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Hitoshi Sakuraba · Yasunori Chiba · Masaharu Kotani
Ikuo Kawashima · Mai Ohsawa · Youichi Tajima
Yuki Takaoka · Yoshifumi Jigami · Hiroshi Takahashi
Yukihiko Hirai · Takashi Shimada
Yasuhiro Hashimoto · Kumiko Ishii
Toshihide Kobayashi · Kazuhiko Watabe
Tomoko Fukushige · Tamotsu Kanzaki

Corrective effect on Fabry mice of yeast recombinant human α-galactosidase with *N*-linked sugar chains suitable for lysosomal delivery

Abstract We have previously reported the production of a recombinant α-galactosidase with engineered *N*-linked sugar chains facilitating uptake and transport to lysosomes in a *Saccharomyces cerevisiae* mutant. In this study, we improved the purification procedure, allowing us to obtain a large amount of highly purified enzyme protein with mannose-6-phosphate residues at the non-reducing ends of sugar chains. The products were incorporated into cultured fibroblasts derived from a patient with Fabry disease via mannose-6-phosphate receptors. The ceramide trihexoside (CTH) accumulated in lysosomes was cleaved dose-dependently, and the disappearance of deposited CTH was maintained for at least 7 days after administration. We next examined the effect of the recombinant α-galactosidase on Fabry mice. Repeated intravascular administration of the enzyme led to successful degradation of CTH accumulated in the liver, kidneys, heart, and spleen. However, cleavage of the accumulated CTH in the dorsal root ganglia was insufficient. As the culture of yeast cells is easy and economical, and does not require fetal calf serum, the recombinant α-galactosidase produced in yeast cells is highly promising as an enzyme source for enzyme replacement therapy in Fabry disease.

Keywords Fabry disease · α-Galactosidase · Ceramide trihexoside · Yeast · Enzyme replacement therapy · Fabry mouse

Introduction

Lysosomal α-galactosidase (EC 3.2.1.22) is a critical enzyme for the cleavage of glycolipids with terminal α-D-galactosyl residues, primarily ceramide trihexoside (CTH; also called globotriaosylceramide, GL-3, and

H. Sakuraba (✉) · M. Kotani · I. Kawashima · M. Ohsawa
Y. Tajima
Department of Clinical Genetics,
The Tokyo Metropolitan Institute of Medical Science,
Tokyo Metropolitan Organization for Medical Research,
3-18-22 Honkomagome,
Bunkyo-ku, Tokyo 113-8613,
Japan
E-mail: sakuraba@rinshoken.or.jp
Tel.: +81-3-38232105
Fax: +81-3-38236008

Y. Chiba · Y. Takaoka · Y. Jigami
Research Center for Glycoscience,
National Institute of Advanced Industrial Science and Technology,
1-1-1 Higashi, Tsukuba 305-0044, Japan

M. Ohsawa
CREST, JST, 4-1-8 Hon-machi,
Kawaguchi 332-0012, Japan

H. Takahashi · Y. Hirai · T. Shimada
Department of Biochemistry and Molecular Biology,
Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku,
Tokyo 113-8602, Japan

Y. Hashimoto · K. Ishii · T. Kobayashi
Supra-Biomolecular System Research Group,
RIKEN Frontier Research System, 2-1 Hirosawa,
Wako 351-0198, Japan

K. Watabe
Department of Molecular Neuropathology,
Tokyo Metropolitan Institute for Neuroscience,
Tokyo Metropolitan Organization for Medical Research,
2-6 Musashidai, Fuchu 183-8526, Japan

T. Fukushige · T. Kanzaki
Department of Dermatology,
Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

Gb$_3$) in lysosomes. Lysosomal α-galactosidase is a glycoprotein, and is synthesized in rough-surfaced endoplasmic reticulum followed by the addition of N-linked high-mannose-type oligosaccharides. The enzyme is then transferred to the Golgi apparatus, where further modification, including addition of mannose-6-phosphate (M6P) residues and binding to M6P receptor, occurs. Subsequently, the enzyme is transported to endosomes via M6P receptors. The enzyme then moves to lysosomes, where it exerts its function. In some type of cells, including cultured fibroblasts, α-galactosidase can be incorporated into the cells from the extracellular milieu via M6P receptors on the plasma membrane and transported to lysosomes (Kornfeld and Sly 2001).

A deficiency of α-galactosidase results in widespread cellular deposition of CTH, thereby causing Fabry disease (MIM 301500) (Desnick et al. 2001). Fabry disease is an X-linked genetic disease exhibiting a wide clinical spectrum. Male patients with classic Fabry disease usually have no α-galactosidase activity and, in childhood or adolescence, there is pain in the peripheral extremities, angiokeratoma, hypohidosis and corneal opacity, followed by renal, cardiac and cerebrovascular involvement with increasing age (Desnick et al. 2003). The incidence of classic Fabry disease has been estimated to be 1 in 40,000 male newborns (Desnick et al. 2001). Patients with variant form Fabry disease have residual α-galactosidase activity and milder clinical manifestations with late onset (Sakuraba et al. 1990; Nakao et al. 1995). Females heterozygous for Fabry disease can be affected to a moderate or severe degree due to random X-chromosomal inactivation (Sakuraba et al. 1986; Fukushima et al. 1995; Itoh et al. 1993, 1996; Lyon 1962). However, a recent survey has revealed that many Fabry females can be affected similarly to Fabry males and thus should be considered as patients rather than carriers of the disease (Mehta et al. 2004). Fabry disease has been under-recognized, and the number of Fabry patients requiring treatment is thought to be much larger than previously assumed.

Recently, two different recombinant α-galactosidases were developed for enzyme replacement therapy for Fabry disease: agalsidase alfa (Replagal; Transkaryotic Therapies, Cambridge, MA) generated in human cultured fibroblasts (Schiffmann et al. 2000), and agalsidase beta (Fabrazyme; Genzyme Therapeutics, Cambridge, MA) produced in Chinese hamster ovary (CHO) cells (Eng et al. 2001a, b). The former has been approved in Europe, and the latter in Europe, the United States, and Japan, and many Fabry disease patients have been successfully treated with these drugs. However, these recombinant enzymes are produced in cultured mammalian cells and thus their production is very expensive. Furthermore, careful monitoring for infection by pathogens is essential because fetal calf serum is usually required for the culture of mammalian cells.

We have constructed a yeast cell line producing a recombinant human α-galactosidase with N-linked high-mannose-type sugar chains (yeast recombinant human α-galactosidase, yr-haGal), as described previously (Chiba et al. 2002). Effective incorporation of the enzyme into affected organs is very important for enzyme replacement therapy, and in Fabry disease successful targeting of α-galactosidase is strongly dependent on the presence of M6P residues on the sugar chains of the enzyme preparations. In this study, we improved the procedures for purification of α-galactosidase from the culture medium of yeast cells to obtain a large amount of highly purified enzyme protein with M6P residues that facilitate incorporation of the enzyme into affected organs, and analyzed the effect of the purified enzyme on cleavage of CTH accumulated in cultured Fabry fibroblasts and organs of Fabry mice.

