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November 7, 2017

Via FedEx

Paulette Gaynor, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Campus Drive
College Park, MD 20740

Re: GRAS Notification for Amano's β-Galactosidase Enzyme Preparation

Dear Dr. Gaynor:

We respectfully submit the enclosed (new) GRAS notification (in electronic format, *i.e.*, CD)[1] on behalf of our client, Amano Enzyme, Inc. (Amano) for a β-galactosidase enzyme preparation derived from *Papiliotrema terrestris* for use as a processing aid in the production of galacto-oligosaccharides (GOS), which, in turn, may be used in a variety of food applications (including products consumed by adults and infants). The enclosed GRAS notification provides detailed information related to the intended uses, manufacturing, and safety of the enzyme.

We look forward to FDA's review of this submission and would be happy to answer any questions. Thank you for your attention to this matter.

Sincerely,

(b) (6)

Melvin S. Drozen

Enclosure

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1 All electronic files included in this submission have been checked and found to be virus free.

GRAS Notification for β-Galactosidase Enzyme Preparation Derived From *Papiliotrema terrestris*

Prepared for: U.S. Food and Drug Administration
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740-3835

Prepared by: Keller and Heckman LLP
1001 G Street, NW
Suite 500 West
Washington, DC 20001

Date: November 7, 2017

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GRAS NOTICE FOR β-GALACTOSIDASE ENZYME PREPARATION DERIVED FROM *Papiliotrema terrestris*

SUBMITTED BY AMANO ENZYME, INC.

Part 1 – Signed statements and certification

1.1 Applicability of 21 C.F.R. part 170, subpart E

We submit this GRAS notice in accordance with 21 C.F.R. part 170, subpart E.

1.2 Name and address of the notifier

Company: Amano Enzyme, Inc.
Name: Mr. Tomonari Ogawa (Quality Assurance Dept. - Nagoya plant)
Address: 2-7, 1-Chome, Nishiki
Naka-ku, Nagoya, Japan 460-8630
Phone: 81 (0) 568.21.4044
Fax: 81 (0) 568.26.6160

1.3 Name of the notified substance

β-Galactosidase enzyme preparation derived from *Papiliotrema terrestris*

1.4 Applicable conditions of use of the notified substance

1.4(a) Foods in which the substance is to be used

Amano's β-galactosidase enzyme preparation ("β-galactosidase") is intended for use in the production of galacto-oligosaccharides (GOS), which is a food ingredient. GOS, in turn, may be used in a variety of conventional foods and infant formulas. For example, GOS made with Amano's β-galactosidase enzyme preparation may be used as an ingredient in infant formula (0.008 g/kg), follow-on formula (0.008 g/kg), meal replacement/ nutritional drinks (0.012 g/kg), juice (0.025 g/kg), yogurt drinks (0.024 g/kg), cereals (0.027 g/kg), snacks (0.143 g/kg), desserts (0.0.27 g/kg), or other baby foods that may contain GOS as an ingredient. Further, as an example of conventional food applications, GOS is also expected to be used in milk (0.020 g/kg), milk drinks (0.030 g/kg), flavored milk beverages (0.030 g/kg), milk substitutes (0.020 g/kg), meal replacement drinks (0.020 g/kg), yogurt (0.033 g/kg), dairy based desserts (0.043 g/kg), frozen dairy desserts (0.043 g/kg), fruit drinks (0.021 g/kg), energy drinks (0.021 g/kg), juices (0.021 g/kg), fitness water and thirst quenchers (0.013 g/kg), fruit pie filling (0.059 g/kg), fruit preparations (0.125 g/kg), cereals (0.125 g/kg), bars (0.125 g/kg), dietary supplements (0.333g/kg), fiber supplements (0.333 g/kg), and any other products that may be used by the general population that may contain GOS as an ingredient.

As listed in **Table 1**, FDA has issued numerous letters indicating that the Agency has "no questions" regarding the GRAS status of GOS produced from β-galactosidase derived

from a variety of microbial sources for use in infant formulas (IF), including follow-on formulas, and/or conventional foods (CF) as follows:

Table 1. Examples of GRAS notices describing foods that may be made with GOS produced from β-galactosidase.

GRN No.	Notifier	Date of FDA Letter	Foods	Source of β-galactosidase
236	Friesland Foods Domo	July 28, 2008	IF & CF	*Bacillus circulans*
285	GTC Nutrition	September 4, 2009	IF & CF	*B. circulans*
286	GTC Nutrition	September 4, 2009	IF	*B. circulans*
334	Yakult Pharmaceutical Industry	October 27, 2010	IF & CF	*Sporobolomyces singularis* and *Kluyveromyces lactis*
484	Clasado	May 8, 2014	CF	*Escherichia coli* BL21 (DE3) with gene from *Bifidobacterium bifidum* NCIMB
489	International Dairy Ingredients	May 22, 2014	IF & CF	*Aspergillus oryza*e
495	Clasado	May 30, 2014	IF	*E. coli* BL21 (DE3) with gene from .*B bifidum* NCIMB
518	New Francisco Biotechnology Company	December 22, 2014	CF	*B. circulans*
569	New Francisco Biotechnology Company	November 25, 2015	IF	*B. circulans*
620	Nestlé Nutrition	July 21, 2016	IF	*A. oryzae*
671	Vitalis Nutrition, Inc.	Evaluation ceased April 11, 2017	IF & CF	*A. oryzae* and *K. lactis*
721	Vitalis Nutrition, Inc.	Pending as of October 23, 2017	IF & CF	*A. oryzae* and *K. lactis*

1.4(b) Levels of use in such foods

Galacto-oligosaccharides (GOS) produced from lactose using Amano's β-galactosidase enzyme preparation maybe used as an ingredient in food at the levels for which GOS is permitted. As an example, foods potentially made with GOS, at the maximum ratios indicated, are listed in **Table 2** below.

Table 2. Maximum levels of GOS in food.

Foods Potentially Made with GOS	Serving size (g)	GOS Maximum Level of Use (g)	GOS (g/kg food)
Milk	244	5	0.020
Milk drinks	250	7.5	0.030
Meal replacement drinks	250	5	0.020
Milk substitutes	245	5	0.020
Yogurt	227	7.5	0.033
Dairy based deserts	70	3	0.043
Frozen dairy deserts	70	3	0.043
Fruit drinks and energy drinks	240	5	0.021
Fitness water and thirst quenchers	240	3	0.013
Juice	240	5	0.021
Fruit pie filling	85	5	0.059
Fruit preparation	40	5	0.125
Food Supplements (Dietary supplements)	15	5	0.333
Food Supplements (Fiber supplements)	15	5	0.333
Bars	40	5	0.125
Cereals	40	5	0.125
Infant formula for term infants	1000	8	0.008
Infant meal replacement drinks	250	3	0.012
Baby juice	120	3	0.025
Baby yogurt drink	125	3	0.024
Baby desert	110	3	0.027
Baby snack	7	1	0.143
Baby cereals	110	3	0.027

Amano's β-galactosidase enzyme preparation is used at the minimum level required to optimize production of the GOS, typically 1.5 lactose units (LU) per gram of lactose; thus, the addition rate of the enzyme equates to approximately 827 mg/kg lactose, based on 1813 LU/g of activity of the enzyme preparation (a typical activity level), as follows:

1.5 x 1000 / 1813 = 0.8274 g, or 827 mg/kg lactose.

Thus, with a total organic solids (TOS) content of 31.26% on average for the enzyme preparation (enzyme + lactose in powder form) and a GOS content of 60% for the finished GOS mixture (dry), the worst-case level of Amano's β galactosidase – in terms of the maximum theoretical level of TOS that could remain in the GOS – was calculated to be approximately 430.9 mg TOS per kg of GOS as follows:

(827 x 0.3126) ÷ 0.6 = 430.867, or 430.9.

1.4(c) Purpose for which the substance is to be used

The notified substance is intended for use in the production of GOS. β-Galactosidase catalyzes the hydrolysis of the β-1,4 glycosidic bond in β-galactosides and releases galactose and residual organic molecules. In the case of lactose as a substrate, it releases galactose and glucose. The enzyme also catalyzes the transgalactosylation of galactose under high substrate concentration conditions. In the case of lactose as the primary substrate, galactose from lactose is bonded to lactose or lactose-related galactooligosaccharide as an acceptor by transgalactosylation in β-1,4, β-1,6, β-1,3 or β-1, 2 binding modes. The enzyme activity is inhibited by Fe^{2+} but is not affected by K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}. The Isoelectric point is pH3.95 and the Km value is 3.03 mM. Amano's β-galactosidase enzyme has excellent acid resistance and temperature resistance as compared with other beta-galactosidases. Thus, heat can be used to optimize solubility of the lactose, and acid can be used to reduce browning of the GOS that is associated with high pH.

1.4(d) Description of the population expected to use the substance

GOS produced using Amano's β-galactosidase enzyme preparation is expected to be consumed by newborns, as well as older infants and toddlers who use infant formula, follow-on formula, nutritional drinks, juice, yogurt, cereals, snacks, desserts, or other baby foods that may contain GOS as an ingredient. Further, GOS produced using Amano's β-galactosidase enzyme preparation is also expected to be consumed by any population that consumes milk, milk drinks, flavored milk beverages, milk substitutes, meal replacement drinks, yogurt, dairy based desserts, frozen dairy desserts, fruit drinks, energy drinks, juices, fortified water, fruit pie filling, fruit preparations, jelly, jam, cereals, or any other products that may contain GOS as an ingredient.

1.5 Basis for the GRAS determination

The statutory basis for our conclusion of GRAS status is through scientific procedures in accordance with 21 C.F.R. §§ 170.30(a) and (b).

1.6 Exclusion from premarket approval

The notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act (FD&C Act) based on our conclusion that the notified substance is GRAS under the conditions of its intended use.

1.7 Availability of data and information

If the Food and Drug Administration (FDA) asks to see the data and information that are the bases for our conclusion of GRAS status, either during or after FDA's evaluation of our notice, we agree to make the data and information available to FDA. Further, upon FDA's request, we will allow the Agency to review and copy the data and information during customary business hours at the above address, and will provide FDA with a complete copy of the data and information, either in an electronic format that is accessible for the Agency's evaluation, or on paper.

1.8 Applicability of FOIA exemptions

This GRAS notice does not contain confidential business information (CBI) exempt from disclosure under the Freedom of Information Act per 5 U.S.C. § 552(b)(4).

1.9 Certification

We certify that, to the best of our knowledge, our GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(b) (6)

Nov. 7, 2017

Name: Tomonari Ogawa
Title: Director, Quality Assurance Division

Date

Please address correspondence to Amano's counsel:

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, N.W., Suite 500 West
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Phone: (202) 434-4222
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Part 2 – Identity, method of manufacture, specifications, and physical or technical effect

2.1 Scientific data and information that identifies the notified substance

The β-galactosidase enzyme preparation contains not less than 1,700 active units per gram in a food grade powder (lactose) that may appropriately be used as a carrier for enzyme preparations in accordance with good manufacturing practice (GMP). The β-galactosidase enzyme component is extracted and purified from the fermentation and processing of a stable non-pathogenic and non-toxicogenic strain of the yeast-like fungus *Papiliotrema terrestris* which was obtained by classical mutation with n-methyl-n'-nitro-n-nitrosoguanidine (NTG). More detailed information regarding the product's identity is provided as follows:

2.1(a) Common or usual name

Beta-galactosidase

2.1(b) International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Nomenclature Chemical name

β-D-galactoside galactohydrolase

2.1(c) IUMB Number

EC 3.2.1.23

2.1(d) Chemical Abstracts Service Registry Number (CASRN)

9031-11-2

2.1(e) European INventory of Existing Commercial chemical Substances (EINECS) Number

232-864-1

2.1(f) Synonyms

Lactase; β-lactosidase; maxilact; hydrolact; β-D-lactosidase; S 2107; lactozym; trilactase; β-D-galactanase; oryzatym; sumiklat

2.1(g) Enzyme specificity

The β-galactosidase enzyme is a hydrolase that can transfer non-reducing β-D-galactose residues from β-D-galactosides (*e.g.*, lactose) to water. Under high lactose concentrations and specific processing conditions, the enzyme can utilize lactose as an alternative acceptor instead of water, resulting in the formation of GOS.

