with a scan of 2 s/step and an interscan duration of 0.1 s/step. In all the ESI-MS experiments, the quadrupole scan mode was used under a capillary needle at 3 kV, a source block temperature of 80 °C, and a desolvation temperature of 150 °C. 100 μl (about 5~10 μg) of purified protein was precipitated by 25% trifluoroacetic acid at 4 °C for 30 min. Centrifugation, the precipitation was washed with acetone twice. The precipitant was further redissolved in 0.1% formic acid for mass analysis.

Enzymatic assays

The h-Fuc activity was determined in sodium acetate buffer (50 mM, pH 5.5, 37 °C) by monitoring (at 348 nm) the hydrolysis of p-nitrophenyl-α-L-fucopyranoside (pNPF, 1 mM) to release p-nitrophenol (or p-nitrophenolate). An enzyme unit was defined as the amount that required for releasing 1 μmol of p-nitrophenol from substrate in 1 min. The pH-dependence of enzyme activity was performed at pH range 2.5-8.0 using pNPF as substrate. Buffers used in this study are sodium acetate (pH 2.5-5.5), morpholinoethanesulfonic acid (pH 5.5-6.5), phosphate (pH 6.5-7.5) and Bicine (pH 7.5-9.0). Enzyme concentrations used for kinetic measurements were 0.06 μM. Reactions were monitored at the isosbestic point (348 nm) of p-nitrophenol and p-nitrophenolate when assay was performed at lower pH (< 6.5), whereas, at higher pH (> 6.5), the monitored wavelength was set at 400 nm. Kinetic constants were calculated based on the molar absorption coefficients determined at each pH values at 37 °C: 348 nm (isosbestic point), $\Delta\varepsilon$ = 3100 $M^{-1}\cdot cm^{-1}$; pH 6.5, 400 nm, $\Delta\varepsilon$ = 6040 $M^{-1}\cdot cm^{-1}$; pH 6.8,

400 nm, $\Delta\varepsilon$ = 7400 $M^{-1}\cdot cm^{-1}$; pH 7.0, 400 nm, $\Delta\varepsilon$ = 10460 $M^{-1}\cdot cm^{-1}$; pH 7.5, 400 nm, $\Delta\varepsilon$ = 15400 $M^{-1}\cdot cm^{-1}$. Reaction rates were determined (in triplicate, at pH 6.8, 37 °C) with each of six substrates at a concentration in a range 0.1-2 mM. The Michaelis parameter was evaluated for each substrate with non-linear regression analysis using EnzFitter program.

pH stability and thermostability

For thermostability experiments, each 500 μl of purified α-L-fucosidase (0.3 μg/mL, 50 mM acetate buffer, pH 5.5) was kept in 50 mM phosphate buffer (pH 6.8) at 25 °C, 35 °C, 45 °C, 55 °C, 60 °C and 75 °C. At appropriate time interval, 30 μl of enzyme solution was employed to assay the residual activity in pH 5.5 at 25 °C. For pH stability investigation, enzyme samples (same as above) were incubated in a series of buffers at pH 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 at 25 °C. The condition for the assay of enzyme residual activity is identical to thermostability study. For the calculation of the residual activity, the activity measured at 25 °C, pH 5.5 was set as 100%. The optimum pH for h-Fuc was determined residual activity by incubating the purified enzyme with pNPF in serious of buffers with pH range 2~11.5. Buffers used in this study were glycine (pH 1.8-3.5 and 9.0-10.0), sodium acetate (pH 4.0-5.5), morpholinoethanesulfonic acid (pH 5.5-6.5), phosphate (pH 6.5-7.5), Tris-HCl (pH 7.5-8.5), Caps (10.5-11.5).

Effect of metal ion

The effects of various metal ions and reagents (Cu^{2+}, Ni^{2+}, Ba^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Hg^{2+}, EDTA and DTT) on h-Fuc activity were investigated by incorporating 2-10 mM ion (or reagents) to the assay system.

