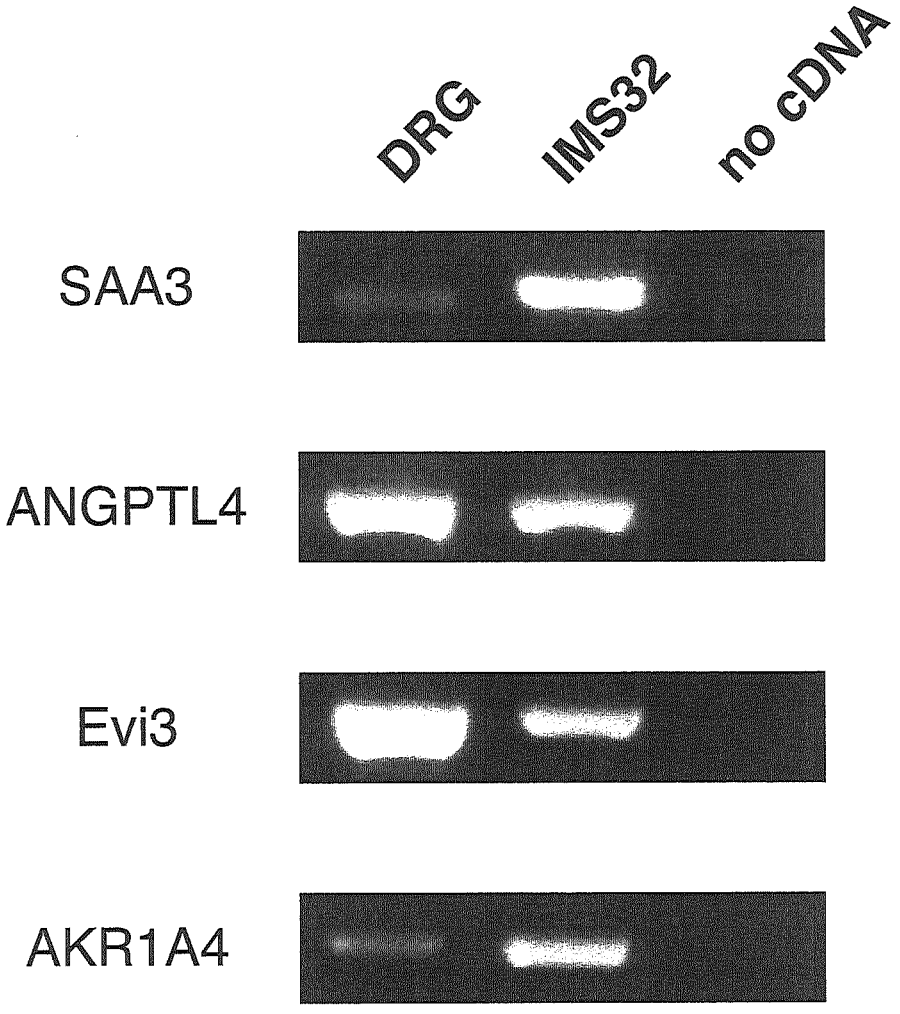
Fig.8



Original article

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

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Abstract

We have reported the production of a recombinant α-galactosidase having engineered *N*-linked sugar chains facilitating uptake and transport to lysosomes in a *Sacchromyces cerevisiae* mutant previously. In this study we improved the purification procedure and it allowed us to obtain a large amount of highly purified enzyme proteins having 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 and the accumulated ceramide trihexoside in lysosomes was cleaved dose-dependently, and the disappearance of deposited ceramide trihexoside held for at least 7 days after the administration. Then we examined the effect of the recombinant α-galactosidase on Fabry mice. Repeated intravascular administration of the enzyme led to successful degradation of the accumulated ceramide trihexoside in the liver, kidneys, heart, and spleen. But cleavage of the accumulated ceramide trihexoside in the dorsal root ganglia was not sufficient. As the culture of yeast cells is easy and economical, and it 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 for Fabry disease.

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 Gb_3) in lysosomes. This enzyme is a glycoprotein, and synthesized in rough-surfaced endoplasmic reticulum followed by the addition of *N*-linked high-mannose type oligosaccharides. Then, the enzyme is transferred to the Golgi apparatus, where further modification including addition of mannose -6-phosphate (M6P) residues and binding to M6P receptor occur. Subsequently the enzyme is transported to endosomes via M6P receptors. Then the enzyme moves to lysosomes and works there. In some type of cells including cultured fibroblasts, α-galactosidase can be incorporated into the cells via M6P receptors on the plasma membrane from the extracellular milieu and transported to lysosomes (Kornfeld and Sly 2001).