2.1(h) Amino acid sequence

Amano's β-galactosidase consists of a mixture of 3 enzymes: the full-size protein consisting of 566 amino acids, and two proteins with N-terminal truncations of 6 and 11 amino acids each, resulting in proteins consisting of 560 and 555 amino acids respectively. Aside from the N-terminal truncations, the 3 proteins are identical in sequence. The full (566 amino acid) β-galactosidase protein sequence is provided in **Figure 1** below.

Figure 1. Amino acid sequence (full) of β-galactosidase from *Papiliotrema terrestris*

```
1    ATTNQDAITP TATGPVGGQG TPAVNFTDYS SSSLEQFWND WVGEVEEPPF AYVPEPPNPY   60

61   PLPNAPPPIY PEYYTKRPKD ILPDYKFPKD FLFGWATAAQ QWEGAVKADG KGPSIWDWAS  120

121  RFPGFIADNT TSDVGDLGYY LYKEDLARIA ALGANVYSFS MFWTRIFPFG KADSPVNQAG  180

181  IDFYHDLIDY SWSLGIEPVV TLFHWDTPLA LQLEYGGFAS ERIIDDYVNY AETVFKAYNG  240

241  SVHKWVTFNE PVVFCSQMAA PVNTTLPPNL NSTIYPYTCS YHLVLAHAKT VKRFRELNIQ  300

301  GQIAFKSDNF VGIPWREGNQ EDIDAVERHQ AYQIGIFAEP IYNTGDWPDI VKNDLSPDIL  360

361  PRFTDDEIAM IKCTADFFPI DGYRDGYVQA VPGGVEACVA NISNPLWPAC NQVNFYDSTP  420

421  AGWAIGTFGN WPTTPWLQNT WQFVRPFLAD LAKRYPTEGG IYLSEFGFSE PFENDKTFIY  480

481  QITQDSGRTA YFNSYLGEVL KGIVEDGIPI KGVFGWSMVD NFEWNSGLST RFGVQYVDYN  540

541  SPTRQRTFKR SALEMSEFWN AHRCSA                                       566
```

2.1(i) Calculated molecular weight

Based on the amino acid sequences shown above, the theoretical molecular weights of the enzyme proteins have been calculated and are estimated to be 63.9, 63.3 and 62.8 using the Compute pI/Mw model using the Expasy Bioinformatics site (http://web.expasy.org/compute_pi/).

2.1(j) Information regarding the source of the notified substance

The β-galactosidase production strain, *Papiliotrema terrestris*, was obtained via conventional mutation with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) from the parent strain. Accordingly, Amano's β-galactosidase production strain does not contain any recombinant, foreign, or non-native DNA.

The parent strain, isolated from soil, was originally identified based on phenotypic properties corresponding to *Cryptococcus terrestris* (Crestani *et al.*, 2009) as follows:

- On YM agar after 3 days at 25℃, colonies are smooth, mucous to butyrous, glistening and cream-colored with a well-defined margin.

- Vegetative cells are sub-spherical to elliptical or oval, and budding is monopolar.
- Formation of sexual reproductive organ was not observed in the plate after 3 weeks of culture.

Further, a search in the National Center for Biotechnology Information's GenBank® database shows 100% homology of the nucleotidesequence of the D1/D2 domain of the 26S rDNA of the parent strain and the type culture, *Cryptococcus terrestris* CBS 10810 (also called *Papiliotrema terrestris*). Additionally, the phenotypic properties listed above were also observed for the producing strain, *P. terrestris*.

More recent work on reconstructing the phylogeny of the majority of described anamorphic and teleomorphic Tremellomycetous yeasts (Liu, *et al.*, 2015a, 2015b) proposed amending the genus *Papiliotrema* to include, among others, *Cryptococcus terrestris* CBS 10810. Accordingly, as reflected in the Mycobank database, *Papiliotrema terrestris* and *Cryptococcus terrestris* are synonymous, while the currently preferred name is *Papiliotrema terrestris*.

As discussed in a bulletin by Tanaka and Matsumoto (1998), the production of GOS using β-galactosidase derived from a closely related species, *P. laurentii* (formerly *C. laurentii*), was patented in Japan (Patent No. 9796) over 25 years ago and commercialized soon afterwards. We are not aware of any adverse effects reported for the use of such GOS products in food.

2.1(k) Taxonomic classification of the production strain

Super Kingdom	Eukaryote
Kingdom	Fungus
Phylum	Basidiomycota
Order	Tremellomycetes
Class	Tremellales
Family	Tremellaceae
Genus	*Papiliotrema*
Species	*Papiliotrema terrestris*

2.1(l) Absence of known toxicants in the source

As noted above, the production organism belongs to a genus, *Papiliotrema*, that was proposed in 2015. Under the conventional classification prior to amendment, the production organism was in a genus, *Cryptococcus*, which contains two major pathogenic species. The major species of *Cryptococcus* that can cause illness (by opportunistic

infection) in humans are *C. neoformans* and, less commonly, *C. gatti*, both of which are most commonly found in pigeon droppings. These species are infectious through inhalation (with no reports of infection by oral exposure). *C. neoformans* and *C. gatti* most commonly infect the lungs or the central nervous system (the brain and spinal cord), but can also affect other parts of the body. Pneumonia-like symptoms (cough, shortness of breath, chest pain, fever) occur from infection of the lungs. Meningitis symptoms (headache, fever, neck pain, nausea, vomiting, sensitivity to light, confusion, or changes in behavior) occur from infection of the brain. Per Cheng *et al.* (2001), two other species, namely *C. laurentii* and *C. albidus* have also been reported to occasionally cause infection in patients with compromised immunity. According to the U.S. Centers for Disease Control and Prevention (CDC), most cases of cryptococcosis (also called cryptococcal disease) occur in people who have weakened immune systems, particularly those who have advanced HIV/AIDS.

Again, however, Liu *et al.* (2015b) have recommended reclassification of *Cryptococcus terrestris* to a different genus (*Papiliotrema*) than the pathogenic species, based on more advanced taxonomical methods. *Papiliotrema* (*Cryptococcus*) are ubiquitous in the soil. Nevertheless, we have searched the publicly available literature and found no reports of pathogenicity associated with Amano's production strain in searching by either name, *Cryptococcus terrestris* or *Papiliotrema terrestris*. Additionally, we note that *C. terrestris* (*P. terrestris*) does not appear on any public registries of hazardous microorganisms, which we have checked as follows: (1) the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agent at work, (2) the list of microbiological hazards of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), and (3) the list of pathogens on the Belgian Biosafety Server (2010). Further, the biosafety level (BSL) classification for *C. terrestris* (*P. terrestris*) by the American Type Culture Collection (ATCC) is BSL-1, which is for organisms that are not known to cause disease in healthy adult humans and, thus, is subject to the least restrictive laboratory practice recommendations established by the CDC. The safety of the production strain is corroborated by the lack of pathogenicity found in toxicological studies, as discussed in Part 6 of this GRAS notice.

2.1(m) Pariza and Johnson decision tree analysis

The β-galactosidase may be "accepted" for the intended use per an analysis using the Pariza and Johnson decision tree (Pariza and Johnson, 2001) as presented in **Figure 2** below.

Figure 2. Pariza and Johnson decision tree analysis of the β-galactosidase from *Papiliotrema terrestris*

1. *Is the production strain genetically modified?*

   YES; The β-galactosidase production strain in this case, *Papiliotrema terrestris*, was obtained via conventional mutation with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) from the parent strain.

If yes, go to 2. If no, go to 6.

2. *Is the production strain modified using rDNA techniques?*

NO

If yes, go to 3. If no, go to 5.

5. *Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects, which may result in the synthesis of toxins or other unsafe metabolites, will not arise due to the genetic modification method that was employed?*

YES; The base production strain is non-pathogenic and non-toxigenic. The final enzyme product is highly purified, which is intended to remove contaminants and the production organism. Further, traditional toxicological studies have been performed that support the safety of the product.

If yes, go to 6. If no, go to 7.

6. *Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?*

NO

If yes, the test article is ACCEPTED. *If no, go to 7.*

7. Is the organism nonpathogenic?

YES

If yes, go to 8. If no, go to 12.

8. Is the test article free of antibiotics?

YES

If yes, go to 9. If no, go to 12.

9. Is the test article free of oral toxin so known to be produced by other members of the same species?

YES

If yes, go to 11. If no, go to 10.

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?

YES

*If yes, the test article is **ACCEPTED**. If no, go to 12.*

2.1(n) Predicted toxicity of peptides

An *in silico* assessment of β-galactosidase from *P. terrestris* was performed to determine what effects simulated digestion would have on the enzyme, and if those effects produced potentially toxigenic peptides. This was accomplished via a two-step process. First, β-galactosidase was subjected to simulated digestion in the form of a set of digestive enzymes using Expasy PeptideCutter (http://web.expasy.org/peptide_cutter/). The results of this analysis were then compared to a database of known toxins and toxic peptides in the Toxic Exposome Database (http://www.t3db.ca/) to determine if exposure to, and subsequent digestion of, β-galactosidase would result in potential exposure to toxigenic peptide sequences. Further, in consideration of the possibility of incomplete digestion of protein by newborn babies who may have less efficient digestive systems, the entire amino acid sequence of the β-galactosidase was screened using ToxinPred (http://crdd.osdd.net/raghava//toxinpred/index.html).

To simulate digestion, the complete amino acid sequence of β-galactosidase was subjected to a set of gastrointestinal peptidases using PeptideCutter. This set of enzymes consisted of pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4), and chymotrypsin (EC 3.4.21.1), which are commonly used to simulate digestion for *in silico* analyses (*see e.g.*, Vercruysse L, *et al.* (2009) and Lafarga T, *et al.* (2014). The analysis returned a total of 231 unique cleavages of β-galactosidase with resulting cleavage products ranging from 1 to 25 amino acids in length. Most peptides produced contained 4 amino acids or less (205 of 231, or ~89%).

The amino acid sequences were further analyzed for toxigenic sequences by comparison to the Toxic Exposome Database. The database is a bioinformatic resource with compound specific information on over 3,600 toxins, including toxigenic proteins, bacterial, and fungal toxins. The database is peer reviewed, and updated periodically as described in Lim E, *et al.* (2010) and Wishart D, *et al.* (2015). An analysis of the peptide sequences produced by the simulated digestion of β-galactosidase using PeptideCutter was performed using standard parameters (*i.e.*, alignment, gap penalties, etc.) and returned no matches to toxins in the Toxic Exposome Database.

ToxinPred was used to further assess the potential of the β-galactosidase to produce toxigenic peptides during infant digestion. Infant digestive systems are not yet fully developed, and as such it is more difficult to predict exactly what peptides will be produced from digestion of proteins. The issue of partial digestion is addressed by the ToxinPred tool, which uses a sliding window approach, described in Gupta *et al.* (2013),

in which every potential peptide produced by cleavage of β-galactosidase, ranging from 10 to 50 amino acids in length, are assessed for toxigenic potential. This analysis indicated no homologies with known toxic peptides.

Thus, *in silico* analysis using publicly available bioinformatics resources indicates the β-galactosidase and its metabolized products are not expected to be toxigenic. The peptides are further digested by various peptidases to free amino acids, which are absorbed in the small intestine. If not digested completely by the digestive system, the peptides would be excreted without adverse effects.

The safety of the enzyme preparation is corroborated by the results of toxicological studies, as discussed in Part 6 of this GRAS notice.

2.1(o) Allergenicity

In contrast to most known food allergens, which are naturally present in a narrow range of foods, a wide variety of enzyme classes (and structures), are naturally present in food. Further, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Such is the case with β-galactosidase. Additionally, Amano found no matches in comparing the amino acid sequence of the β-galactosidase to the primary sequences of known allergens based on a search of the Structural Database of Allergenic Proteins, including a search for ≥ 35% homology over a sliding "window" of 80 amino acids, and an exact match of 8 contiguous amino acids.