Transglycosylation activity

h-Fuc (~2 units) was added in 1 mL ammonium acetate buffer, pH 5.5, containing 20% alcohol and 10 mM substrate. 3 substrates, pNPF, pCPF and CNPF, were examined separately. The reaction proceeded at 37 °C, overnight. The reaction mixture was concentrated to dryness and the crude solid was resuspended in 0.5 mL of water. The solution was then subjected to extraction by chloroform (0.5 mL, once) and ethyl acetate (0.5 mL, twice) to remove p-nitrophenol. The resulting aqueous solution was lyophilized and exchanged with D_2O for ^{1}H-NMR analysis. The product of transglycosylation was determined by ^{1}H-NMR performed at 50 °C in a Bruker DRX 300 spectrometer equipped with a ^{1}H 5 mm probe. The product ratio was calculated based on the integration of the signal of C1 proton on sugar ring.

RESULTS AND DISCUSSION

Enzyme expression and purification

α-L-Fucosidase is a glycosidase involved in the degradation of fucose-containing glycoconjugates. Though human tissue α-L-fucosidase (h-Fuc) gene had been cloned and expressed in *E. coli*, the recombinant enzyme was an inclusion body and inactive.[44] In this study, by controlling the growing condition of *E. coli*, the recombinant h-Fuc was successfully expressed as a soluble active form. To begin with, the vector pCMV.SPORT6 containing a human tissue α-L-fucosidase gene was amplified with two designed primers by PCR. The mature *h-fuc* gene, without the sequence of signal peptide (MRSRPAGPALLLLLLFLGAAES), was further inserted into pET22b(+) vector. This new clone, pET22b(+)_*h-fuc* was transferred into *E. coli* BL21 (DE3) for expression. The recombinant protein with an extra Met residue added at the N-terminus was obtained as a soluble form in the cytosol of *E. coli*. Note that the pH condition of the culture medium is an important factor for the expression of active protein. Better activity of h-Fuc was retained when bacteria were grown in LB medium at pH 6.0, whereas a greater pH (≥ 7.0) yielded enzyme with low activity. The recombinant α-L-fucosidase, with estimated pI value 6.0, was induced by adding isopropyl thio-β-D-galactoside (2 mM) for 20 h. Cells were harvested and the recombinant protein was purified by the applications of three chromatographic steps including, strong cation-exchange column (HiTrap SP) (Fig. 1a), anion-exchange column (HiTrap Q) (Fig. 1b), and size exclusion Sephadex_G-75 column (not shown). The recovery yield of each step of purification is summarized in Table 1. The final yield of h-Fuc was 5% with 33-fold purification. The homogeneity (> 95%) and molecular weight of the purified enzyme was confirmed by SDS-PAGE (Fig. 2a) and ESI/Mass spectrometry (50895 KDa) (Fig. 2b).

Substrate specificity

To analyse the catalytic activity and the substrate specificity of the recombinant enzyme, a variety of glycosides, including p-nitrophenyl-α-L-fucopyranoside (pNPF), p-nitrophenyl-β-D-arabinopyranoside (pNPbA), p-nitrophenyl-α-L-arabinopyranoside (pNPaA), p-nitrophenyl-α-L-arabinofuranoside (p-NPAF), p-nitrophenyl-β-D-glucopyranoside (pNPGlc), p-nitrophenyl-β-D-N-acetylglucosaminide (pNPGlcNAc), p-nitrophenyl-β-D-galactopyranoside (pNPGal) were tested as substrate. The kinetic pa-

Table 1. Purification of recombinant human α-L-fucosidase

Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purity Fold	Yield (%)
crude enzyme extract*	102	651	6.4	1	100
HiTrap SP (pH 5.5)	34.8	342	9.8	1.5	34
HiTrap Q (pH 6.2)	6.7	266	39.7	6.2	20
Superdex G-75 (pH 5.5)	0.34	72	211.7	33.1	5.1

* A crude enzyme extract was obtained from liquid culture (1 L, OD_{600} = 17) on 20%~85% saturation of ammonium sulfate precipitation.