A deficiency of α-galactosidase results in widespread cellular deposition of CTH and thereby causes 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 one in 40,000 male newborn (Desnick et al. 2001). There are also variant form Fabry patients with residual α-galactosidase activity and milder clinical manifestations with late onset (Sakuraba et al. 1990; Nakao et al. 1995). As to females heterozygous for Fabry disease, they 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 who need treatment is thought to be much larger than that assumed.

Recently, two different recombinant α-galactosidases were developed for enzyme replacement therapy for Fabry disease: agalsidase alfa (Replagal®; Transkaryotic Therapies, Inc., 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, 2001b). The former has been approved in Europe, and the latter in Europe, the U.S.A., and Japan, and many Fabry disease patients have been successfully treated with these drugs. However, these recombinant enzymes have been 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 and produced a recombinant human α-galactosidase having *N*-linked high-mannose-type sugar chains in the yeast cells (yeast recombinant human α-galactosidase, yr-haGal), as described previously (Chiba et al. 2002). An effective incorporation of 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 culture medium of the yeast cells to obtain a large amount of highly purified enzyme proteins having M6P residues which facilitate incorporation of the enzyme into affected organs, and analyzed the effect of the purified enzyme on cleavage of the CTH accumulated in cultured Fabry fibroblasts and organs of Fabry mice.

Materials and methods

Purification of the recombinant human α-galactosidase (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 cereviae* strain, HPY21, as described previously (Chiba et al. 2002). A large-scale culture (100 L) was performed to examine the effect of the yr-haGal on Fabry mice in this study. We had used Blue-Sepharose and ConA-Sepharose columns to purify the yr-haGal in the previous study (Chiba et al. 2002). However these columns are very expensive and had weak binding ability because of the characteristic of the affinity chromatography. So, we improved the purification procedure. All column materials used in this experiment were purchased from Amersham Biosciences Japan (Tokyo, Japan). The culture medium of the HPY21G strain was collected and concentrated, and then ammonium sulfate was added slowly to the supernatant to a final concentration of 55%. The precipitate was recovered by centrifugation, resolved in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, and then dialyzed against the same buffer. Then, a sample was applied to a HiLoad Q 16/10 Sepharose HP column equilibrated with the same buffer. After the column had been washed, α-galactosidase was eluted with a 0~1 M NaCl gradient in the same buffer. The fractions containing the enzyme activity were pooled, and then a 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 the column had been washed, α-galactosidase

was eluted with a 0.3~0 M ammonium sulfate gradient in the same buffer. The fractions containing the enzyme activity were dialyzed against 20 mM Tris-HC1 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. Then the fractions containing the enzyme activity 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 in the previous paper (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 same conditions as described above.

Biochemical analyses of enzymatic properties of the yeast recombinant human α-galactosidase (yr-haGal)

The purity and molecular mass of the yr-haGal produced in yeast cells were determined by means of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in the previous report (Chiba et al. 2002). Reversed-phase high-performance liquid chromatography (HPLC) analysis of the purified recombinant α-galactosidase was

performed on a Cosmosyl $5C_4$-AR-300 (4.6 x 150 mm) column (Nacalai Tesque, Inc., Kyoto, Japan). The protein was eluted with a linear trifluoroacetic acid/acetonitrile gradient at the flow rate of 1 ml/min with ultraviolet detection at 215 nm. Deglycosylation of the recombinant α-galactosidase with *N*-glycanase F (Takara Bio, Inc., 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 as a standard.

Sugar chain analysis of the 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 hours, and then monosaccharides derived from the sugar chains were quantitated by means of capillary electrophoresis using a P/ACE MDQ equipped with a laser-induce fluorescence detector (Beckman Coulter, Fullerton, CA), authentic

monosaccharides being used as standards for the quantitation.

Examination of the effect of the yeast recombinant human α-galactosidase (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 a incubator containing 5% CO_2. The study involving the cultured human fibroblasts was approved by the Ethical Committee of our institute.

For examination of the uptake of the recombinant α-galactosidase by Fabry fibroblasts, the recombinant α-galactosidase produced in yeast cells was added to the culture medium of Fabry fibroblasts to give the 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 the yr-haGal, Fabry fibroblasts were cultured in the culture medium containing 5 mM of M6P and 1.0 μg/ml of the yr-haGal. After 18 hours 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. Then, an appropriate amount of water was added to the pellet and the cells were sonicated, and the resulting homogenate was used for the α-galactosidase

assay and protein determination.

To examine the degradation of the accumulated CTH by the incorporated recombinant α-galactosidase, Fabry fibroblasts were cultured with culture medium containing the recombinant α-galactosidase at the concentrations of 0, 0.5, 1.0, 2.0 and 3.0 μ g/ml for 3 days. Alternatively, Fabry fibroblasts were cultured with the culture medium containing 3.0 μ g/ml of the 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% bovine serum albumin in PBS for 1 hour. Then the cells were 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 hour. After washing, they were reacted for 1 hour with a fluorescent isothiocyanate-conjugated goat anti-mouse IgG $F(ab')_2$ (1:200 diluted; Jackson Immuno Research, West Grove, PA) and a rhodamine-conjugated goat anti-rabbit IgG $F(ab')_2$ (1:400 diluted; 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 of 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; Carl Zeiss, Oberkochen, Germany) equipped with a confocal laser scanning imaging system (LSM510; Carl Zeiss).