Regarding potential impurities, the manufacturing process for β-galactosidase enzyme preparation employs standard materials that are routinely used in the enzyme industry. No ingredients used in production of the enzyme contain protein derived from one of the "major food allergens" designated by the Food Allergen Labeling and Consumer Protection Act (FALCPA). Furthermore, the production media will be consumed by the microorganism as the nutrient in the fermentation process. Additionally, levels of any protein residue remaining after fermentation will be reduced by the ultrafiltration procedure described in Table 3 (Detailed description of the manufacturing process) below.

Additional indicators of a lack of allergenic potential for the β galactosidase from *Papiliotrema terrestris* are noted as follows:

- Bindslev-Jensen *et al.* (2006) concluded that food allergy is not likely to be a concern regarding ingestion of food enzymes in general based on a study of enzymes produced by wild-type and genetically modified strains, as well as wild-type enzymes and Protein Engineered variants in 400 patients diagnosed with allergies to inhalation allergens, food allergens, bees, or wasps.

- An expert group convened by the Association of Manufacturers & Formulators of Enzyme Products (AMFEP), *i.e.*, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food, evaluated the existing scientific data and concluded that for exposure by ingestion, as opposed to exposure by inhalation, enzyme proteins are not potent allergens and sensitization to ingested enzymes is rare.

- Wüthrich (1996) published a list of enzymes that are often ingested daily over many years as digestive aids, at much higher amounts compared to enzymes present in food (up to 1 million times more), and concluded that such enzymes are not potent allergens by ingestion.

Thus, scientific data do not indicate that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers. Additional considerations supporting the conclusion that allergenicity is not a concern for ingestion of enzymes in foods are outlined as follows:

- Most proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens.

- Whereas food enzymes are used in small amounts during food processing, resulting in very small amounts of the enzyme protein, if any, in the final food, we noted that a high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk. *See e.g.*, Goodman *et al.* (2008) and the World Health Organization (WHO; 2001).

- Where proteins are denatured, as is β-galactosidase used to produce GOS in this case, the tertiary conformation of the enzyme molecule is destroyed, which is an alteration in protein conformation that is generally associated with a decrease in the antigenic reactivity in humans in the vast majority of investigated cases where denatured proteins are found to be much less immunogenic than the corresponding native proteins. *See e.g.*, Kikuchi *et al.* (2006), Nakazawa *et al.* (2005), Takai *et al.* (1997), and Valenta (2002a and 2002b).

2.1(p) Inactivation of any residual β-galactosidase in GOS

No β-galactosidase activity was found at a limit of detection (LOD) of 0.005 Lactose Units (LU) per gram in GOS produced under the expected manufacturing conditions, as demonstrated in the analytical report provided in **Appendix 1 (Report of residual enzyme activities in GOS)**. The analytical report also provides details on validation of the LOD.

2.2 Description of the method of manufacture

The β-galactosidase enzyme preparation is manufactured using a pure culture (*i.e.*, *Papiliotrema terrestris*) under controlled fermentation conditions. Production of the enzyme complies with FDA's current good manufacturing practice (CGMP), hazard analysis, and risk-based preventive controls (HARPC) for human food regulations set forth in 21 C.F.R. Part 117. Further, the manufacturing process is performed in accordance with the FSSC 22000 Food Safety System Certification.

Table 3: Detailed description of the manufacturing process

Step		Control points
Fermentation	Seed fermentation	Temperature pH Microbial observation Agitation Air supply
	Main fermentation	
Filtration	Filter press	Temperature
Concentration	Ultrafiltration	Temperature
Filtration	Filter press	Temperature
	Micro filtration	Extrusion pressure Temperature
Drying	Spray drying	Temperature Degree of vacuum
Sifting and Magnet		
Bulk Powder		Enzyme activity Lead Total viable aerobic count Coliforms
Formulation (with lactose powder)		Enzyme activity Lead Coliforms *Escherichia coli* *Salmonella*

Each raw material used in the manufacturing steps outlined above has an appropriate FDA regulatory status for its intended use and is of a grade suitable for use in producing food. In the future, should Amano choose to modify the raw materials or processing aids used in the production of the β-galactosidase enzyme preparation, the Company will ensure that all such components are safe and suitable for their intended use.

2.3 Specifications of the food-grade material

Specifications for the β-galactosidase enzyme preparation are in keeping with the requirements of the Food Chemicals Codex (10th Edition) and Joint FAO/WHO Expert Committee on Food Additives (JECFA 2006) monographs for enzyme preparations used in food processing. Table 4 below identifies the specifications for the β-galactosidase enzyme preparation, as well as the results of analyses for three batches.

Table 4. Specifications for the β-galactosidase enzyme preparation.

Item (Method)	Unit:	Lot number (Manufacturing date)			Target value
		GFEO1250131SDR (Dec. 1, 2016)	GFEO1250133SDR (Dec. 1, 2016)	GFEO1250531SDR (Dec. 5, 2016)	
β-galactosidase activity	(U/g)	1,850	1,750	1,840	≥ 1,700
Loss on drying	(%)	3.8	3.4	3.9	< 10
Lead	(mg/g)	0.009	0.027	0.015	< 5
Total viable aerobic count	(CFU/g)	< 10	< 10	< 10	< 1000
Coliforms	(CFU/g)	< 10	< 10	< 10	< 30
E. coli	(in 25 g)	Negative	Negative	Negative	Negative
Salmonella	(in 25 g)	Negative	Negative	Negative	Negative

β-galactosidase activity is measured by Amano's in-house lactose substrate method, which is provided in **Appendix 2 (Lactose substrate method)**. Loss on drying is measured by the difference in weight before-and-after drying a 1 gram sample at 105°C for 4 hours. Lead is measured by the JECFA method (Measurement of minerals and metals by Inductively Coupled Plasma –Atomic Emission spectrophotometric (ICP-AES) Technique), *see* Combined Compendium of Food Additive Specifications (Volume 4) at page 66. Microbial testing is conducted per FDA's Bacteriological Analytical Manual (BAM), except for total viable aerobic count (*i.e.*, plate count), which is conducted using soybean casein digest (SCD) agar.

The specification for lead (*i.e.*, Max. 5 ppm) is the same as the acceptance criteria for lead of NMT 5 mg/kg that is specified in the FCC 10 and JECFA 2006 monographs for enzyme preparations used in food processing. Likewise, the microbial specifications, *i.e.*, for coliforms (30 cfu/g), *E. coli* (Absent in 25 g in JECFA; no FCC specification), and *Salmonella* (Negative/25 g) are the same or more stringent (in the case of *E. coli*) than the specifications for these impurities in the FCC 10 or JECFA 2006 monographs. In addition to meeting the FCC and JECFA monographs for enzyme preparations, the Notifier has additionally specified limits for total viable aerobic plate count, which is commonly identified as of concern to the food industry. There are no extraordinary concerns, however, for any impurities in the Notifier's β-galactosidase enzyme preparation.

2.4 Data and information bearing on the physical or other technical effect

The mode of action of the enzyme is presented at Part 1.4(c) above. Our GRAS notice does not otherwise include data and other information bearing on a physical or other technical effect because such data and other information are not necessary to demonstrate safety.

Part 3 – Dietary exposure

As described above in Part 1.4(b), the concentration of β-galactosidase enzyme preparation would be approximately 430.9 mg TOS per kg of GOS if all the enzyme used to produce GOS were to remain in the finished food ingredient. Under this worst-case assumption, we have calculated the theoretical estimated daily intake (EDI) of β-galactosidase for (1) the general population, as relevant to conventional foods and dietary supplements made with GOS, (2) infants (<6 months), as relevant to infant formula, and (3) small children (≤ 2 years), as relevant to infant meal replacement drinks and "baby" products (juice, yogurt drink, desert, snack, and cereal).

3.1 Theoretical EDI for the general population

Under the exaggerative pretext that GOS contains the enzyme preparation at a level of 430.9 mg TOS/kg, and conservatively assuming that all the food and beverage consumed by a person each day will contain GOS and that all such food and beverage will contain GOS at a use level of 0.125 GOS/kg food (12.5%), which is the maximum use level at which we understand GOS could potentially be used in conventional foods (*see* Table 2 at Part 1.4(b) above), based on FDA's assumptions for average daily intake (*i.e.*, 3 kg/person/day, which includes both solid and liquid food) and average body weight (*i.e.*, 60 kg), we calculate the EDI of β-galactosidase as follows:

3 kg/p/day x 12.5% x 430.9 mg TOS/kg GOS ÷ 60 kg bw = **2.69 mg TOS/kg bw/day**.

Additionally, we have also assumed that a person may additionally consume dietary supplements and fiber supplements, each made with GOS at a level of 0.333 GOS/kg (33.3%), and that a person consumes one 15 g serving of dietary supplement and one 15 g serving of fiber supplements (30 g total) daily. Thus, we calculate the EDI of β-galactosidase from the consumption of dietary supplements as follows:

30 g/p/day x 33.3% x 430.9 mg TOS/kg GOS ÷ 60 kg bw = **0.072 mg TOS/kg bw/day**.

Based on the foregoing, we calculate the worst-case EDI of β-galactosidase for the general population as follows:

2.69 mg TOS/kg bw/day + 0.072 mg TOS/kg bw/day = **2.76 mg TOS/kg bw/day**.

3.2 Theoretical EDI for infants

We have assumed that infant formula made with GOS produced with Amano's enzyme preparation may be the sole source of nutrition for infants for a duration of 6 months and that the average body weight for an infant is 6.3 kg. Where FDA assumes an average daily food intake of 0.9 kg/p/day for infants, however, we have assumed the average daily food intake for an infant is 1 kg/p/day. Regarding the corresponding intake level of β-galactosidase, we have assumed that GOS is used in infant formula for term infants at a level of 0.8%, a level which is roughly the same as the level addressed in GRN 620 (noted in Table 1 at Section 1.4(a) above) for the use of GOS as an ingredient in non-exempt term infant and toddler formula at a use level providing up to 7.8 g of GOS per liter of formula as

consumed (*i.e.*, 0.78%). Based on the foregoing, we calculate the worst-case EDI of β-galactosidase for infants (0 to 6 months) as follows:

1 kg/p/day x 0.8% x 430.9 mg TOS/kg GOS ÷ 6.3 kg bw = **0.55 mg TOS/kg bw/day**

3.3 Theoretical EDI for small children (≤ 2 years)

While older infants (6-12 months) and small children (≤ 2 years) may consume conventional foods made with GOS, this is not likely to happen concurrently with the consumption of "baby" foods made with GOS due to physical limitations on how much food can be consumed each day. Further, dietary supplements made with GOS are targeted to adults and, thus, are not likely to be consumed by children 2 years old or younger. Accordingly, we have conservatively assumed that small children will consume one serving of infant formula (8 g GOS) and one serving each of infant meal replacement drinks (3 g GOS), baby juice (3 g GOS), baby yogurt drink (3 g GOS), baby desert (3 g GOS), baby snack (1 g GOS), and baby cereal (3 GOS) each day. We have also adopted FDA's assumption regarding the body weight for a 2-year old (*i.e.*, 12 kg). In this regard, smaller children, weighing less, would also eat less; moreover, it is exaggerative to assume that a child of any age or size would consume all of the foods made with GOS each day. Thus, based on these exaggerations and the average body weight of a 2-year old, we calculate the worst-case EDI of β-galactosidase for infants (6 to 12 months) and small children (≤ 2 years) would be:

(8 + 3 + 3 + 3 + 3 + 1 + 3) g GOS/p/day x 0.001 g/kg x 430.9 mg TOS/kg GOS ÷ 12 kg bw = **0.86 mg TOS/kg bw/day.**

Part 4 – Self-limiting levels of use

The use of Amano's β-galactosidase enzyme preparation is not self-limiting. Due to the cost of the product, however, the amount of enzyme used is not expected to be significantly higher than the minimum level required for optimal synthesis of galacto-oligosaccharides (GOS).