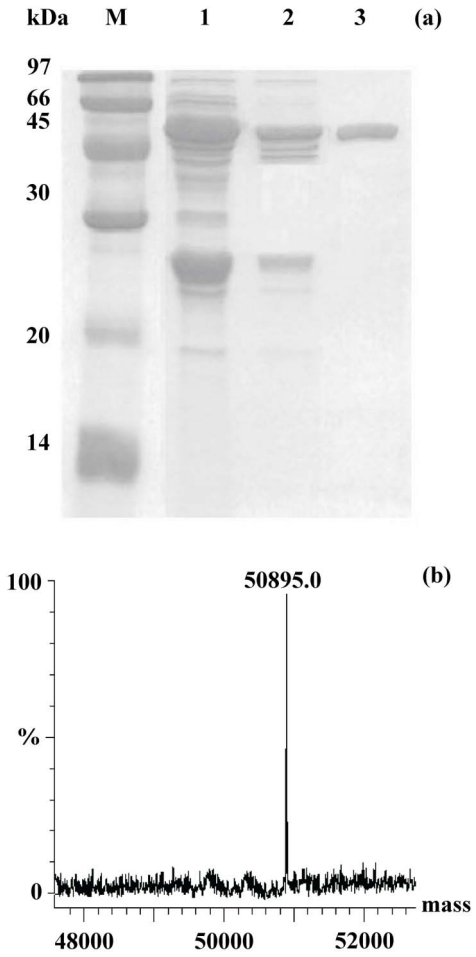

Fig. 2. Molecular mass analysis of h-Fuc. obtained from different step of purified protein. (A) By SDS-PAGE. Lanes: M, molecular mass markers; 1, the collection of active fractions of recombinant enzyme from HiTrap-SP column; 2, the collection of active fractions from HiTrap-Q column; 3, the collection of active fractions from Sephadex_G-75 column. (B) By ESI-MS. The mass spectrum of the purified recombinant h-Fuc to give molar mass = 50895.0 ± 2 Da (calculator: 50897 Da).

rameters (k_{cat} and K_m) determined at 37 °C, pH 6.8 were summarized in Table 2. For all tested substrates, only pNPF and pNPbA can be effectively hydrolyzed with k_{cat} and K_m being 17.1 s^{-1}, 0.28 mM and 1.9 s^{-1}, 0.73 mM, respectively. The catalytic activities (k_{cat}/K_m) of pNPbA substrate was about 23-fold weaker than pNPF. Since the structures of both substrates can be distinguished at the C5 position of the sugar ring, the lower relative activity of h-Fuc toward pNPbA suggests that methyl group on C5 of α-L-fucoside plays a significant role for interaction with protein. Although, h-Fuc can promote the hydrolysis of β-D-arabinopyranoside, which can be considered as the structural analogue of α-L-fucopyranoside, it has considerable specificity for α-L-fucosides over all other tested substrates.

Characteristics of recombinant h-Fuc

The stability of the enzyme to temperature (25 °C-75

Table 2. Michaelis-Menten parameters for the hydrolysis of a series of p-Nitrophenyl Glycopyranosides by recombinant h-Fuc[a]

Phenyl substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
pNPF	0.284 ± 0.002	17.07 ± 0.02	60.11
pNPbA	0.73 ± 0.02	1.9 ± 0.1	2.60
pNPaA	ND[b]	ND	
pNPAF	ND	ND	
pNPGal	ND	ND	
pNPGlc	ND	ND	
pNPGlcNAc	ND	ND	

[a] Assays were performed at 37 °C, sodium phosphate buffer (50 mM, pH 7.0).