Materials and methods

Purification of yr-haGal secreted into the culture medium of yeast cells

Here we used a yeast strain, HPY21G, constructed by introducing the human α-galactosidase cDNA into a *Saccharomyces cerevisiae* strain, HPY21, as described previously (Chiba et al. 2002). A large-scale culture (100 l) was performed to examine the effect of yr-haGal on Fabry mice. We had previously used Blue-Sepharose and ConA-Sepharose columns to purify yr-haGal (Chiba et al. 2002). However, these columns are very expensive and had only weak binding ability because of the characteristics of the affinity chromatography so we improved the purification procedure. All column materials used in the experiments reported here were purchased from Amersham Biosciences Japan (Tokyo, Japan). The culture medium of the HPY21G strain was collected and concentrated, and ammonium sulfate was added slowly to the supernatant to a final concentration of 55%. The precipitate was recovered by centrifugation, re-dissolved in 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, and then dialyzed against the same buffer. A sample was then applied to a HiLoad Q 16/10 Sepharose HP column equilibrated with the same buffer. After washing the column, α-galactosidase was eluted with a 0–1 M NaCl gradient in the same buffer. Fractions containing enzyme activity were pooled, and then a one-tenth volume of 3 M ammonium sulfate was added. A sample was then applied to a HiLoad 26/10 Phenyl HP column equilibrated with 25 mM MES buffer, pH 6.0, containing 0.3 M ammonium sulfate. After washing the column, α-galactosidase was eluted with a 0.3–0 M ammonium sulfate gradient in the same buffer. Fractions containing enzyme activity were dialyzed against 20 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, and then were concentrated with an Amicon Ultra-4 (13,000 MWCO; Millipore, Bedford, MA). A sample was then applied to a HiLoad 16/60 Superdex 200pg column. Fractions containing enzyme activity then were pooled and subjected to α-mannosidase treatment to expose M6P residues at

the non-reducing ends of the sugar chains. Treatment of the recombinant α-galactosidase with the culture supernatant of SO-5, a new bacterium producing an α-mannosidase, was performed as described previously (Chiba et al. 2002). After the α-mannosidase treatment, the α-galactosidase protein was re-purified on HiLoad Q and HiLoad 16/60 Superdex 200pg columns under the conditions described above.

Biochemical analyses of the enzymatic properties of yr-haGal

The purity and molecular mass of yr-haGal produced in yeast cells were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Chiba et al. 2002). Reversed-phase high-performance liquid chromatography (HPLC) analysis of the purified yr-haGal was performed on a Cosmosyl $5C_4$-AR-300 (4.6×150 mm) column (Nacalai Tesque, Kyoto, Japan). The protein was eluted with a linear trifluoroacetic acid/acetonitrile gradient at a flow rate of 1 ml/min with ultraviolet detection at 215 nm. Deglycosylation of yr-haGal with N-glycanase F (Takara Bio, Shiga, Japan) was performed according to the method recommended by the manufacturer, and N-terminal amino acid sequence analysis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis were performed by Shimazu Corporation (Kyoto, Japan).

α-Galactosidase activity was measured fluorometrically with 4-methylumbelliferyl-α-galactopyranoside (Calbiochem, San Diego, CA) as a substrate in the presence of N-acetylgalactosamine (Sigma, St. Louis, MO), a specific inhibitor of α-N-acetylgalactosaminidase (Mayes et al. 1981). The protein concentration was determined with a DC assay kit (Bio-Rad, Richmond, CA), using bovine serum albumin (BSA) as a standard.

Sugar chain analysis of yr-haGal was performed according to the method reported previously (Takashiba et al. 2004). Briefly, the enzyme was hydrolyzed with 2 M trifluoroacetic acid and L-rhamnose, as an internal standard, at 100°C for 2 h, and monosaccharides derived from the sugar chains were then quantitated by means of capillary electrophoresis using a P/ACE MDQ equipped with a laser-induced fluorescence detector (Beckman Coulter, Fullerton, CA); authentic monosaccharides were used as standards for quantitation.

Examination of the effect of yr-haGal on cultured human Fabry fibroblasts

Cultured fibroblasts from a patient with Fabry disease and a normal control subject were established and maintained in our laboratory. The cells were cultured in Ham's F-10 medium containing 10% fetal calf serum and antibiotics at 37°C in an incubator containing 5% CO_2. The study involving the cultured human fibroblasts was approved by the Ethical Committee of our institute.

To examine uptake of yr-haGal by Fabry fibroblasts, yr-haGal produced in yeast cells was added to the culture medium of Fabry fibroblasts to give concentrations of 0, 0.25, 0.5, 1.0, 3.0 and 6.0 μg/ml. For examination of the inhibitory effect of M6P on the cellular uptake of yr-haGal, Fabry fibroblasts were cultured in medium containing 5 mM M6P and 1.0μg/ml yr-haGal. After 18 h culture, the cells were harvested mechanically, washed three times with phosphate-buffered saline (PBS), pH 7.4, and then collected as a pellet by centrifugation. An appropriate amount of water was then added to the pellet and the cells were sonicated; the resulting homogenate was used for α-galactosidase assay and protein determination.

To examine degradation of accumulated CTH by the incorporated recombinant α-galactosidase, Fabry fibroblasts were cultured with culture medium containing the recombinant α-galactosidase at concentrations of 0, 0.5, 1.0, 2.0 and 3.0μg/ml for 3 days. Alternatively, Fabry fibroblasts were cultured in medium containing 3.0μg/ml recombinant α-galactosidase for 0, 1, 3, 5, and 7 days. Cells grown on a Lab-Tek chamber slide (Nunc, Naperville, IL) were fixed with 2% paraformaldehyde in PBS, pH 7.4, for 10 min, followed by blocking with 5% BSA in PBS for 1 h. The cells were then incubated with a mouse monoclonal anti-CTH antibody (culture supernatant; IgG isotype) (Kotani et al. 1994) and rabbit polyclonal anti-α-galactosidase antibodies (1:100 diluted; IgG isotype) (Ishii et al. 1994) for 1 h. After washing, they were reacted for 1 h with a fluorescent isothiocyanate-conjugated goat anti-mouse IgG F(ab')₂ (diluted 1:200; Jackson Immuno Research, West Grove, PA) and a rhodamine-conjugated goat anti-rabbit IgG F(ab')₂ (diluted 1:400; Jackson Immuno Research). To determine the localization of the accumulated CTH, double staining with the anti-CTH antibody and a mouse monoclonal antibody to lysosome-associated membrane protein-1 (LAMP-1; Southern Biotechnology, Birmingham, AL), a marker for lysosome, was also performed according to a modified method described elsewhere (Kotani et al. 2004). The stained cells were observed under a microscope (Axiovert 100M; Zeiss, Oberkochen, Germany) equipped with a confocal laser scanning imaging system (LSM510; Zeiss).