Part 5 – Experience based on common use in food before 1958

N/A

Part 6 – Narrative

6.1 Introduction

Amano has determined that its β-galactosidase enzyme preparation is GRAS based on the following:

- The identity and specifications for the β-galactosidase enzyme preparation;
- The safety of the production microorganism;
- Acceptability by analysis under the Pariza & Johnson decision tree;
- Lack of homology of digestion products of the enzyme with known toxins by i*n silico* analysis;
- Low allergenicity risk for enzymes in general and especially denatured enzymes;
- The manufacturing process for the β-galactosidase enzyme preparation;
- The intended use of the β-galactosidase enzyme;
- Supportive evidence regarding the GRAS status of other β-galactosidase enzyme preparations used in the production of GOS and β-galactosidase

As discussed in detail below, Amano's GRAS conclusion is corroborated by existing clearances for comparable substances and by toxicological data, which indicate the source organism is not pathogenic and establish a no-observed-adverse effects level (NOAEL) for the enzyme that is well over a 100-times greater than the theoretical worst-case EDIs for all populations that may consume foods made with GOS.

6.2 Existing clearances for comparable substances

FDA has issued numerous letters indicating that the Agency has "no questions" regarding the GRAS status of GOS produced from β-galactosidase derived from a variety of microbial sources for use in infant formulas (IF), including follow-on formulas, and/or conventional foods (CF) as presented in As noted in Table 1 at Part 1.4(a) above.

FDA has also issued letters stating the Agency has "no questions" about the GRAS status of three β-galactosidase enzyme preparations for use in the production of GOS as follows:

- GRN 485: β-galactosidase derived from recombinant *Escherichia coli* BL21 (DE3) (Clasado; effective April 15, 2014)
- GRN 579: Lactase from *Bifidobacterium bifidum* produced in *Bacillus subtilis* (effective November 5, 2015)

- GRN 649: β-galactosidase derived from *Bacillus circulans* produced in *Bacillus subtilis* (GenoFocus; effective November 28, 2016)

6.3 Safety data

The production organism of the β-galactosidase enzyme, *P. terrestris*, belongs to a genus (*Papiliotrema*) of organisms that is ubiquitous in the soil with no reports of pathogenicity, the organism is removed from the enzyme during production, and any enzyme remaining in GOS that is produced using the enzyme is inactive and the amino acid sequences of the enzyme and its digestion products are not homologous to any known toxins. Further, a closely related strain of *Papiliotrema* has been used to safely produce β-galactosidase for the manufacture of GOS for over 25 years in Japan.

Additionally, active β-galactosidases of microbial and human origin are naturally present in the gastrointestinal tract. Further, Pariza and Foster (1983) have noted that the results of exhaustive literature reviews, conducted by FDA, relating to the safety of microbial and non-microbial enzymes used in food production, support the position that enzymes from non-toxicogenic, non-pathogenic organisms are safe to consume. Nevertheless, in corroboration of the GRAS conclusion for the intended use of the enzyme, Amano sponsored a series of toxicological tests of the β-galactosidase concentrate and pathogenicity study for the source organism (*Papiliotrema terrestris* and *Cryptococcus terrestris* are synonymous, while the currently preferred name is *Papiliotrema terrestris*). The study reports are provided as follows:

Appendix 3. Single intravenous inoculation of *Cryptococcus terrestris* suspensions and single oral inoculation of *Cryptococcus terrestris* culture in rats. Study No 360030, Nihon Bioresearch Inc., August 22, 2016.

Appendix 4. A bacterial reverse mutation test of β-galactosidase concentrate, Study No. T-2095, BoZo Research Center, March 9, 2016.

Appendix 5. Chromosome aberration test in cultured mammalian cells treated with β-galactosidase concentrate, Study No. T-G212, BoZo Research Center, September 15, 2016.

Appendix 6. A 13-week oral gavage toxicity study of beta-galactosidase concentrate in rats, Experiment No. TT-160003, BoZo Research Center, January 12, 2017.

6.3(a) Summary of pathogenicity study

Male and female Slc: ICR mice were inoculated once, either intravenously with *P. terrestris* suspensions or orally with a *P. terrestris* culture. Washed yeast suspensions in saline were used for the intravenous inoculations, and a yeast suspension in medium was used for the oral inoculations. For intravenous inoculation, concentrations of the yeast were set at 2.2×10^7, 2.5×10^8, and 2.4×10^9 CFU/mL, and the inoculation volume was set at 0.1 mL/body. For oral inoculation, a concentration of the yeast was set at 2.5×10^9 CFU/mL, and the inoculation volume was set at 0.5 mL/body. Each group consisted of 5 animals of each sex. A group treated with physiological saline, the vehicle for the

P. terrestris suspensions, at the same dosing volume was employed as a control for intravenous inoculation, and a group treated with culture medium for *P. terrestris*, the vehicle for the *P. terrestris* culture, at the same dosing volume was employed as a control for oral inoculation.

The animals were given free access to feeders containing solid feed and to tap water with water bottles. The animals in the oral inoculation groups were fasted from the evening on the day of grouping until about 4 hours after inoculation. All groups were observed for 14 days post inoculation for mortality and changes in body weight. At the end of the 14-day period, all animals were sacrificed. Brain, lungs, liver, spleen, and kidneys were collected from each animal and observed for macroscopic abnormalities. Each was also used to determine remaining viable yeast by growth on YM agar and sectioned for histopathologic examination.

No animals died in any of the groups treated intravenously with the *P. terrestris* suspensions. In the group treated at 2.4×10^9 CFU/mL, decreased locomotor activity was noted in both sexes on the inoculation day and inhibited body weight gain 1 day after inoculation. These changes were transient, and no abnormalities were noted thereafter. In the other groups treated with the *P. terrestris* suspensions, no abnormalities were noted in clinical signs or body weight changes in either sex. No abnormal findings were noted at necropsy in either sex in any group. In the histopathological examination, mild granuloma was noted in the liver in 4 of the 5 males and in all 5 females of the group treated at 2.4×10^9 CFU/mL. However, no remaining viable yeast was noted in the brain, lungs, liver, spleen, or kidneys in either sex in any of the groups treated with the *P. terrestris* suspensions. Therefore, the mild granuloma formation was attributable to a foreign body (killed yeast) removal reaction to a large amount of the inoculated test yeast; this finding was not judged to be a change suggesting pathogenicity.

No animals died in the group treated with the *P. terrestris* culture. No abnormalities attributable to the test yeast were noted in the clinical signs, body weight, necropsy findings, or histopathological findings. No remaining viable yeast was noted in the brain, lungs, liver, spleen, or kidneys in either sex in either of the oral inoculation groups.

Conclusion: *P. terrestris* was non-pathogenic upon one single intravenous administration to male and female Slc: ICR mice (five of each sex) of 2.4×10^8 cfu and oral administration to male and female Slc: ICR mice (five of each sex) of 1.3×10^9 cfu.

6.3(b) Summary of reverse mutation test

A bacterial reverse mutation assay (Ames test) of the gene mutation inducibility of β-galactosidase concentrate was performed following Good Laboratory Practice (GLP) standards and OECD test guidelines 471 in *Salmonella typhimurium* (*S. typhimurium*) TA100, TA1535, TA98 and TA1537, and *Escherichia coli* (*E. coli*) WP2 *uvrA* in the presence or absence of metabolic activation by S9 mix.

A dose-range finding test and main test were conducted with and without metabolic activation by the pre-incubation method. Five dose levels (5000, 1250, 313, 78.1,

19.5 µg/plate) were set for the dose-range finding test. The dose finding test noted growth inhibition at the highest dosage in the absence of S9 metabolic activation (5000 µg /plate), however no growth inhibition was present at this dosage with S9 metabolic activation. Therefore, this dosage was used as the maximum dosage for the duplicate main tests.

In the main tests, 6 dose levels (5000, 2500, 1250, 625, 313 and 156 µg/plate) were conducted for all strains in the absence of metabolic activation; and 5 dose levels (5000, 2500, 1250, 625, and 313 µg/plate) were conducted for all strains in the presence of metabolic activation because no growth inhibition was observed. The main test was conducted twice at the same dose levels.

The positive control used for *S. typhimurium* TA100, *E. coli* WP2 *uvrA*, and *S. typhimurium* TA98 was 2-(2-furyl)-3-(5-nitrol-2-furyl)-acrylamide (AF-2) at levels of 50 µg/mL (5.0 µg/plate), 100 µg/mL (10.0 µg/plate), and 50 µg/mL (5.0 µg/plate), respectively. Sodium azide (SAZ) was used a level of 20 µg/mL (2.0 µg/plate) as the positive control for *S. typhimurium*TA1535. Acridine mutagen ICR-191 was used at a level of 50 µg/mL (5.0 µg/plate) as a positive control for *S. typhimurium* TA1537. Distilled water was used as a negative control for all tester strains.

Test article, positive or negative control article (0.1 mL of each) was placed into a sterilized test tube with either 0.5 mL of 0.1M phosphate buffer (pH 7.4) without metabolic activation or 0.5 mL of S9 Mix for metabolic activation was added, and then 0.1 mL of ~ 4 x 10^9 cfu/mL bacterial solution was added to each tube. Each mixture was then pre-incubated while shaking (80 rpm) at 37°C for 20 minutes. After pre-incubation, 2.0 mL of Bacto agar with 0.6 wt% agar, 0.6wt % NaCl, 0.05 mM D-biotin, 0.05 mM L-histidine, and 0.05 mM L-tryptophan was added to each tube, and this mixture was shaken and overlaid uniformly on minimal glucose agar plates. Plates were incubated at 37°C for 48 hours and examined for revertant colonies. The test article was judged to be positive if a two-fold or more increase in the number of revertant colonies compared to that of spontaneous revertant colonies (the negative control value) and dose-response and reproducibility were noted, or even if no clear dose-response was observed but there was at least a two-fold increase in the number of revertant colonies and reproducibility was noted.

Neither precipitation nor coloration of the test article on the plate was observed at any dose concentration at the time of stratification on the plate at any dose level irrespective of the presence/absence of metabolic activation. In the observation of bacterial background lawn using a stereoscopic microscope, growth inhibition was observed at 2500 µg/plate or more for all strains in the absence of metabolic activation.

Conclusion: No biologically or statistically significant increases in the number of revertant colonies were observed in any tester strain, either in the absence or presence of the metabolic activation, in the preliminary and both two main tests. Based on the test results, it is concluded that test article did not show any reverse mutation activity.

6.3(c) Summary of chromosome aberration test

A mammalian cell chromosome aberration test in Chinese hamster lung fibroblast (CHL/IU) cells was undertaken following GLP standards and OECD test guidelines 473. Cells were used at passage numbers from 9-20. Cells were maintained at 37°C with 5% CO_2 in Minimum Essential Medium (MEM) with 10% bovine serum (BS) and sub-cultured every 1-4 days. The test article was as previously described, β-galactosidase concentrate prepared from *P. terrestris*. Short term tests (6-hour treatment) were conducted both with and without S9 metabolic activation, while continuous treatments (24 and 48 hours) were conducted without S9 activation. Distilled water was used as a negative control. Mitomycin C (MMC) at 0.075 µg/mL (without S9 activation) and cyclophosphamide (CP) at 14 µg/mL (with S9 activation) were used as positive controls. A cell growth inhibition test was conducted to determine dosage levels for the chromosome aberration tests. Plates were seeded with approximately 2 $x10^4$ cells and cultured for 72 hours at 37ºC with humidity and 5% CO_2. Cells were confirmed to have no abnormalities and were treated with test article at 5000, 2500, 1250, 625, 313, 156, 78.1 and 39.1 µg/mL with and without S9 metabolic activation for 6 hours and without S9 for 24 hours. The short term exposures were washed with isotonic NaCl solution, refilled with media, and cultured for another 18 hours. Cells were then enumerated using an automated cell counter. Cell population doubling (PD) and relative population doubling (RPD) were determined by the following formulae:

PD= [log (Post-treatment number of cells/ Initial number of cells)] *I* log 2

RPD= [PD in treated cultures/PD in negative control cultures] x 100

The cell growth inhibition ratio (100-RPD) was then calculated and the approximate value of 50% cell-growth inhibitory concentration was used to determine concentrations used for chromosome aberration tests.