[b] ND: no significant activity can be detected. The abbreviation of the substrates: p-nitrophenyl-α-L-fucopyranoside, pNPF; p-nitrophenyl-α-L-arabinofuranoside, p-NPAF; p-nitrophenyl-β-D-arabinopyranosid, pNPbA; p-nitrophenyl-α-L-arabinopyranosid, pNPaA; p-nitrophenyl-β-D-N-acetylglucosaminide, pNPGlcNAc; p-nitrophenyl-β-D-galactopyranoside, pNPGal; p-nitrophenyl-β-D-glucopyranoside, pNPGlc.

°C) and acidity (pH 2.5-9) was investigated with pNPF as the substrate. In general, the recombinant h-Fuc was stable up to 55 °C with incubation at pH 6.8 for 2 h and lost its activity rapidly at 75 °C (Fig. 3); the optimum temperature for h-Fuc is approximately 55 °C (data not shown). The recombinant protein was stable in pH range of 2.5-7.0 (at 25 °C for at least 2 h) but lost its catalytic activity greatly for pH ≥ 8.0 or < 2.5 (Fig. 4). The study of pH-dependent activity (k_{cat}) showed that the recombinant enzyme had optimum activity at two regions near at pH 4.5 and pH 6.5 (Fig. 5). The pH-dependent activity on (k_{cat}/K_m) showed that the recombinant enzyme had optimum activity at pH 4.8-5.2 (Fig. 5). Although these features exhibited above are comparable to the native α-L-fucosidase purified directly from human liver, the activity is somewhat distinguishable. For instance, at pH 5.0, the catalytic activity of recombinant h-Fuc (K_m = 0.105 mM, k_{cat} = 48.6 s^{-1}) with pNPF as substrate was 12 times that of a counterpart purified from human liver tissue (K_m = 0.43 mM, k_{cat} = 16.3 s^{-1}).[27,40] The characteristic of two optimal peaks in the pH-dependent activity may be attributed to the stages of structure perturbations that provide appropriate active-domain for catalysis.

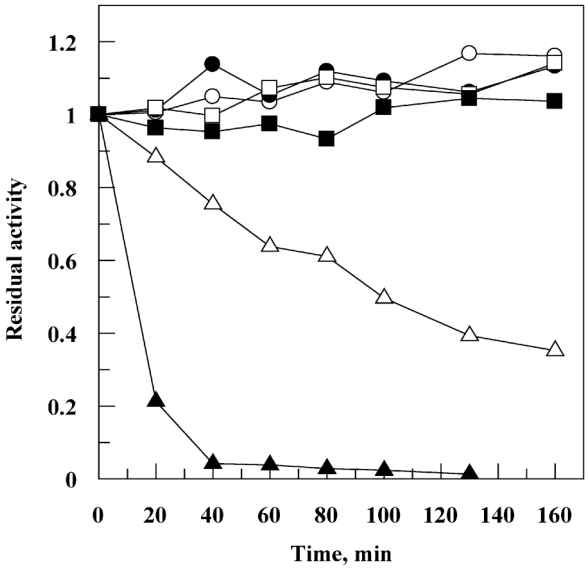

Fig. 3. Thermal stability of the recombinant enzyme assay as h-Fuc. Enzyme was incubated at pH 6.8 in various temperature: 25 °C (○), 37 °C (●), 45 °C (□), 55 °C (■), 60 °C (△), and 75 °C (▲). An aliquot of enzyme was removed for the assay of the residual activity at different time intervals: 1, 20, 40, 60, 80, 100, 130, 160 min. The reaction rate assayed at 25 °C, pH 5.5 was set as 100%.