Examination of the effect of yr-haGal on Fabry mice

Fabry mice (α-galactosidase knock-out mice, donated by Ashok B. Kulkarini and Toshio Oshima) and wild type C57BL/6 mice were used in this experiment according to the rules drawn up by the Animal Care Committee of our institute.

To examine the pharmacokinetics and biodistribution of the recombinant α-galactosidase, a single dose, 3.0 mg/kg body weight, of recombinant α-galactosidase was injected into the tail veins of Fabry mice. As a control, a single dose, 2.0 mg/kg body weight of agalsidase beta (purchased from Genzyme Japan, Tokyo, Japan)

was injected into litter-matched Fabry mice so that the injected enzyme activity was almost the same (6.0–6.4 mmol h^{-1} kg^{-1} body weight). Each group consisted of two mice. Blood samples were collected at 0, 1, 3, 5, 10, 20, 30, and 40 min after injection of the enzymes, and a time course of changes in α-galactosidase activity in plasma was determined. The mice were sacrificed at 1 h after administration of the enzymes, and their livers, kidneys, hearts, and spleens were then removed. Tissue samples were then homogenized in citrate-phosphate buffer, pH 4.6, and centrifuged. The resulting supernatants were assayed for α-galactosidase activity.

To examine cleavage of the CTH accumulated in organs, two groups of litter-matched Fabry mice, each consisting of three mice, were repeatedly injected with the recombinant α-galactosidase, 3.0 mg/kg body weight, and agalsidase beta, 2.0 mg/kg body weight, separately every week for four doses, and then sacrificed 6 days after the last injection. Their livers, kidneys, hearts, spleens, and dorsal root ganglia were then removed, and used as samples for biochemical and/or morphological analyses.

For immunohistochemical analysis, the mouse tissues were stored at $-80°C$ before use, and then frozen sections of 10μm thickness were fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. The specimens were incubated with PBS containing 5% (w/v) BSA for 30 min at room temperature to block nonspecific binding. Subsequently, the samples were treated with a mouse monoclonal anti-CTH antibody (culture supernatant; IgG isotype) for 1 h at room temperature, and then treated with fluorescent isothiocyanate conjugated goat anti-mouse IgG F(ab')$_2$ (diluted 1:200; Jackson Immuno Research). The stained tissues were examined under a confocal laser scanning microscope as described above.

For determination of CTH levels, tissues, including liver, kidney, heart, and spleen, were analyzed by means of thin-layer chromatography, followed by densitometry according to the method described previously (Takahashi et al. 2002).

For morphological examination, kidney tissues were cut into small pieces, and then fixed in cold 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The specimens were rinsed in PBS, and then postfixed with 2% osmium tetraoxide in 0.2 M sucrose in PBS for 1 h and dehydrated with graded concentrations of ethanol, 50% through absolute, and glycidyl n-butyl ether. Dehydrated specimens were then embedded in Epon 812 resin. Sections of 0.1μm thickness were prepared and stained with 2% uranyl acetate in 50% ethanol for 5 min, restained with Reynolds lead citrate for 3 min, and finally examined under an electron microscope (Hitachi H-7100; Hitachi, Tokyo, Japan).

Examination of the anti-α-galactosidase immune reaction

To determine whether or not Fabry mice injected with the enzymes produced antibodies against the enzymes, solid-phase enzyme-linked immunosorbent assay (ELISA) was performed. Serum samples were obtained from Fabry mice repeatedly injected with yr-haGal (3.0 mg/kg body weight), and agalsidase beta (2.0 mg/kg body weight) separately every week for four doses. Briefly, a 96-well flat bottom microplate for ELISA (Immulon 2 HB; Thermo Lab Systems, Flanklin, MA) was coated with 1.0 μg/ml of the enzymes in PBS. After washing 5 times with 1% BSA in PBS, 200 μl 1% BSA in PBS was added to each well as a blocking solution, followed by incubation for 1 h at room temperature. After removing the blocking solution, 100 μl of the mouse sera or rabbit anti-α-galactosidase antibodies (Ishii et al. 1994) diluted to various concentrations was added to each well, followed by incubation for 1 h. The wells were then washed, incubated in 100 μl peroxidase-conjugated anti-mouse IgG F(ab')$_2$ (diluted 1: 2,000; Jackson Immuno Research) for 45 min, washed again, and finally incubated in 100 μl O-phenylenediamine (Sigma) generated as 0.4 mg/ml 0.05 M citrate-phosphate buffer, pH 5.0. After incubation with the chromogenic substrate for 10 min, the optical density of each well was measured by means of an ELISA reader (Bio-Rad, Hercules, CA).

Results

Properties of yr-haGal

The new purification method described in this paper allowed us to treat a large volume of culture medium and obtain highly purified yr-haGal with 870-fold purifica-

Table 1 Monosaccharide analysis. *M6P* Mannose-6-phosphate, *yr-haGal* yeast recombinant human α-galactosidase

	yr-haGal[a] (mol/mol protein)	Agalsidase beta[b] (mol/mol protein)	Agalsidase alfa[b] (mol/mol protein)
M6P	3.8 ± 0.2	3.1 ± 0.1	1.8 ± 0.0
Galactose	ND[c]	8.0 ± 0.4	12.2 ± 1.0
Fucose	ND	1.8 ± 0.1	3.0 ± 0.3
N-Acetylglucosamine	0.8 ± 0.6	18.4 ± 0.4	22.5 ± 2.3
Mannose	53.8 ± 2.6	25.7 ± 1.8	27.6 ± 0.5
Sialic acid	ND	7.0 ± 1.0	6.9 ± 0.6

[a]Values expressed as means $\pm$ SD, $n = 3$
[b]Lee et al. 2003
[c]Not detected

tion. The amount of recombinant α-galactosidase secreted from the HPY21G strain, a *S. cerevisiae* mutant harboring a human α-galactosidase cDNA, into the culture medium was approximately 290 μg per 1 l culture. The recovery of the enzyme through the purification procedure was 30%. Before treatment with α-mannosidase the purified enzyme was detected as a single band on SDS-PAGE, and its apparent molecular mass was determined to be 51 kDa on MALDI/TOF-MS. The molecular mass changed to 46 kDa following α-mannosidase digestion. The HPLC profile on a reversed-phase column contained a single peak. However, the *N*-terminal amino acid sequence could not be determined, indicating that some modification occurred at the *N*-terminus of yr-haGal. The specific enzyme activity of yr-haGal was 2.0 mmol h^{-1} mg protein^{-1}, which was a little higher than that purified by the previously described method (1.7 mmol h^{-1} mg protein^{-1}).

Monosaccharide composition of yr-haGal

The monosaccharide composition of the recombinant α-galactosidase was determined, and then compared with those of agalsidase alfa and agalsidase beta, which have been reported elsewhere (Lee et al. 2003). The results are shown in Table 1. The recombinant α-galactosidase produced in yeast has high-mannose-type sugar chains and contains no fucose or galactose residues. The content of M6P residues is 3.8 mol/mol protein, this value being a little higher than those of agalsidase alfa (1.8 mol/mol protein) and agalsidase beta (3.1 mol/mol protein).