For the chromosome aberration tests, cells were prepared as for the cell growth inhibition assay. Based on the results of the previous cell growth inhibition assay, cells were treated with 5000, 2500, or 1250 µg/mL (6 hours, without S9), 5000, 2500, or 1250 µg/mL (6 hours, with S9), or 2000, 1600, 1200, 800, or 400 µg/mL (24 hours) of the test article. 2 Hours prior to the end of the short and long term growth studies, 0.1 mL of colcemid (demecocine solution 10 µg/mL) was added to 2 plates in each group. Cells were removed from plates with 0.25% trypsin solution, washed with 0.075M potassium chloride, fixed with a 3:1 alcohol:acetic acid solution, and finally stained with 2% Giemsa. Cells were then observed for chromosomal anomalies. An additional 48 hour continuous test was conducted, with test article concentrations of 50, 33.3, 22.2, and 14.8 µg/mL.

A test was considered negative when no significant differences were seen between the test group and negative control group by Fisher's exact test (one-tailed test, $p>0.05$) and by the Cochran-Armitage trend test (one-tailed test, $p>0.05$), and any of the results are inside the 95% probability distribution of the historical negative control data.

For the initial cell growth inhibition test, cell growth inhibition that exceeded 50% was observed at 2500 µg/mL in the 24 hour treatment, but was not observed in the short-term treatment at any dosage level, with or without metabolic activation. The 50% cell growth inhibitory concentration (approximate value) was calculated to be 1420 µg/mL for continuous treatment. As such, the maximum dose used was 5000 µg/mL and 2000 µg/mL for short-term and continuous treatments, respectively.

A significant increase in the number of ski-pair formations was noted for the initial 24 hours (continuous) treatment ($p<0.05$). Because this can be an indication of cell-cycle delay, which can lead to false negatives, a 48-hour continuous treatment was conducted. For this study, the 50% cell growth inhibitory concentration (approximate value) was calculated to be 38 µg/mL. Thus, concentrations of 50, 33.3, 22.2, and 14.8 µg/mL were used for the 48-hour continuous treatment chromosomal aberration study. The 48-hour treatment produced a slight, but not statistically significant, increase in ski-pair formation. The data for the negative controls were within 95% probability distributions of historical negative control data, and the positive controls induced a statistically significant increase in aberrations when compared to negative controls.

Conclusion: No β-galactosidase treatment dose produced a statistically significant increase in aberrations, indicating that the enzyme concentrate is negative for production of structural and numerical chromosomal aberrations.

6.3(d) Summary of 13-week oral gavage toxicity study

Sprague-Dawley SPF rats [Crl:CD(SD)] were obtained from Charles River Laboratories Japan, Inc. at 5 weeks of age. Male and female rats were acclimated for 8 or 9 days respectively. 48 Rats of each sex were assigned by a combination of the block placement method and random sampling method into groups with comparable mean body weight. Individual body weights ranged from 209 to 246g for males and 144 to 179g for females. Animals were allowed free access to a commercial diet (CR-LPF) and tap water. Animals were housed 2 per sex per cage, at 22^{o}C ±3, relative humidity of 50 ± 20%, 10 to 20 air changes per hour, and a 12-hour day/night cycle.

The test article was prepared a maximum of 7 days in advance and in an aqueous solution of 100 mg/mL. Groups of 12 animals of each sex were fed by oral gavage daily for 13 weeks. Dosages consisted of negative control (water), low (500 mg/kg bw/day), middle (1000 mg/kg bw/day), and high (2000 mg/kg bw/day). Dosages were selected based upon a previous 2-week oral toxicity study that showed no apparent toxicity at the highest dosage tested (2000 mg/kg bw/day).

Animals were observed for clinical signs such as abnormalities in appearance, nutritional condition, posture, behavior, and excretions 3 times a day: before dosing, immediately after, and 1 to 3 hours after dosing. Body weights were recorded twice in the first week of the study, once a week thereafter, and at necropsy. Food consumption was recorded twice during the initial week of the study, and once a week thereafter. Animals were administered ophthalmologic examinations using an indirect ophthalmoscope before and at the beginning of week 13 of the study. Urinalysis was conducted on day 87 and 88 for

males and day 86 and 87 for females. 4-Hour urine samples were collected under deprivation of food but free access to water, and 20-hour urine samples were collected with free access to both food and water. At the end of the study, all animals were sacrificed and necropsied. At the time of necropsy, blood was collected from the abdominal aorta. The following organs were weighed and absolute and relative organ weights were determined: brain, pituitary, thyroid, adrenal, thymus, spleen, heart, lung, salivary gland, liver, kidney, testis, prostate, seminal vesicle, ovary and uterus. Additional histopathological studies were conducted on organs after fixation and staining. Fixation was in 10% formalin except for eyeballs and optic nerves (3% glutaraldehyde/ 2.5% formalin) and testes and epididymides (Bouin's solution then 10% formalin). All organs were embedded in paraffin and sectioned, and stained with hematoxylin and eosin. Organs were examined microscopically from control and high dosage groups. Organs examined included: cerebrum, cerebellum, spinal cord (thoracic), sciatic nerve, eyeball, optic nerve, harderian gland, pituitary, thyroid, parathyroid, adrenal, thymus, spleen, submandibular lymph node, mesenteric lymph node, heart, thoracic aorta, trachea, lung, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, submandibular gland, sublingual gland, liver, pancreas, kidney, urinary bladder, testis, epididymis, prostate, seminal vesicle, ovary, uterus, oviduct, vagina, mammary gland, sternum, femur, femoral skeletal muscle, skin, nasal cavity, and zymbal gland.

Statistical analysis of body and organ weights, food and water consumption, urinalysis, and hematology were calculated as the mean ± standard deviation. An analysis of variance was conducted by the Bartlett test ($p>0.01$). Homogenous data were then compared (control and test groups) by the Dunnett's test ($p>0.05$ and $p>0.01$, two tailed) while heterogeneous data were compared by the Steel's test ($p>0.05$ and $p>0.01$, two tailed). All analyses were performed by SAS Release 9.1.3 (SAS Institute, Inc.).

No animal deaths occurred in any group, and there was no significant change in body weight between treatment and control groups. Exposed animals additionally showed no treatment related differences in food consumption or clinical signs. Ophthalmological examination revealed no treatment related changes. Urinalysis revealed a statistically significant increase in chloride in males at 2000 mg/kg bw/day (TOS 1800 mg/kg bw/day) and decrease in potassium in males at 500 and 2000 mg/kg bw/day (TOS 450 and 1800 mg/kg bw/day). However, this change is considered to be of no toxicological significance due to the minimal change and the lack of changes in plasma levels of chloride and potassium.

Hematology and blood chemistry analyses revealed no treatment related differences in either sex. A statistically significant decrease in reticulocytes was noted in females at 500 and 1000 mg/kg bw/day (TOS 450 and 900 mg/kg bw/day), as well as a decrease in hematocrit in females at 1000 mg/kg bw/day. However, these changes were deemed incidental due to the lack of a dose response. Additionally, a statistically significant decrease in chloride and increase in phosphate were recorded in males at 2000 mg/kg bw/day (TOS 1800 mg/kg bw/day), but this result was deemed to be of no toxicological significance because the individual values were mostly within the historical control data.

There were no treatment related changes observed at study termination and animal necropsy, including organ weights, gross necropsy, and histopathological examination. A statistically significant increase in relative liver weight in females in the 2000 mg/kg bw/day (TOS 1800 mg/kg bw/day) group was noted. However, this increase was considered to be of no toxicological relevance because it was a minimal change only in the relative weight. Incidental findings on gross necropsy and histopathological examination included thyroid cysts, extramedullary hematopoiesis in the spleen, mild alveolar macrophage aggregation, stomach and intestinal ulcers, and minimal pancreatic fibrosis. These findings were considered to be incidental and of no toxicological significance.

Conclusion: Repeated oral dosing of Sprague-Dawley rats with β-galactosidase concentrate produced no findings of toxicological concern at any dosage level tested. Therefore, the no observed adverse effect level (NOAEL) is considered to be the high dose of 2000 mg/kg bw/day (TOS 1800 mg/kg bw/day) in both male and female rats under the stated test conditions.

6.3(e) Additional data on safety of β-galactosidase

Studies of β-galactosidase produced by other microorganisms likewise indicate no toxicological concerns, as noted below:

Flood and Kondo (2004) reported on the safety of a β-galactosidase enzyme preparation produced by *Penicillium multicolor* (called tilactase). Adult and juvenile rats administered 0, 500, 1000, or 4000 mg/kg bw/day of the enzyme preparation by gavage for 35 days, and dogs administered 0, 200, 500, or 1000 mg/kg bw/day in capsules for 30 days, exhibited no significant dose-related changes in body weights, feed consumption, organ weights, urinalysis, hematological profiles, clinical chemistry, or histopathological profiles. Rats receiving the same doses for 6 months also exhibited no dose-related effects, except for a small increase in the weight of the large intestine, an effect considered to be a physiological reaction to passage of a large amount of a non-absorbable substance. The NOAEL was 4000 mg/kg bw/day for rats and 1000 mg/kg bw/day for dogs. In three separate studies to examine reproductive and developmental toxicity, rats received 0, 250, 1000 or 4000 mg/kg bw/day by gavage up to the 7th day of pregnancy, during days 7-17 of pregnancy, and from day 17 of pregnancy to 21 days after delivery. There were no treatment-related effects on the dams, gestation period, numbers of implantations, parturition rates, sex ratios, or survival of offspring in any of the studies. No treatment-related external, internal, or skeletal abnormalities were observed in fetuses from any of the three studies. The NOAEL was 4000 mg/kg bw/day. In addition to the three rat studies, rabbits received 0, 250, 500, or 1000 mg/kg bw/day by gavage from the 6th to 18th day of pregnancy. No treatment-related changes were observed in the dams, or fertility indices; nor were there any treatment-related fetal abnormalities. The NOAEL was 1000 mg/kg bw/day. When viable *P. multicolor* spores were injected into the tail veins of mice, no deaths occurred, no fungal cells were observed in various organs, and histopathology showed only focal necrosis in the liver of some of the animals, including the controls. Similar effects were observed when spores were administered to mice in a single dose by gavage.

Additionally, as discussed in GRN 285 (**see page 56**), an unpublished study by Yamaguchi *et al.* (2005) found no toxicologically significant effects in Sprague-Dawley rats at the highest dose of 13,150 mg/kg bw of β-galactosidase concentrate from *Bacillus circulans* LOB 377 administered daily for 91 days.

6.4 Safety margins between NOAEL and theoretical EDIs for Amano's β-galactosidase

The safety margins between the NOAEL for Amano's β-galactosidase and the EDI of GOS would be 652 for the general population, 3,273for infants (< 6 months), and 2,093for small children (≤ 2 years); calculated as follows:

General Population: 1800 mg TOS/kg bw/day ÷ 2.76 mg TOS/kg bw/day = 652

Infants: 1800 mg TOS/kg bw/day ÷ 0.55 mg TOS/kg bw/day = 3,273

Small Children: 1800 mg TOS/kg bw/day ÷ 0.86 mg TOS/kg bw/day = 2,093

Safety margins greater than 100 suggest that the toxicological data demonstrate the safety of the proposed uses for Amano's food production enzyme. Therefore, even with the extremely conservative assumptions described above, there are still adequate safety margins for potential dietary exposure to any residual enzyme when Amano's β-galactosidase is used in the production of GOS. Further, as noted regarding the lack of concern for allergenicity at Part 2.1(o) above, the non-detection of β-galactosidase activity in the GOS further diminishes the risk of any toxicity as well.

6.5 Conclusion

Based on the documentation provided in this GRAS notification, and as discussed above, Amano has concluded that its β-galactosidase enzyme preparation is GRAS via scientific procedures for use as a processing aid in the production of galacto-oligosaccharides (GOS).