For the investigation of the effect of metal ion, various divalent metal cations (Cu^{2+}, Ni^{2+}, Ba^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Hg^{2+}) and dithiothreitol (DTT) were added up to 10 mM in enzymatic assay. No sig-

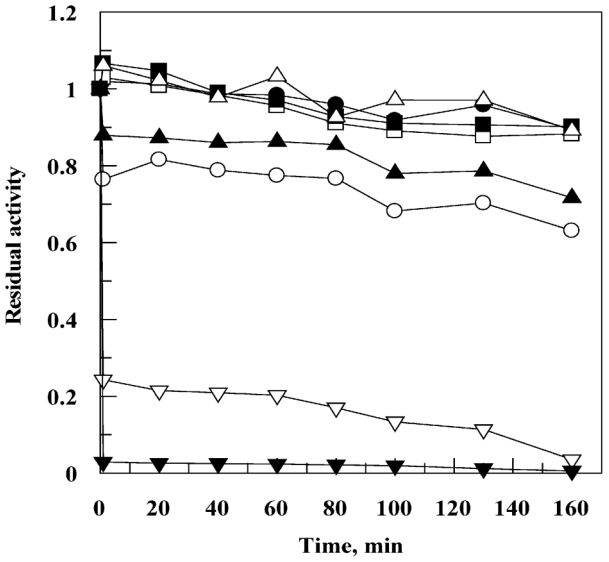

Fig. 4. pH stability of the recombinant h-Fuc. Enzyme was incubated in a series of buffers with pH 2.5 (○), 3.0 (●), 4.0 (□), 5.0 (■), 6.0 (△), 7.0 (▲), 8.0 (◊) and 9.0 (▼) at 25 °C. An aliquot of enzyme was removed for the assay of the residual activity at different time intervals: 1, 20, 40, 60, 80, 100, 130, 160 min. The reaction rate assayed at 25 °C, pH 5.5 was set as 100%.

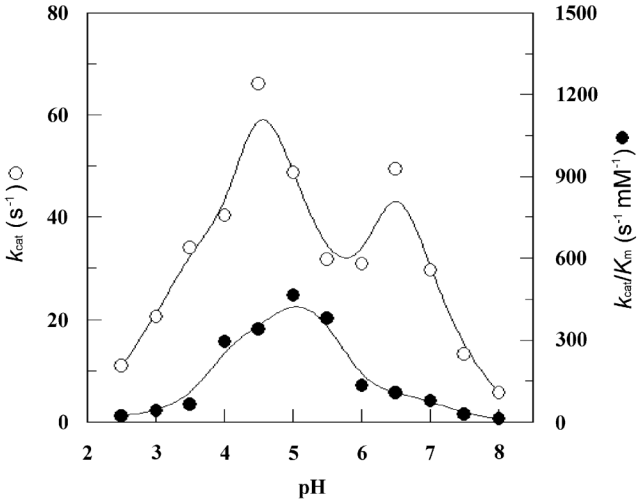

Fig. 5. pH dependence of k_{cat} (○) and k_{cat}/K_m (●) of recombinant h-Fuc. All reactions were performed at 37 °C in a series of pH values (2.5-8.0) with pNPF as substrate and monitored at 348 nm.

nificant activity loss can be observed for the tested ions and reagents except for Hg^{2+} (> 10 μM) and DTT (> 50 μM) for which 90-95% inhibition was obtained, suggesting that sulfhydryl group plays an important role in either the catalytic function or protein structure. Similarly, the process involved in extensive dialysis of enzyme solution against EDTA or inclusion of 10 mM EDTA in the reaction mixture could not affect h-Fuc activity, indicating that h-Fuc is unlikely to be a metalloenzyme.