Effect of yr-haGal on cultured human Fabry fibroblasts

Fabry fibroblasts were cultured in culture medium containing the indicated concentrations of the recombinant α-galactosidase for 18 h. Uptake of the enzyme by the Fabry fibroblasts was then investigated. The results are shown in Fig. 1. The α-galactosidase activity in untreated Fabry fibroblasts was almost nil, but it increased in response to addition of recombinant α-galactosidase dose-dependently, reaching a normal level when the recombinant α-galactosidase was added to the culture medium at a concentration of 0.25–1.0 μg/ml. Uptake of yr-haGal was decreased to 15% of the control level by the addition of 5 mM M6P, suggesting that incorporation of the enzyme depends largely on the M6P receptor.

We also investigated incorporation of the recombinant α-galactosidase protein into cultured Fabry fibroblasts, and its effect on cleavage of accumulated CTH by means of double staining for α-galactosidase and CTH. At first, double staining for CTH and LAMP-1, a lysosomal marker, was performed, which revealed that the accumulated CTH was localized in lysosomes of untreated Fabry cells (data not shown). The results of time-course analysis are shown in Fig. 2a. The recombinant α-galactosidase was added to the culture medium at a concentration of 3.0 μg/ml. After a 1-day incubation, immunofluorescence of α-galactosidase was detected and that of CTH was apparently decreased. After 3 days of culture, the maximum immunofluorescence for α-galactosidase was observed and the accumulated CTH was completely degraded. Thereafter, the immunofluorescence of α-galactosidase gradually decreased, but the disappearance of deposited CTH was maintained for at least 7 days. Next, we added recombinant α-galactosidase to the culture medium of Fabry fibroblasts at various concentrations from 0.5 to 3.0 μg/ml, and then cultured the cells for 3 days. Double staining for α-galactosidase and CTH revealed that clearance of the accumulated CTH in response to incorporation of the enzyme occurred dose-dependently (Fig. 2b).

Fig. 1 Uptake by Fabry fibroblasts of the recombinant α-galactosidase produced in yeast cells (yr-haGal). Fabry fibroblasts were cultured in culture medium containing yr-haGal at concentrations of 0, 0.25, 0.5, 1.0, 3.0 and 6.0 μg/ml. After 18 h incubation, α-galactosidase activity in the cells was measured. The original α-galactosidase activity in Fabry fibroblasts was <1 nmol h^{-1} mg protein^{-1}, and the normal range was 15–35 nmol h^{-1} mg protein^{-1}
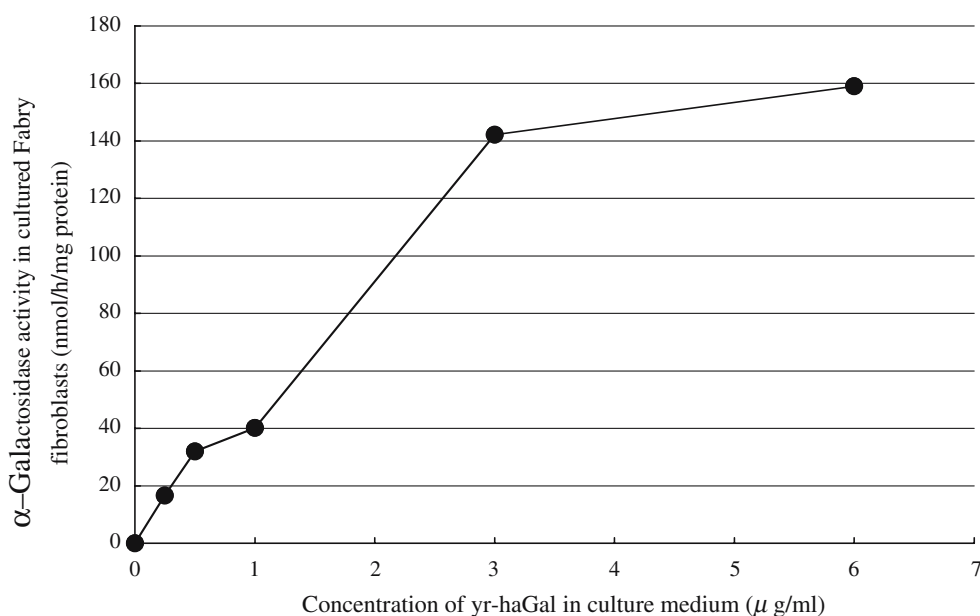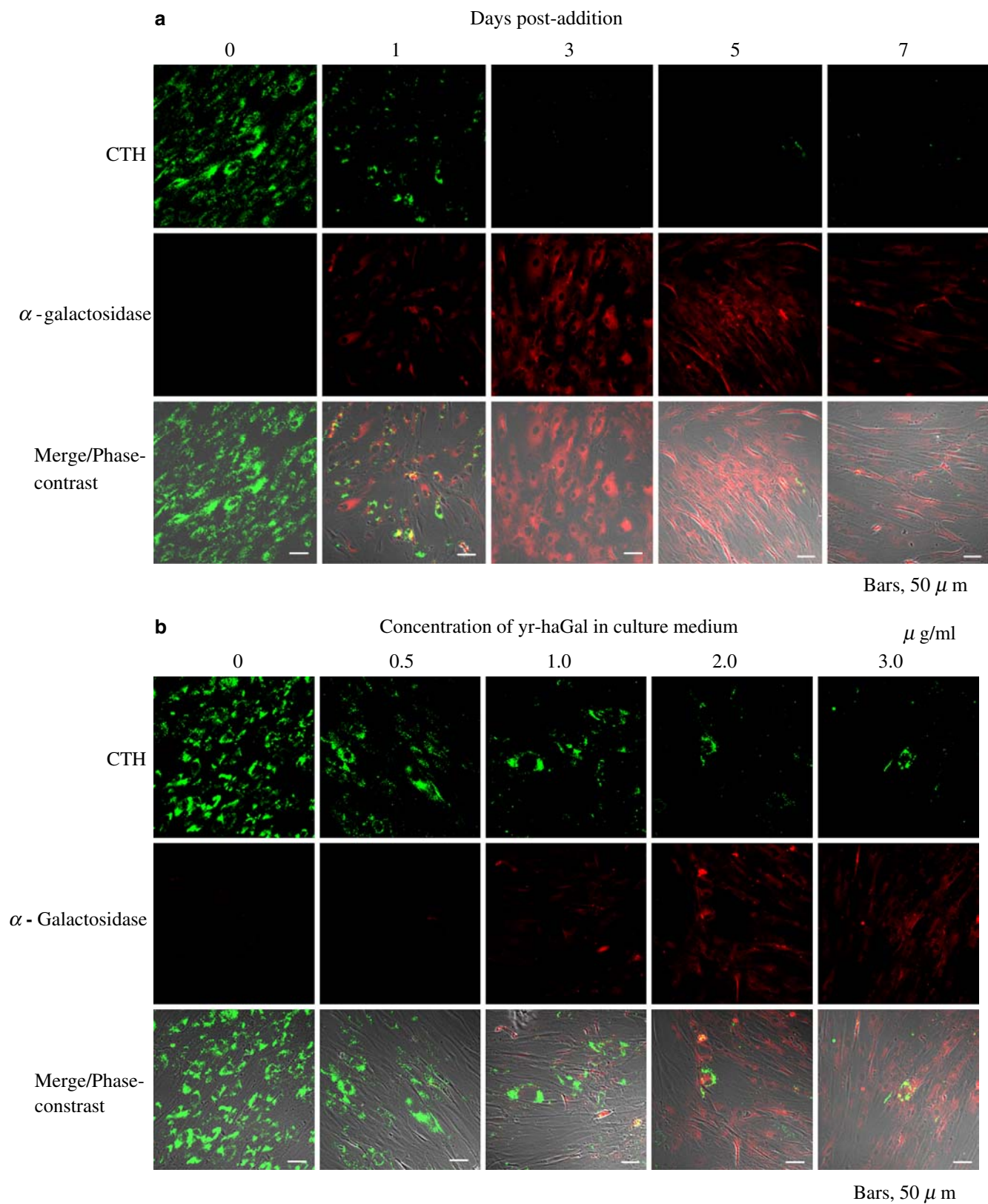
Fig. 2a,b Immunostaining for CTH and α-galactosidase in Fabry fibroblasts after addition of the recombinant α-galactosidase produced in yeast cells (*yr-haGal*). CTH, stained with an anti-CTH antibody (*green*); α-galactosidase, stained with anti-α-galactosidase antibodies (*red*); *Merge/Phase-contrast* overlapping images with these two fluorescent probes and phase-contrast images. Time-course (**a**) and dose-dependency (**b**). *Bars* 50μm