Part 7 – List of supporting data and information

7.1 References

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7.2 Tables

7.3 Figures

7.4 Appendices

APPENDIX 1

May 4, 2017

R&D Dept., Gifu Laboratory

Amano Enzyme Inc.

Report of Residual Enzyme Activities in GOS

1. Purpose

The purpose of this report is to demonstrate the absence of the residual enzyme activities in the galactooligosaccharides (GOS) products.

2. Material and Method

2-1. Material

GOS: Manufactured in commercial scale by the process described in the section 4.4.1 of the safety dossier.

1) Lot No. 2016.09.14
2) Lot No. 2017.01.16
3) Lot No. 2016.10.26

2-2. Methods

- Assay Method of Residual Beta-galactosidase Activity (details are indicated later)
- Assay method for Protease activity (Folin method pH 6.0)

3. Result

Lot No.	Beta-galactosidase Activity (u/g)	Protease activity (u/g)
2016.09.14	ND	ND
2017.01.16	ND	ND
2016.10.26	ND	ND

ND: not detected

Any enzyme activities were not detected in any lot of the GOS products.

Assay Method of Residual Beta-galactosidase Activity

Principle

When lactose is hydrolyzed by beta-galactosidase, it is converted into glucose and galactose. The enzyme activity is determined by measuring the amount of liberated glucose.

This assay method was designed not to be affected by exogenous glucose by cancelling the amount of glucose derived from the sample preparation.

Definition of activity unit

One beta-galactosidase unit is defined as the amount of enzyme that liberates 1 μmol of glucose per min at the early stage of the reaction at 40℃, pH 6.0.

Reagents and solutions

(1) 1 mol/L acetic acid solution

Dilute 60 g of acetic acid with distilled water and bring to 1,000 mL.

(2) 1 mol/L sodium acetate solution

Dissolve 136 g of sodium acetate trihydrate in distilled water and bring to 1,000 mL.

(3) 1 mol/L acetate buffer (pH 6.0)

Add 1 mol/L acetic acid solution to 1 mo/L sodium acetate solution to give a pH of 6.0.

(4) 20 mmol/L acetate buffer (pH 6.0)

Dilute 20 mL of 1 mol/L acetate buffer (pH6.0) with distilled water and bring to 1,000 mL.

(5) 10% Triton X-100 solution

Dissolve 10 g of Triton X-100 in distilled water by heating and bring to 100 mL.

(6) Diluent

Dissolve 11.69 g of sodium chloride in distilled water, add 100 mL of 1 mol/L acetate buffer and 1.0 mL of 10% Triton X-100 solution, and add distilled water to bring to 1,000 mL.

(7) Substrate (12% lactose solution)

Accurately weigh 12.63 g of lactose monohydrate into a 200 mL Erlenmeyer flask, add about 80 mL of distilled water, and heat the flask in a boiling water bath to dissolve. Then cool it with a running water, quantitatively transfer the mixture into a 100 mL volumetric flask, add 10 mL of 1 mol/L acetate buffer (pH 6.0), and bring to volume with distilled water. Prepare before use.

Sample preparation

For removal glucose from GOS solution

- Dilute GOS solution 5 times with water
- Equilibrate the desalting column PD-10 (GE Healthcare) with 20 mmol/L acetate buffer (pH6.0)

according to instructions for use

- Apply 2.5 mL of diluted GOS solution to the column
- Elute with 3.5 mL of 20 mmol/L acetate buffer (pH6.0)

Procedure

(Test)

Pipet 5 mL portion of the substrate into a 18φ×180mm test tube and preincubate it in a water bath at 40±0.5℃ for 10 min. Rapidly pipet 1 mL of the test preparation into the equilibrated substrate and then mix by swirling, starting the stopwatch at zero time. Allow to stand at 40±0.5℃ for exactly 5 hr, and immediately immerse the test tube in ice water.

(Blank)

Pipet 5 mL portion of the substrate into a 18φ×180mm test tube, add 1 mL of the test preparation and immediately mix by swirling and immerse the test tube in ice water.

Immediately determine glucose content for each reaction mixture according to modified method using AUTOKIT GLUCOSE C2** (Wako Diagnostics, details are indicated later).

Calculation

Calculate the activity of the enzyme preparation taken for analysis as follows:

$$\text{LU/g, mL} = \frac{(G_T - G_B)}{0.18} \times \frac{6}{1} \times \frac{1}{300} \times \frac{1}{W}$$

In which,

G_T is glucose content, in mg/mL, for Test;

G_B is glucose content, in mg/mL, for Blank;

0.18 is the amount of glucose, in mg, equivalent to 1 μmol;

6 is the total volume, in mL, of the reaction mixture;

1 is the test preparation volume, in mL, in the reaction mixture;

300 is the reaction time,in min;

W is the weight, in g, of the GOS solution contained in 1 mL of the test preparation.

****: Determination of glucose content – modified AUTOKIT GLUCOSE C2**

Principle

When a test sample is allowed to react with the reagent, α-D-glucose existing in the sample is converted rapidly to the β-isomer by the action of mutarotase and is then oxidized by Glucose oxidase to produce hydrogen peroxide.

α-D-Glucose

$\downarrow$ mutarotase

β-D-Glucose + O_2 $\xrightarrow{\text{glucose oxidase}}$ D-Glucono-δ-lactone + H_2O_2

$2H_2O_2$ +4-aminoantipyrine + Phenol $\xrightarrow{\text{peroxidase}}$ Quinoneimine dye + $4H_2O$

The appearance of quinoneimine dye formed by coupling with 4-aminoantipyrine and phenol is measured at 505 nm by spectrophotometry.

Reagents and solutions

(1) Working solution

Prepare according to instructions for AUTOKIT GLUCOSE

Procedure

Accurately pipette 0.2 mL portion of sample or standard into a test tube. Rapidly add 3 mL of working solution and then mix by swirling, starting the stopwatch at zero time. Allow to stand at 37-40℃ for exactly 5 min, and measure the absorbance at 505nm.

Calculation

Calculate the glucose concentration as follows:

$$\text{Glucose (mg/dL)} = \frac{A_S}{A_{Std}} \times C_{Std}$$

In which,

A_S is absorbance of sample

A_{Std} is absorbance of standard

C_{Std} is concentration of standard, in mg/dL

Addition and Recovery test

Addition and recovery test was conducted in order to set the detection limit of the Assay Method of Residual Beta-galactosidase Activity.

GOS solution

The GOS solution for addition and recovery test was made under below conditions.

Lactose solution (50% Dry Matter of lactose)

↓

↓← Beta-galactosidase* (1.0 u/g Lactose)

↓

Incubate at pH 5, 65℃ for 24 hours

↓

Heat treatment (Boiled for 5 min)

*: Beta-galactosidase from *Papiliotrema terrestris* (Lot. GFEO1151531SDR)

Procedure

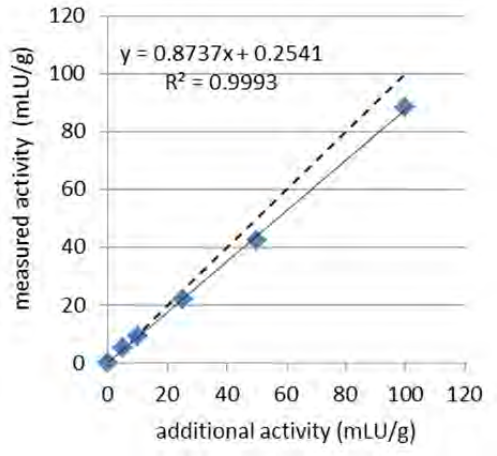
Added the enzyme solution to above GOS solution to desired final concentration (0, 5, 10, 25, 50 and 100mLU/g)

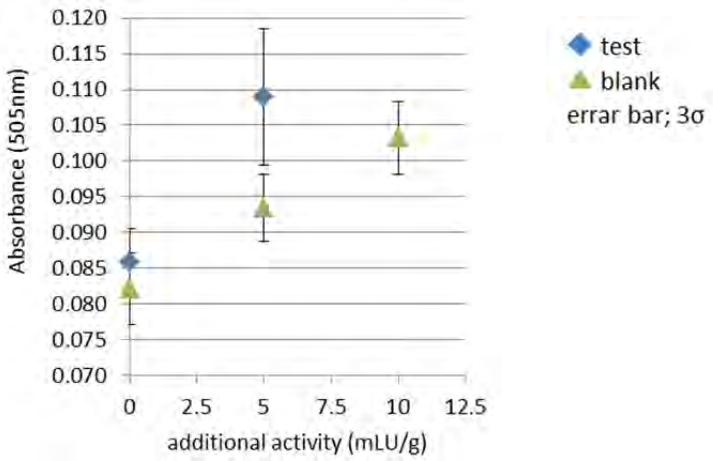
Measured the activity in each mixture according to above described Assay Method of Residual Beta-galactosidase Activity.

○ **Result**

The activities could be detectable in the low activity range although the values were around 85% of additional activities. With regard to the detection limit, it could be estimated that the value was 5 mLU/g because the absorbance of the test sample was significantly higher than it of blank sample.

additional activity mLU/g	measured activity mLU/g
0	0
5	5
10	9
25	22
50	42
100	88





Conclusion

The newly developed beta-galactosidase activity assay method has sufficient sensitivity. The detection limit was determined at 5mLU/g (0.005LU/g).

APPENDIX 2

Assay method for β-Galactosidase activity (Lactose substrate method)

Reagents and Solutions

1) 2 mol/L Hydrochloric acid solution

Add 180 mL of Hydrochloric acid to about 500 mL of water, and dilute to 1000 mL with water.

2) 2 mol/L Sodium hydroxide solution

Dissolve 86.0 g of Sodium hydroxide in about 700 mL of water, and cool. Dilute to 1000 mL with water.

3) Acetic acid solution

Add 60.1 g of Acetic acid to water, and dilute to 1000 mL with water.

4) Sodium acetate solution

Dissolve 82.04 g of Sodium acetate in about 800 mL of water, and dilute to 1000 mL with water.

5) 1 mol/L Acetic acid・Sodium acetate buffer pH6.0

Add Acetic acid solution to about 40 mL of Sodium acetate solution until the pH stabilizes at 6.00.

6) GTW solution

Add Coloring solution (Glucose CⅡ test Wako : Wako Pure Chemical Industries, Ltd, Osaka Jpan) to Buffer solution (Glucose CⅡ test Wako) and mix.

7) Glucose standard solution (0.4 mg/mL)

Add 1 mL of Glucose standard solution Ⅰ (200 mg/dL : Glucose CⅡ test Wako) to 4 mL of water, and mix.

8) Substrate solution

Weigh 12.63 g of Lactose monohydrate into a 200 mL beaker, and add about 60 mL of water. Heat the beaker in boiling water for 5 minites, and cool. Add 10 mL of 1 mol/L Acetic acid・Sodium acetate buffer pH6.0, and dilute to 100 mL with water.

Preparation of the sample solution

Dissolve the sample in an appropriate amount of water or a buffer solution. This enzyme solution should be prepared to make the value of (A_S-A_{SB}) into 0.2～1.0.

PROCEDURE

Assay

Pipet 5 mL of Substrate solution into test tubes and place the tubes in water bath maintained at 40±0.5 ℃ for 10 minutes. Add 1 mL of sample solution and mix, place the tubes in water bath at 40±0.5 ℃ for exactly 10 minutes. Add 1 mL of 2 mol/L Sodium hydroxide solution and mix, place the tubes in water bath at 40±0.5 ℃ for 5 minutes. Cool in iced water. Add 1 mL of 2 mol/L Hydrochloric acid solution and mix. (Reaction solution) Store in iced water until use.

As the blank, pipet 5 mL of Substrate solution into test tubes. Add 1 mL of 2 mol/L Sodium hydroxide solution and mix. Place the tubes in water bath at 40±0.5 ℃ for 10 minutes, add 1 mL of sample solution and mix. Place the tubes in water bath at 40±0.5 ℃ for 5 minutes and cool in iced water. Add 1 mL of 2 mol/L Hydrochloric acid solution and mix. (Blank solution) Store in iced water until use.