Examination of the effect of the yeast recombinant human α-galactosidase (yr-haGal) on Fabry mice

Fabry mice (α-galactosidase knock-out mice, denoted by Drs. 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 the 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, Co., 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/kg 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 the time course changes in the α-galactosidase activity in plasma were determined. The mice were sacrificed at 1 hour after administration of the enzymes, and then their livers, kidneys, hearts, and

spleens were removed. Then tissue samples were homogenized in citrate-phosphate buffer, pH 4.6, and centrifuged. Then the resulting supernatants were assayed for α-galactosidase activity.

To examine the 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 4 doses, and then sacrificed 6 days after the last injection. Then their livers, kidneys, hearts, spleens, and dorsal root ganglia were removed, and used as samples for biochemical and/or morphological analyses.

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

For determination of the 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, the 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 hour and dehydrated with graded concentrations of ethanol, 50% through absolute, and glycidyl n-butyl ether. Then dehydrated specimens were embedded in Epon 812 resin. Sections of 0.1 μ m 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 the Fabry mice repeatedly injected with the yr-haGal, 3.0 mg/kg body weight, and agalsidase beta, 2.0 mg/kg body weight, separately

every week for 4 doses. Briefly, a 96-well flat bottom microplate for ELISA (Immulon 2 HB; Thermo Lab Systems, Flanklin, MA) was coated with 1.0 μ g/ml of the enzymes in PBS. After washing 5 times with 1% bovine serum albumin in PBS, 200 μ l of 1% bovine serum albumin in PBS was added to each well as a blocking solution, followed by incubation for 1 hour at room temperature. After removing the blocking solution, 100 μ l 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 hour. Then the wells were washed, incubated in 100 μ l of peroxidase-conjugated anti-mouse IgG $F(ab')_2$ (1: 2,000 diluted; Jackson Immuno Research) for 45 min, washed again, and finally incubated in 100 μ l of *O*-phenylenediamine (Sigma) generated as 0.4 mg/ml of 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 the yeast recombinant human α-galactosidase (yr-haGal)

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

purification. The amount of the recombinant α-galactosidase secreted from the HPY21G strain, a *Saccharomyces cerevisiae* mutant with human α-galactosidase cDNA introduced, into the culture medium was approximately 290 μg per 1 L culture. The recovery of the enzyme through the purification procedure was 30%. The purified enzyme before treatment with α-mannosidase 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 on 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. Specific enzyme activity of the yr-haGal was 2.0 mmol/h/mg protein, which was a little higher than that purified by the previously described method (1.7 mmol/h/mg protein).

Monosaccharide composition of the yeast recombinant human α-galactosidase (yr-haGal)

The monosaccharide composition of the recombinant α-galactosidase was determined, and then compared with those of agalsidase alfa and agalsidase beta, which were 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 does not have any 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 agalisidase beta (3.1 mol/mol protein).

Effect of the yeast recombinant human α-galactosidase (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 hours. Then, the uptake of the enzyme by the Fabry fibroblasts was 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 the addition of the recombinant α-galactosidase dose-dependently and reached a normal level when the recombinant α-galactosidase was added to the culture medium at a concentration of 0.25~1.0 μ g/ml. Uptake of the yr-haGal was decreased to 15% of the control level by the addition of 5 mM M6P, suggesting that the incorporation of the enzyme may largely depend on the M6P receptor.

We also investigated the 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 1 day's incubation, immunofluorescence of α-galactosidase was detected and that of CTH was apparently decreased. After 3 days' culture, the maximum immunofluoescence 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 held for at least 7 days. Next, we added the 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. Then, double staining for α-galactosidase and CTH was performed. The results are shown in Fig. 2b. The clearance of the accumulated CTH in response to incorporation of the enzyme occurred dose-dependently.

Effect of the yeast recombinant human α-galactosidase (yr-haGal) on Fabry mice

We injected the recombinant α-galactosidase into Fabry mice to examine its therapeutic effect. We used agalsidase beta as a control enzyme because

it had been reported that agalsidase beta was 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/mg protein) was a little lower than that of the agalsidase beta sample (3.2 mmol/h/mg protein), 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, reached the maximum level at 3 min after the injection and then gradually decreased. The pattern of the pharmacokinetics was essentially the same as in the case of agalsidase beta (Fig. 3). The biodistribution of yr-haGal and agalsidase beta after the 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 when yr-haGal was administered. 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 that in the case of 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 4 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).