Effect of yr-haGal on Fabry mice

We injected recombinant α-galactosidase into Fabry mice to examine its therapeutic effect. We used agalsidase beta as a control enzyme because it has been reported that agalsidase beta is incorporated into affected organs in Fabry mice more than agalsidase alfa (Lee et al. 2003). As the specific enzyme activity in the yr-haGal we used (2.0 mmol h^{-1} mg protein^{-1}) was a little lower than that of the agalsidase beta sample (3.2 mmol h^{-1} mg protein^{-1}), we injected almost the same activity of the enzyme preparations into litter-matched Fabry mice for comparison.

After a single dose, 3.0 mg/kg body weight, of yr-haGal had been injected, its pharmacokinetics and biodistribution were examined and compared with those of agalsidase beta. The enzyme activity in plasma quickly increased, reaching a maximum level at 3 min after injection and then gradually decreasing. The pattern of the pharmacokinetics was essentially the same as that of agalsidase beta (Fig. 3). The biodistribution of yr-haGal and agalsidase beta after administration of a single dose is shown in Table 2. An apparent increase in α-galactosidase activity was observed in the organs of Fabry mice following yr-haGal administration. The degree of the enzyme activity increase in the kidneys and heart was almost the same as in the case of agalsidase beta, although that in the liver and spleen was a little lower than with agalsidase beta. The effect of yr-haGal on the degradation of tissue CTH was examined after repeated administration of the enzyme at 3.0 mg/kg body weight every week for four doses, followed by killing of the mice 6 days after the last injection. Immunohistochemical analysis revealed that the CTH deposited in the liver was cleaved (Fig. 4a). In kidney tissues, CTH immunofluorescence in the renal tubular cells was apparently decreased. Cleavage of the accumulated CTH in the glomeruli was insufficient although immunofluorescence was slightly decreased (Fig. 4b). In the heart, immunofluorescence of CTH accumulated in the tissue was apparently decreased after repeated injection of yr-haGal (Fig. 4c), as was also the case in the spleen (data not shown). In the dorsal root ganglia, no apparent degradation of accumulated CTH was observed (Fig. 4d). These findings were essentially the same as those found following administration of agalsidase beta. The results of quantitative analysis of CTH are shown in Table 3. Repeated administration of yr-haGal decreased the accumulated CTH in the liver to the level found in wild type mice, and decreased it to 70 and 30% of the levels in untreated Fabry mice in the kidneys and heart, respectively. The degree of degradation of CTH deposited in these tissues was almost the same as in the case of agalsidase beta. The decrease in accumulated CTH in the spleen upon administration of yr-haGal (to 30% of the untreated Fabry mice level) was smaller than that seen upon administration of agalsidase beta (to 10% of the untreated Fabry mice level). Morphological analysis revealed that many lamellar inclusion bodies, exhibiting accumulation of CTH, were present in the renal tubular cells of untreated Fabry mice (Fig. 5a), and that their number was markedly decreased after repeated administration of yr-haGal (Fig. 5b).

Immunoreactivity

The antigenicity of yr-haGal and agalsidase beta was examined by analyzing the cross-reactivity of antisera

Fig. 3 Pharmacokinetics of yr-haGal and agalsidase beta. A single dose of yr-haGal or agalsidase beta was injected into Fabry mice; blood samples were collected and time courses of changes in plasma α-galactosidase activity were determined. Each value represents the mean from two mice
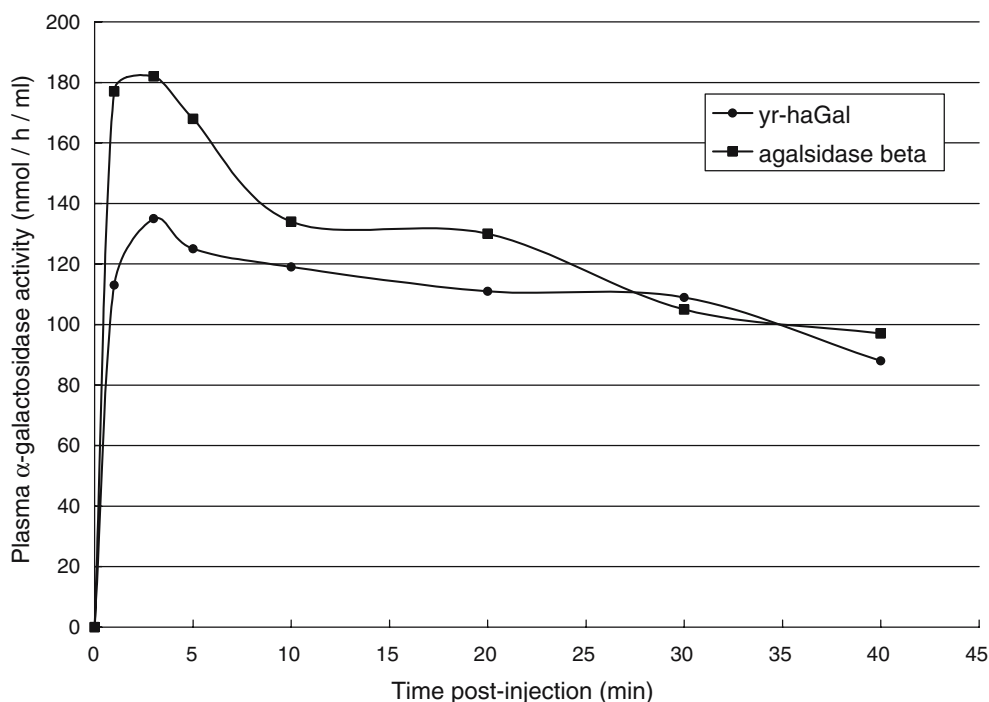
Table 2 α-Galactosidase activities (nmol h^{-1} mg protein^{-1}) in organs from Fabry mice treated with a single dose of yr-haGal or agalsidase beta. Fabry mice were injected with a single dose (almost the same enzyme activity) of yr-haGal (3 mg/kg body weight) or agalsidase beta (2 mg/kg body weight), and then were sacrificed 1 h after the administration. *Wild type* Wild type mice, *Fabry* untreated Fabry mice, *yr-haGal* Fabry mice treated with yr-haGal, *agalsidase beta* Fabry mice treated with agalsidase beta. Values are expressed as the means for two mice