Pipet 3 mL of GTW solution in test tubes and add each 0.2 mL of Reaction solution or Blank solution or Glucose standard solution(0.4 mg/mL) or water, and mix. Place the tubes in water bath at 40±0.5 ℃ for 5 minutes. Measure the absorbance at 505 nm.

Definition of Activity Unit

One unit is defined as the quantity of enzyme required to liberate 1 μmol of glucose per 1 minute under the conditions of the assay.

CALCULATION

$$\beta\text{-Galactosidase activity unit/g} = (A_S - A_{SB}) \;/\; (A_R - A_{RB}) \times 0.4 \times 8 \;/\; 0.18 \;/\; 10 \times n$$

A_S : Absorbance of the solution added Reaction solution
A_{SB} : Absorbance of the solution added Blank solution
A_R : Absorbance of the solution added Glucose standard solution(0.4 mg/mL)
A_{RB} : Absorbance of the solution added water
0.4 :Concentration of Glucose standard solution (0.4 mg/mL)
8 : Volume of solution (Reaction solution)
0.18 :Molecular weight of glucose (mg/μmol)
10 : Reaction time (minutes)
n : Dilution factor of the enzyme

APPENDIX 3

Study No. 360030

Single Intravenous Inoculation of *Cryptococcus terrestris* Suspensions and Single Oral Inoculation of *Cryptococcus terrestris* Culture

FINAL REPORT

Prepared: August 22, 2016

Hashima Laboratory, Nihon Bioresearch Inc.

1. Contents

2. Signature of Person Preparing Final Report

Study No.: 360030

Title: Single Intravenous Inoculation of *Cryptococcus terrestris* Suspensions and Single Oral Inoculation of *Cryptococcus terrestris* Culture

Hashima Laboratory, Nihon Bioresearch Inc.

Study Director _____(signed)_____ (seal) August 22, 2016
Takahiko Nagase

3. Title of the Study

Single Intravenous Inoculation of *Cryptococcus terrestris* Suspensions and Single Oral Inoculation of *Cryptococcus terrestris* Culture

4. Study No.

360030

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8. Objective of the Study

To assess the pathogenic potential of *Cryptococcus terrestris*, male and female mice were inoculated once, intravenously or orally, with *Cryptococcus terrestris*.

9. Standards Followed

Standards for the Reliability of Application Data (Regulations for Enforcement of Laws for Securing the Quality, Efficacy, and Safety of Drugs and Medical Appliances)

10. Guidelines Followed for Animal Welfare

Basic Guidelines for the Use of Experimental Animals in Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare (Notification No. 0601001 of the Science Bureau, Japanese Ministry of Health, Labour and Welfare, June 1, 2006)

Guidelines for Management and Welfare of Experimental Animals (Nihon Bioresearch Inc., April 2, 2007)

The protocol of the present study was evaluated by the Animal Care and Use Committee at the testing facility.

11. Study Director

Takahiko Nagase

12. Study Schedule

Start of the study	April 28, 2016
Receipt of animals	May 11, 2016
Grouping	May 16, 2016
Intravenous inoculation	May 16, 2016
Oral inoculation	May 17, 2016
Completion of observation (necropsy in the intravenous inoculation groups)	May 30, 2016
Completion of observation (necropsy in the oral inoculation groups)	May 31, 2016
Finalization of histopathological findings	July 25, 2016
Completion of the study	August 22, 2016

13. Archives

All data obtained in the present study will be stored in the archives of the testing facility for 5 years from the day of conclusion of this contract. Their subsequent disposition will be determined by mutual agreement with the sponsor.

14. Unforeseeable Circumstances That Might Have Affected the Reliability of the Study and Deviations from the Protocol

There were no unforeseeable circumstances that might have affected the reliability of the study, and there were no deviations from the protocol.

15. Study Personnel and Work Responsibilities

Takahiko Nagase

Preparation of the protocol, supervision of test operations, and preparation of the final report

Eiji Matsui, Tatsumi Inoue, Tetsuya Yamada, Miki Sugiyama, and Ryusuke Sakuma

Inoculation with dosing preparations, observation for clinical signs, body weight measurement, husbandry of animals, and culture of remaining viable yeast

Tadashi Itoh, Jun Imai, Hisami Matsushita, Katsumi Endo, and Hitoshi Kimura

Necropsy, preparation of histopathological specimens, and histopathological examination

Makoto Kunieda

Statistical analysis

16. Summary

To assess the pathogenic potential of *Cryptococcus terrestris*, male and female Slc: ICR mice were inoculated once, either intravenously with *Cryptococcus terrestris* suspensions or orally with a *Cryptococcus terrestris* culture. Washed yeast suspensions in saline were used for the intravenous inoculations, and a yeast suspension in medium was used for the oral inoculations. For intravenous inoculation, concentrations of the yeast were set at 2.2×10^7, 2.5×10^8, and 2.4×10^9 CFU/mL, and the inoculation volume was set at 0.1 mL/body. For oral inoculation, a concentration of the yeast was set at 2.5×10^9 CFU/mL, and the inoculation volume was set at 0.5 mL/body. Each group consisted of 5 animals of each sex. A group treated with physiological saline, the vehicle for the *Cryptococcus terrestris* suspensions, at the same dosing volume was employed as a control for intravenous inoculation, and a group treated with culture medium for *Cryptococcus terrestris*, the vehicle for the *Cryptococcus terrestris* culture, at the same dosing volume was employed as a control for oral inoculation.

<Intravenous inoculation>

No animals died in any of the groups treated with the *Cryptococcus terrestris* suspensions. In the group treated at 2.4×10^9 CFU/mL, the following findings were noted in both sexes: decreased locomotor activity on the inoculation day and inhibited body weight gain 1 day after inoculation. These changes were transient, and no abnormalities were noted thereafter. In the other groups treated with the *Cryptococcus terrestris* suspensions, no abnormalities were noted in clinical signs or body weight changes in either sex. No abnormal findings were noted at necropsy in either sex in any group. In the histopathological examination, mild granuloma was noted in the liver in 4 of the 5 males and in all 5 females of the group treated at 2.4×10^9 CFU/mL. However, no remaining viable yeast was noted in the brain, lungs, liver, spleen, or kidneys in either sex in any of the groups treated with the *Cryptococcus terrestris* suspensions. Therefore, the mild granuloma formation was attributable to a foreign body (killed yeast) removal reaction to a large amount of the inoculated test yeast; this finding was not judged to be a change suggesting pathogenicity.

<Oral inoculation>

No animals died in the group treated with the *Cryptococcus terrestris* culture. No abnormalities attributable to the test yeast were noted in the clinical signs, body weight, necropsy findings, or histopathological findings. No remaining viable yeast

was noted in the brain, lungs, liver, spleen, or kidneys in either sex in either of the oral inoculation groups.

From the above results, it was surmised that the *Cryptococcus terrestris* suspensions and the *Cryptococcus terrestris* culture, with which mice had been inoculated once intravenously and orally, respectively, were not infectious under the conditions of the present study, and that the *Cryptococcus terrestris* suspensions and the *Cryptococcus terrestris* culture were not pathogenic.

17. Introduction

To assess the pathogenic potential of *Cryptococcus terrestris*, male and female mice were inoculated once, either intravenously with *Cryptococcus terrestris* suspensions or orally with a *Cryptococcus terrestris* culture.

18. Test Articles and Control Articles

18.1. Test Article 1 (for Intravenous Inoculation)

Name:	*Cryptococcus terrestris* suspension (washed yeast suspension in saline)
Lot Nos.:	20160516-1 (2.2×10^7 CFU/mL) 20160516-2 (2.5×10^8 CFU/mL) 20160516-3 (2.4×10^9 CFU/mL)
Description:	Pale light yellow to light yellow
Expiry:	2:00 p.m. on May 16, 2016
Storage conditions:	Stored in ice.
Site of storage:	Not stored at the testing facility, since test article 1 was received on the day of inoculation and used immediately after receipt.
Supplier:	Amano Enzyme Inc.

18.2. Test Article 2 (for Oral Inoculation)

Name:	*Cryptococcus terrestris* culture (yeast suspension in medium)
Lot No.:	20160517-1 (2.5×10^9 CFU/mL)
Description:	Light yellow
Expiry:	1:00 p.m. on May 17, 2016
Storage conditions:	Stored in ice.
Site of storage:	Not stored at the testing facility, since test article 2 was received on the day of inoculation and used immediately after receipt.
Supplier:	Amano Enzyme Inc.

18.3. Control Article (Vehicle) 1 (for Intravenous Inoculation)

Name:	Physiological saline (Normal Saline Syringe Otsuka 20 mL)

Lot No.:	15K70A
Expiry:	November 2018
Storage conditions:	Stored in ice.
Site of storage:	Not stored at the testing facility, since control article 1 was received on the day of inoculation and used immediately after receipt.
Supplier:	Amano Enzyme Inc.

18.4. Control Article (Vehicle) 2 (for Oral Inoculation)

Name:	Culture medium for *Cryptococcus terrestris*
Lot No.:	20160517CONT
Expiry:	May 17, 2016
Storage conditions:	Stored in ice.
Site of storage:	Not stored at the testing facility, since control article 2 was received on the day of inoculation and used immediately after receipt.
Supplier:	Amano Enzyme Inc.

18.5. Confirmation of Stability

The stability test (incubation on plates: at 27°C for 2 days) was performed before and after inoculation using samples of the *Cryptococcus terrestris* suspension at each concentration and the *Cryptococcus terrestris* culture dispensed at the time of formulation by the sponsor. The results of these tests were obtained from the sponsor (Attachment 1). It was ascertained that there were no problems with the stability of the test articles.

18.6. Handling of Leftover Test Articles and Control Articles

The test articles and control articles remaining after inoculation were returned to the sponsor.

19. Dosing Preparations

19.1. Methods of Formulation

The test articles and control articles prepared by the sponsor on the day of inoculation were received and used unmodified.

20. Test System

20.1. Species, Strain, and Reason for Selection

Species: Mice (SPF)

Strain: Slc: ICR

Reason for selection: Following "Development of safety assessment method for animal and microorganism feeds"[1] established by Agriculture, Forestry and Fisheries Research Council.

20.2. Date of Animal Receipt, Sex, Age in Weeks, and Number of Animals Received

On May 11, 2016, 37 males and 37 females at 5 weeks of age were received.

20.3. Body Weight Ranges 1 Day after Receipt

Males: 27.1 to 31.2 g

Females: 24.2 to 28.1 g

20.4. Supplier

Japan SLC, Inc.

20.5. Quarantine/Acclimatization

The animals were quarantined/acclimatized for 5 days. During the quarantine/acclimatization period, the animals were weighed (electronic balance: PB3002-S/FACT, Mettler-Toledo GmbH) twice (the day after animal receipt and on the day of completion of quarantine/acclimatization) and observed daily for clinical signs. Animals which had no abnormalities during the quarantine/acclimatization period in either body weight changes or clinical signs were used for group assignment.

20.6. Group Assignment

Body weights of the animals were stratified using a computer program (IBUKI, Nihon Bioresearch Inc.), and the animals were grouped on the day of intravenous inoculation by a random sampling method to assure that mean body weights and variances were as equal as possible among the groups.

Animals remaining after group assignment were euthanized on the day of group assignment by bleeding from the abdominal aorta under isoflurane anesthesia.

20.7. Individual Identification

The animals were individually identified on the day of receipt both by marking the tail and by dyeing the fur of the limbs with oil-based red ink. After grouping, each animal was individually identified by marking the animal number (the last 3 figures) on the tail with oil-based ink of the same color as the label of the cage in which the animal was housed; control animals were marked with oil-based black ink.

For individual identification of the cages, an identifying label bearing the study number, date of animal receipt, and quarantine/acclimatization animal number was attached to each cage during the quarantine/acclimatization period. For individual identification of the cages after group assignment, an identifying label with a different color for each group, bearing the study number, name of the group, inoculation route, yeast concentration, and animal number, was attached to each cage.