Transglycosylation of h-Fuc and its possible catalytic mechanism

In general, glycosyl hydrolases cleave the glycosidic bond of the substrate by two different mechanisms, the retention and the inversion of the anomeric configuration. Both mechanisms require two essential residues, which in most glycosyl hydrolases are residues with carboxylic acid side-chain such as aspartate or glutamate.[46,47] α-L-Fucosidases are exo-glycosidases, based on general catalytic mechanism of cleavage of glycosidic bonds, were classified into two families as glycohydrolyase family GH-29 and GH-95. Two different mechanisms have been reported for the catalysis of α-L-fucosidase, leading to release glycose with either retention (for GH-29) or inversion (for GH-95) of the anomeric configuration. A time-course ^{1}H-NMR study is commonly employed to examine the anomeric preference in the catalytic reaction of a GH. Though such work provides unequivocally stereochemical data, the process is tedious, as equilibration with a deuterated buffer system is required and the enzyme concentration must be carefully controlled. Also, a significant limitation of this technique is that the rate of product mutarotation must be relatively slow so that NMR detection of authentic product is feasible. To overcome the limitation, a method involving transglycosylation of h-Fuc using methanol as the glycosyl acceptor was employed. The advantage of this strategy is the formation of a methyl glycoside that cannot mutarotate. For most retaining enzymes, though the formation of a covalent enzyme intermediate is expected, it is difficult to detect as the lifetime is short. In the past, 2-fluoroglycosides have been employed for a specific glycosyl-enzyme trapping technique.[48-50] In this study, we used an alternative method to indirectly prove the formation of glycosyl-enzyme intermediate. If a constant chemical bias may be noted in different enzyme products, the formation of a common intermediate in an enzymatic reaction

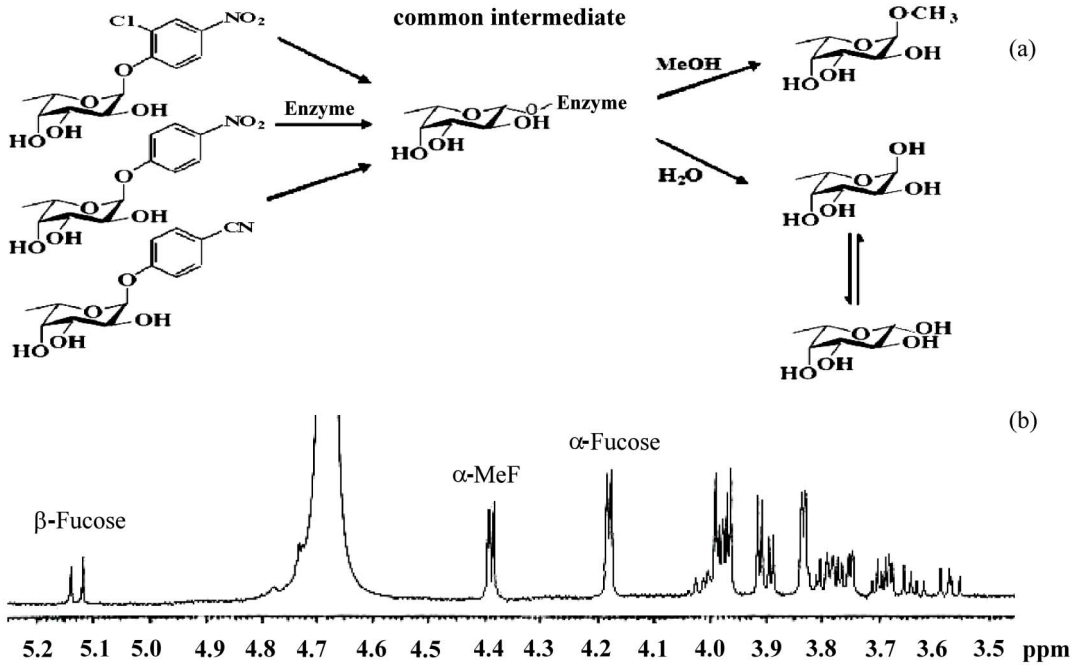

Fig. 6. Stereochemical properties and common intermediates of h-Fuc catalysis. (a) Enzymatic reactions, using various substrates, in the presence of methanol. (b) A partial NMR spectrum (chemical shift 3.5-5.2 ppm) of the end-products. Peak assignment is given in the text. The integrations of the signals of the C1 protons on each sugar ring were used to calculate the end-product ratio (Fucose/MeF).