	Liver	Kidney	Heart	Spleen
Wild type	30	11	7	29
Fabry	1	1	1	1
yr-haGal	57	52	90	42
agalsidase beta	174	87	76	100

derived from Fabry mice repeatedly injected with these preparations. The results are shown in Fig. 6. No significant antibodies unique to either enzyme preparation were found under the experimental conditions used.

Discussion

Production of recombinant human α-galactosidases in yeast cells has considerable advantages over production in mammalian cells, i.e., culture is easy, economical, and does not require fetal calf serum. Here, we used as a host a *S. cerevisiae* mutant in which two genes involved in *N*-linked mannan biosynthesis, *OCH1* and *MNN1*, are disrupted. The glycoprotein expressed in this cell line has mammalian-like *N*-linked high-mannose-type sugar chains and has no β-linked mannoside residues that are antigenic in humans (Chiba et al. 1998, 2002). As previously reported, treatment of the recombinant α-galactosidase expressed in this cell line with a bacterial α-mannosidase results in the exposure of M6P residues at the non-reducing ends of sugar chains (Chiba et al. 2002). As M6P residues are essential for incorporation of α-galactosidase into human cells via M6P receptors on the cell membrane (Kornfeld and Sly 2001), an α-galactosidase having many M6P residues would be beneficial for enzyme replacement therapy for Fabry disease. The yeast cell line also has a mutation in the *MNN4* gene, which regulates mannosylphosphorylation (Odani et al. 1996), resulting in the production of recombinant α-galactosidase with highly phosphorylated sugar chains. Productivity of the recombinant enzyme could be further improved in the future by careful choice of a host cell strain. We have preliminarily prepared a methylotrophic yeast cell line secreting recombinant α-galactosidase into the culture medium at a concentration of 12 mg/l, and attempts to obtain abundant enzyme protein using the improved purification method described in this report are underway.

The recombinant α-galactosidase added to the culture medium of Fabry fibroblasts was well incorporated into the cells. The accumulated CTH was cleaved, and the disappearance of deposited CTH held for at least 7 days. Incorporation of the enzyme was strongly inhibited in the presence of M6P. This finding allowed us to examine the effect of the recombinant α-galactosidase on Fabry mice.

Lee et al. (2003) reported that agalsidase beta has a higher percentage of phosphorylated oligomannose chains than agalsidase alfa, which results in improved binding of agalsidase beta to M6P receptors, and higher enzyme levels in the kidneys and heart, which are the organs most affected in Fabry disease, when tested at the same dose. Considering these results, we injected agalsidase beta into Fabry mice as a control in the experiment on the incorporation of yr-haGal into organs of Fabry mice, and its CTH-degrading activity. yr-haGal was successfully incorporated into the liver, kidneys, heart and spleen, and the CTH deposited in these organs was cleaved as in the case of agalsidase beta. However, degradation by these recombinant enzymes of CTH accumulated in the glomeruli was insufficient, although that in renal tubular cells was almost complete. Clinical trials using agalsidase beta have revealed that clearance of the CTH accumulated in podocytes was more limited than that observed in other cell types in kidney tissues (Thurnberg et al. 2002), suggesting that uptake of the recombinant α-galactosidases by podocytes is very low. There was a difference between yr-haGal and agalsidase beta in the degree of enzyme activity increase in the liver and spleen. This is probably due to differences in their sugar chain compositions. Asialylated complex-type oligosaccharides are involved in the uptake of lysosomal enzymes by hepatocytes in the liver via asialoglycoprotein receptors (Rosenfeld et al. 1986). Unlike agalsidase beta, the recombinant α-galactosidase produced in yeast cells does not have any complex-type sugar chains. Why agalsidase beta was incorporated into the spleen more than yr-haGal remains obscure. However, as Fabry disease does not affect the liver or spleen, the relatively low uptake of yr-haGal is thought not to be disadvantageous for enzyme replacement therapy for Fabry disease. Immunohistochemical analysis revealed that administration of either enzyme did not reduce granular immunofluorescence in the dorsal root ganglia from Fabry mice. Recently, we found that recombinant human β-hexosaminidases A and B, which are the lysosomal enzymes responsible for Sandhoff disease, produced in CHO cells, could be incorporated into cultured Schwann cells derived from dorsal root ganglia and adjacent peripheral nerves from Sandhoff mice via M6P receptors, but incorporation was apparently lower than that in the case of cultured Sandhoff fibroblasts
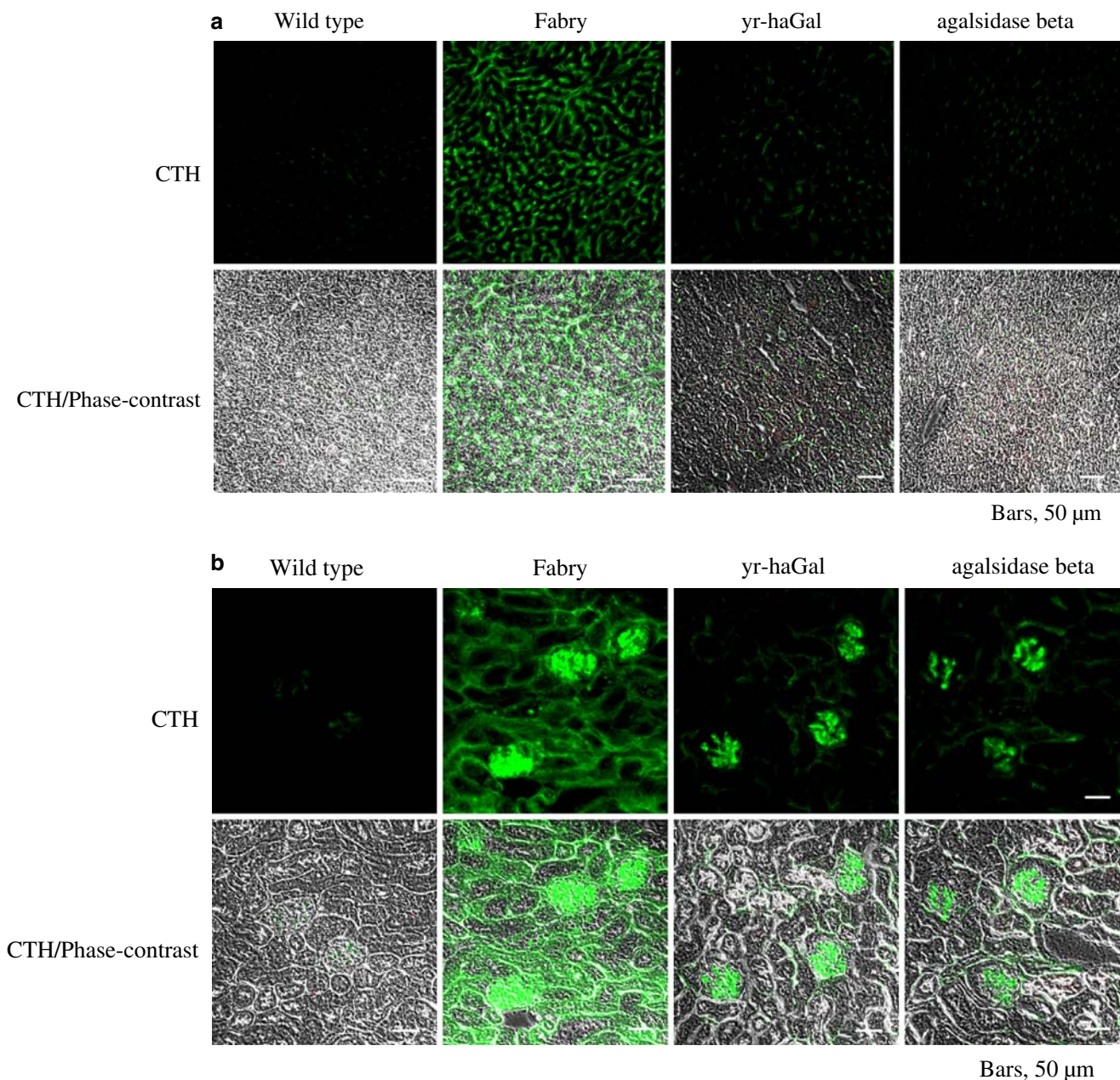
Fig. 4 Immunohistochemical analyses of the accumulated CTH in organs of Fabry mice, and its degradation by yr-haGal and agalsidase beta. Fabry mice were repeatedly injected with yr-haGal and agalsidase beta separately, and then immunostaining for CTH was performed. *CTH* Stained with an anti-CTH antibody (*green*), *CTH/Phase-contrast* overlapping CTH and phase-contrast images, *Phase-contrast* phase-contrast images. *Wild type* A wild type mouse, *Fabry* an untreated Fabry mouse, *yr-haGal* a Fabry mouse treated with yr-haGal, *agalsidase beta* a Fabry mouse treated with agalsidase beta. **a** Liver, **b** kidneys, **c** heart, **d** dorsal root ganglia. *Bars* 50 μm

(Ohsawa et al. 2005). The total number of M6P receptors on the surface of neural cells might be less than that on non-neural cells. Intravascularly administered lysosomal enzymes are barely incorporated into neural cells. Phase 3 clinical trials for agalsidase beta revealed that there were no significant differences in improvement of pain in the peripheral extremities between a group of patients treated with agalsidase beta and the placebo group (Eng et al. 2001). Some improvement is required for targeting of the enzyme to neural cells, i.e., the

production of a recombinant α-galactosidase with abundant M6P residues.

It is known that the administration of agalsidase alfa and agalsidase beta frequently causes infusion reactions, mainly allergic reactions (Schiffmann et al. 2000; Eng et al. 2001). Thus, we examined the levels of antibodies against yr-haGal in sera of recurrently injected Fabry mice by means of ELISA, but no specific antibodies for yr-haGal were detected, as was also the case of agalsidase beta under the experimental conditions used.
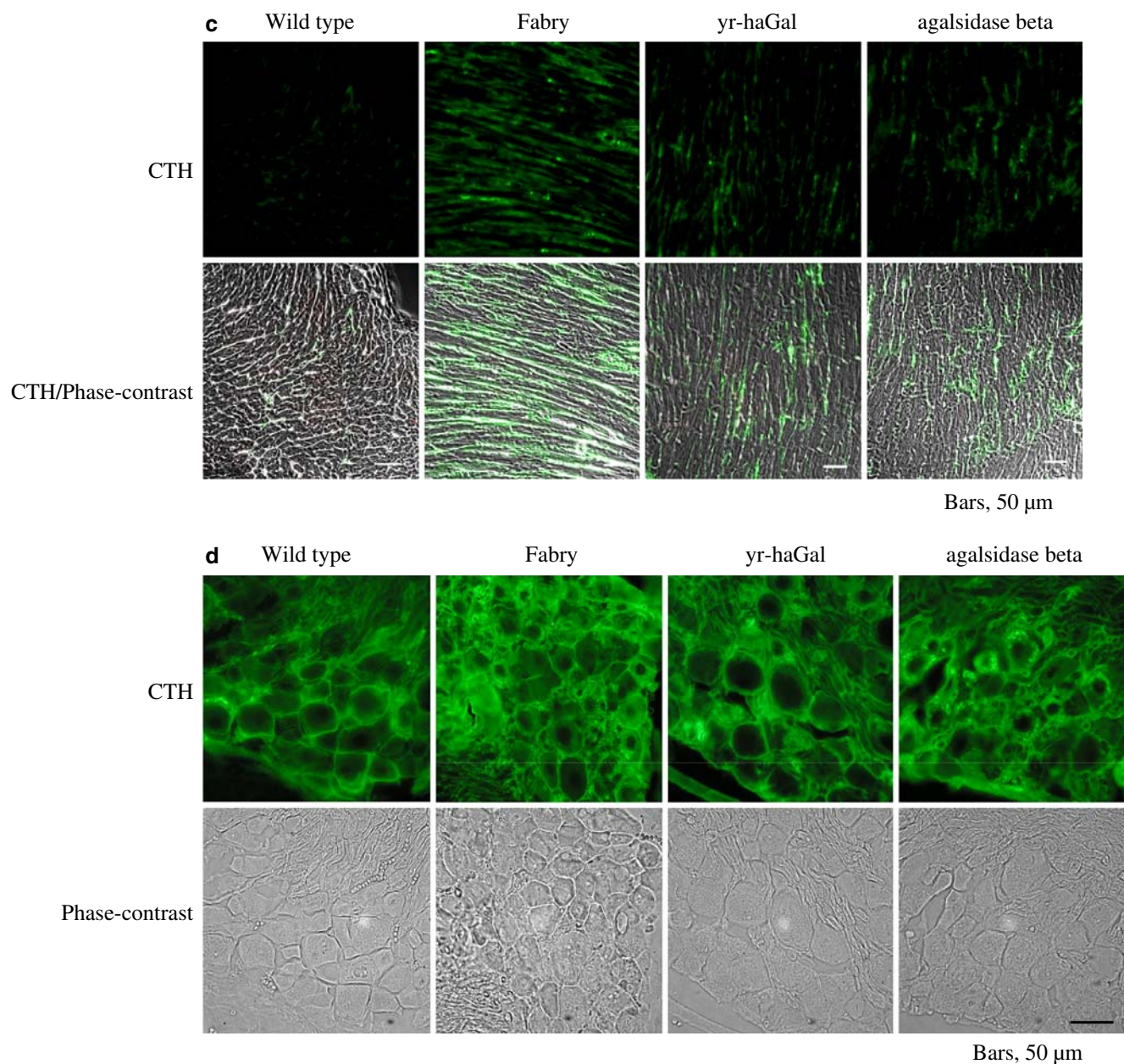
c