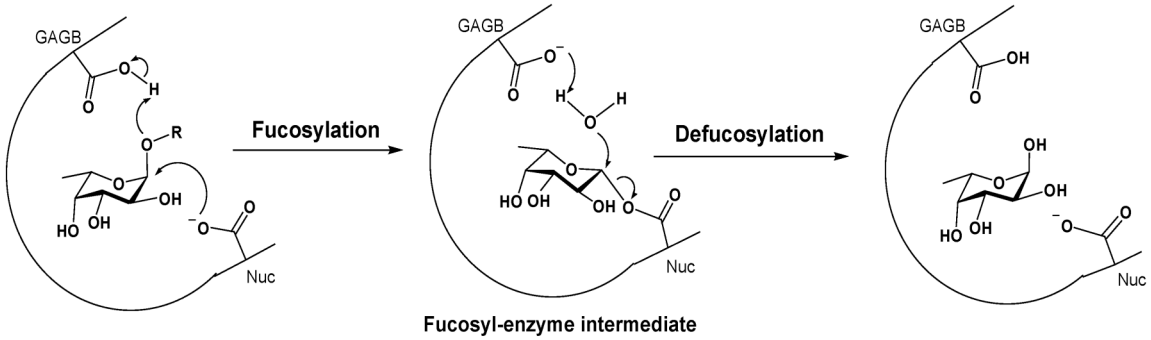

Fig. 7. Proposed mechanism of h-Fuc. A two-step, double-displacement mechanism is proposed involving two essential amino-acid residues, one functioning as a nucleophile (Nuc) and the other as a general acid/base (GAGB).

may be inferred. The present study showed that h-Fuc exhibited strong transglycosylation activity when methanol was used as a fucosyl acceptor. Here, a suitable amount of CNPF, pNPF and pCPF were enzymatically hydrolyzed in acetate buffer (pH 5.7) containing 20% (v/v) methanol. The solution was dried and exchanged with D_2O several times before ^{1}H-NMR analysis. For all three reactions, the ^{1}H-NMR spectra (measured at 50 °C) of the sugar moieties (in the range of 3-6 ppm) were nearly identical. In principle, 3 different end-products with fucosyl ring structures should be observed if the catalysis follows the retaining mechanism (Fig. 6a). In the present study, the C1 proton of each sugar ring was assigned as follows: methyl-α-L-fucopyranoside (MeF) (4.39 ppm, J = 3.4 Hz), α-fucopyranose (4.18 ppm, J = 3.9 Hz) and β-fucopyranose (5.13 ppm, J = 8.2 Hz) (Fig. 6b). Based on peak assignment and the integration of the C1 proton on the sugar ring, the ratio of fucose/MeF was calculated from each spectrum. Regardless of the substrates, these ratios were nearly constant, and averaged 1.31 ± 0.02 (1.31 for CNPF, 1.33 for pNPF and 1.30 for pCPF). This suggests that a common intermediate, most likely a fucosyl-enzyme structure, occurs in the reaction pathways. As the product of transglycosylation in this experiment is MeF, we can confirm that the h-Fuc is indeed a "retaining" enzyme. Propose retaining glycosidases catalyze the hydrolysis via a two-step, double-displacement mechanism, as shown in Fig. 7. Two key active-site carboxylic acid residues are involved. One functions as the nucleophile and the other functions as the general acid/base. In the first step (the glycosylation step), the nucleophile attacks the anomeric carbon of the glycoside, while the acid/base catalyst protonates the glycosidic oxygen, thereby assisting the leaving of the aglycon moiety. This leads to the formation of a covalent glycosyl-enzyme intermediate. In the second step, breakdown of the glycosyl-enzyme intermediate proceeds through a general base-catalyzed attack of water at the anomeric center to release the glycose with the retention of the anomeric configuration. The detailed mechanistic action and the catalytic essential residues of h-Fuc will be discussed elsewhere.

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