| | Wild type | Fabry | yr-haGal | agalsidase beta |

CTH

CTH/Phase-contrast

Bars, 50 μm

d

| | Wild type | Fabry | yr-haGal | agalsidase beta |

CTH

Phase-contrast

Bars, 50 μm

Fig. 4 (Contd.)

Table 3 Ceramide trihexoside (CTH) levels (nmol/mg protein) in organs from Fabry mice treated with four doses of yr-haGal or agalsidase beta. Fabry mice were injected with almost the same enzyme activity of yr-haGal (3 mg/kg body weight) or agalsidase beta (2 mg/kg body weight) separately every week for four doses, and were sacrificed 6 days after the last injection. *Wild type* Wild type mice, *Fabry* untreated Fabry mice, *yr-haGal* Fabry mice treated with yr-haGal, *agalsidase beta* Fabry mice treated with agalsidase beta. Values are expressed as means $\pm$ SEM ($n = 3$)

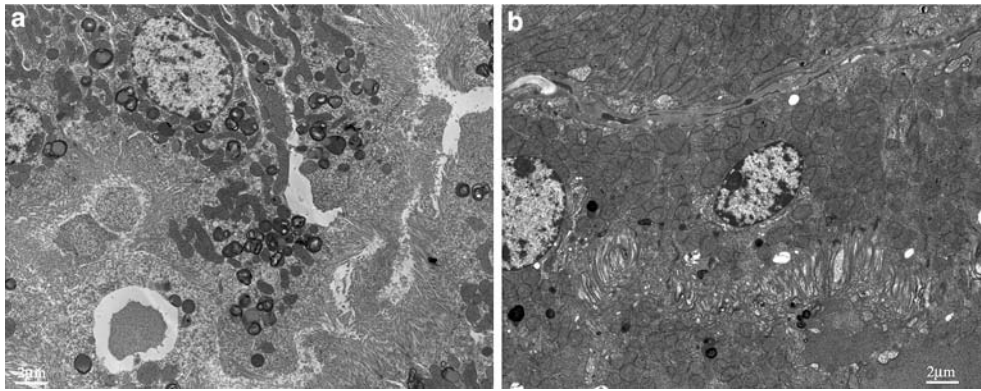|	Liver	Kidney	Heart	Spleen
Wild type	0.07 ± 0.01	0.78 ± 0.10	0.00 ± 0.00	0.34 ± 0.11
Fabry	1.40 ± 0.41	5.36 ± 0.08	2.01 ± 0.21	3.05 ± 0.33
yr-haGal	0.03 ± 0.02	3.89 ± 0.36	0.58 ± 0.16	0.96 ± 0.41
agalsidase beta	0.09 ± 0.06	3.39 ± 0.14	0.67 ± 0.34	0.37 ± 0.06

Fig. 5a,b Morphological effects of repeated administration of yr-haGal on renal tubular cells of Fabry mice. yr-haGal was repeatedly injected into Fabry mice; kidney tissues were then examined by electron microscopy. **a** An untreated Fabry mouse.

b A Fabry mouse treated with yr-haGal. Many lamellar inclusion bodies can be seen in the renal tubular cells of the untreated Fabry mice, and the number of lamellar inclusion bodies is apparently decreased after repeated administration of yr-haGal. *Bar* 2 μm

In conclusion, we produced in yeast cells a recombinant α-galactosidase with M6P residues at the non-reducing ends of *N*-linked sugar chains. This recombinant enzyme was incorporated into the liver, kidneys, heart and spleen, and degraded the accumulated CTH in these tissues, although cleavage of the CTH accumulated in the dorsal root ganglia was insufficient. As production of recombinant α-galactosidase in yeast is easy and economical, and does not require fetal calf serum, yr-haGal is highly promising as an enzyme source for enzyme replacement therapy for Fabry disease.
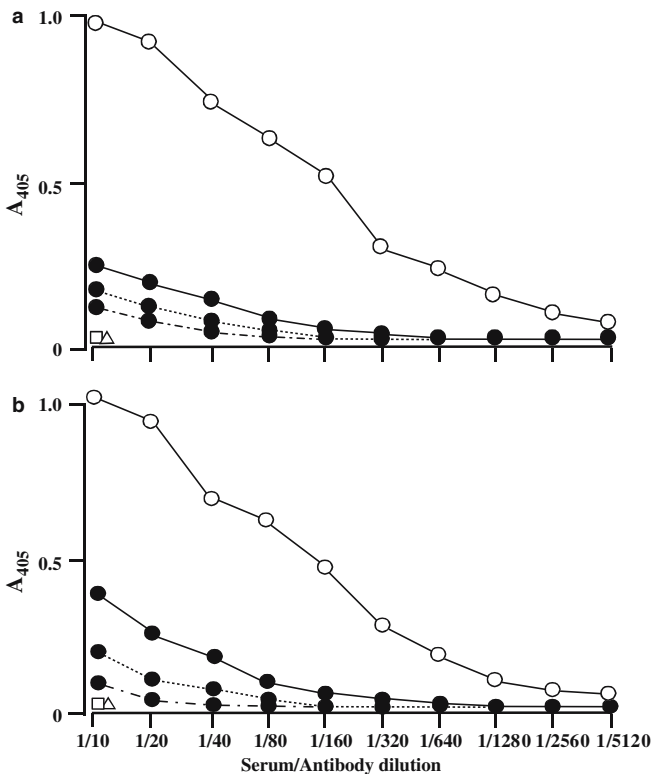
Fig. 6 Antigenicity of yr-haGal and agalsidase beta. ELISA was performed to determine whether Fabry mice recurrently injected with yr-haGal (**a**) or agalsidase beta (**b**) produced antibodies to the enzymes or not. *Open circles* Rabbit anti-α-galactosidase antibodies, *closed circles* Fabry mouse sera treated with the enzymes (yr-haGal and agalsidase beta), *open squares* serum from an untreated Fabry mouse, *open triangles* serum from an untreated wild type mouse

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