20.8. Environmental Conditions and Husbandry of Animals

The animals were kept in an animal room (Animal Room No. 1, Kisosansen Laboratory) with the temperature maintained at 18 to 28°C (values by actual measurement: 19 to 24°C) and the relative humidity held at 30 to 80% (values by actual measurement: 53 to 79%) on a 12-hour light and dark cycle (lighting: 6:00 a.m. to 6:00 p.m.). The animals were housed in sterilized TPX plastic cages (W: 170 × D: 250 × H: 140 mm) set on a rack with an isolator (W: 1500 × D: 750 × H: 1810 mm). The animals were housed in groups of 3 to 5 per cage during the quarantine/acclimatization period and in groups of 5 per cage after group assignment.

The cages and water bottles were changed at least once a week. The floor of the animal room was disinfected daily by mopping with an antiseptic solution (sodium hypochlorite diluted 250 times).

20.9. Feed

The animals were given free access to feeders containing solid feed (CRF-1, Oriental Yeast Co., Ltd.) that had been manufactured less than 5 months earlier. The animals in the oral inoculation groups were fasted from the evening on the day of grouping until about 4 hours after inoculation. Feed of the same lot number as that of the feed used for the present study was analyzed for concentrations of contaminants, the number of bacteria, and nutrient contents, and the results of these analyses were obtained. It was ascertained that the values obtained from the analyses were within the standard ranges of values established by the testing facility.

Facilities for analysis: Eurofins Scientific Analytics (concentrations of contaminants) and Oriental Yeast Co., Ltd. (the number of bacteria and nutrient contents)

20.10. Drinking Water

The animals were given free access to tap water with water bottles. Concentrations of contaminants and the number of bacteria in the water were analyzed approximately every 6 months, and the results of these analyses were obtained. It was ascertained that the values obtained from the analyses were within the standard ranges of values established by the testing facility.

Facilities for analysis: Tohzai Chemical Industry Co., Ltd.

20.11. Wood Chips

Wood chips for test animals (Sunflake, Charles River Laboratories Japan, Inc.) were used. Concentrations of contaminants and trace metals in the wood chips were analyzed approximately every 6 months, and the results of these analyses were obtained. It was ascertained that values obtained from the analyses were within the standard ranges of values established by the testing facility.

Facilities for analysis: Eurofins Scientific Analytics

21. Inoculation

21.1. Inoculation Routes and Reasons for Selection

Inoculation route: Intravenous.
Reason for selection: Following "Development of safety assessment method for animal and microorganism feeds"[1].

Inoculation route: Oral.
Reason for selection: This is the most practical, as well as being the natural, route of infection.

21.2. Inoculation Methods and Reason for Selection

21.2.1. Intravenous Inoculation

Inoculation method: The dosing preparation was intravenously inoculated into the caudal vein using a 1.0-mL disposable polypropylene hypodermic syringe (Terumo Corporation) with a 27G winged hypodermic needle (Terumo Corporation). The dosing preparation was stirred well with the container upside down and immediately used for inoculation.

Reason for selection: This is the usual method employed at the testing facility.

21.2.2. Oral Inoculation

Inoculation method: The dosing preparation was orally inoculated by gavage using a disposable polypropylene hypodermic syringe (Terumo Corporation) with a disposable gastric tube for mice (Fuchigami Kikai Ltd.). The dosing preparation was stirred well with the container upside down and immediately used for inoculation.

Reason for selection: This is the usual method employed at the testing facility.

21.3. Inoculation Volume, Time, and Frequency

Inoculation volume: Set at 0.1 mL/body for intravenous inoculation and 0.5 mL/body for oral inoculation.

Inoculation time: Between 10:55 a.m. and 11:13 a.m. for intravenous inoculation and between 10:21 a.m. and 10:33 a.m. for oral inoculation.

Inoculation frequency: Once each for intravenous and oral inoculation

22. Group Composition and Inoculation Volume

A total of 6 test groups, 4 for intravenous inoculation and 2 for oral inoculation, were employed as shown below. Each test group consisted of 5 animals of each sex.

Group No.	Test group	Yeast concentration[a] (CFU[b]/mL)	Color of label	No. of animals (Animal No.) Males	Females
Single intravenous inoculation					
1.	Control (vehicle)	0	White	5 (M01101 to M01105)	5 (F01151 to F01155)
2.	*Cryptococcus terrestris* suspension	2.2×10^7	Green	5 (M02201 to M02205)	5 (F02251 to F02255)
3.	*Cryptococcus terrestris* suspension	2.5×10^8	Blue	5 (M03301 to M03305)	5 (F03351 to F03355)
4.	*Cryptococcus terrestris* suspension	2.4×10^9	Red	5 (M04401 to M04405)	5 (F04451 to F04455)
Single oral inoculation					
5.	Control (vehicle)	0	Yellow	5 (M05501 to M05505)	5 (F05551 to F05555)
6.	*Cryptococcus terrestris* culture	2.5×10^9	Brown	5 (M06601 to M06605)	5 (F06651 to F06655)

a): Actual yeast concentration.

b): Colony forming unit.

23. Reason for Selection of Inoculation Volume

In reference to "Development of safety assessment method for animal and microorganism feeds"[1], the target maximum volume was set at 10^7 CFU/body for intravenous inoculation, and lower concentrations were calculated using a common ratio of 10; 3 concentrations were thereby determined. The target volume was set at 10^7 CFU/body for oral inoculation. A group treated with the vehicle at the same dosing volume as the test article was employed as a control group for each inoculation route.

24. Observation and Examination

24.1. Observation Period

The observation period was set at 14 days after inoculation; the day after inoculation was defined as Day 1 after inoculation.

24.2. Observation for Clinical Signs

The animals were observed for clinical signs and mortality before inoculation (observation for clinical signs at grouping) and for 4 hours after inoculation (at the start of inoculation to 1 hour after inoculation, 1 to 2 hours after inoculation, and 2 to 4 hours after inoculation) on the day of inoculation. They were observed in the morning once a day thereafter during the observation period.

24.3. Body Weight Measurement

The animals were weighed before inoculation on the day of inoculation (body weights at grouping were used for the oral inoculation groups) and on Days 1, 3, 7, 10, and 14 after inoculation (electronic balance: MS3002S/02 or PB3002-S/FACT, Mettler-Toledo GmbH).

24.4. Necropsy

The animals were euthanized on completion of the observation period by bleeding from the abdominal aorta under isoflurane anesthesia and necropsied.

After observation for macroscopic abnormalities of each organ and tissue, the brain, lungs, liver, spleen, and kidneys were collected and put on petri plates for yeast cultivation. Each organ was divided into 2 on the plate. Specifically, the brain was divided sagittally, the lungs were divided into the left lobe and all other parts, the lateral left lobe of the liver was cut longitudinally, the spleen was cut transversally in the center, and the right and left kidneys were each cut transversally. For the brain, liver, spleen, and kidneys, one of the parts yielded by these divisions was used for counting remaining viable yeast. For the lungs, all parts other than the left lobe were used for counting remaining viable yeast. The remaining parts of each organ were fixed in 20 vol% neutral buffered formalin for histopathological examination. The instruments used for necropsy were cauterized for each animal and for each organ.

24.5. Counting of Remaining Viable Yeast

Remaining viable yeast in each organ and tissue specimen obtained at necropsy was counted. Specifically, the specimen for counting remaining viable yeast was cut into small pieces with scissors and spread evenly over the agar medium for counting remaining viable yeast with a bacteria spreader. A piece of the tissue was then collected and incubated in an incubator (set at 30°C, MTR-251, SANYO Electric Co., Ltd.) for 3 days. No yeast growth was noted in any culture medium. Therefore, the

remaining viable yeast was recorded as 0.

24.6. Histopathological Examination

According to the usual method, the histopathological specimens obtained at necropsy were embedded in paraffin, and HE-stained tissue specimens were prepared for histopathological examination. Organs and tissues remaining after resection were preserved in 10 vol% neutral buffered formalin. Since no remaining viable yeast was noted, Gram-stained specimens (Brown-Hopps method) were not prepared.

25. Statistical Methods

Group mean values with standard deviations were calculated for body weights. Significance tests were conducted at a significance level of 5%, and probabilities are shown as $p<0.05$ (less than 5%) or $p<0.01$ (less than 1%).

<Intravenous inoculation>

The test was performed for comparisons between the control group and each of the groups treated with the *Cryptococcus terrestris* suspensions. Specifically, Bartlett's test was performed for homogeneity of variance. Since the variances were homogeneous, Dunnett's test was performed. For the remaining viable yeast, Jonckheere's rank test was performed for dose response.

<Oral inoculation>

The test was performed for comparison between the control group and the group treated with the *Cryptococcus terrestris* culture. Specifically, an F test was performed for homogeneity of variance. Since the variances were homogeneous, Student's t test was performed.

26. Results

26.1. Clinical Signs

Results of the observation for clinical signs in the intravenous inoculation groups are shown in Tables 1-1 and 1-2 (Appendices 1-1-1 to 1-1-4 and 1-2-1 to 1-2-4), and those in the oral inoculation groups are shown in Tables 2-1 and 2-2 (Appendices 2-1-1, 2-1-2, 2-2-1, and 2-2-2).

<Intravenous inoculation>

No animals died or became moribund in the control group or in any of the groups treated with the *Cryptococcus terrestris* suspensions. Decreased locomotor activity was noted in both sexes in the group treated at 2.4×10^9 CFU/mL; this finding was

noted in all 5 males 0 to 2 hours after inoculation and in all 5 females 0 to 4 hours after inoculation. No abnormal clinical signs were noted in the control group or in the other groups treated with the *Cryptococcus terrestris* suspensions.

<Oral inoculation>

No animals died or became moribund in the control group or in the group treated with the *Cryptococcus terrestris* culture. No abnormal clinical signs were noted in either sex in either group.

26.2. Body Weight

Results of the body weight measurement in the intravenous inoculation groups are shown in Tables 3-1 and 3-2 (Appendices 3-1-1 to 3-1-4 and 3-2-1 to 3-2-4) and Figs. 1-1 and 1-2, and those in the oral inoculation groups are shown in Tables 4-1 and 4-2 (Appendices 4-1-1, 4-1-2, 4-2-1, and 4-2-2) and Figs. 2-1 and 2-2.

<Intravenous inoculation>

In both sexes in the group treated at 2.4×10^9 CFU/mL, body weight was significantly lower than in the control group on Day 1 after inoculation.

In the groups treated at 2.2×10^7 CFU/mL and 2.5×10^8 CFU/mL, body weights changed normally in both sexes; no significant differences from the control group were seen.

<Oral inoculation>

In the group treated with the *Cryptococcus terrestris* culture, body weights changed normally in both sexes; no significant difference from the control group was seen.

26.3. Necropsy

Results of the necropsy in the intravenous inoculation groups are shown in Tables 5-1 and 5-2 (Appendices 5-1-1 to 5-1-4 and 5-2-1 to 5-2-4), and those in the oral inoculation groups are shown in Tables 6-1 and 6-2 (Appendices 6-1-1, 6-1-2, 6-2-1, and 6-2-2).

<Intravenous inoculation>

No abnormalities were noted in either sex in the control group or in any of the groups treated with the *Cryptococcus terrestris* suspensions.

<Oral inoculation>

No abnormalities were noted in either sex in the control group or in the group treated with the *Cryptococcus terrestris* culture.

26.4. Counting of Remaining Viable Yeast

Results of the counting of remaining viable yeast in the intravenous inoculation groups are shown in Tables 7-1 and 7-2 (Appendices 7-1-1 to 7-1-4 and 7-2-1 to 7-2-4), and those in the oral inoculation groups are shown in Tables 8-1 and 8-2 (Appendices 8-1-1, 8-1-2, 8-2-1, and 8-2-2).

<Intravenous inoculation>

No remaining viable yeast was noted in the brain, lungs, liver, spleen, or kidneys in either sex in any of the groups treated with the *Cryptococcus terrestris* suspensions.

<Oral inoculation>

No remaining viable yeast was noted in the brain, lungs, liver, spleen, or kidneys in either sex in the group treated with the *Cryptococcus terrestris* culture.

26.5. Histopathological Examination

Results of the histopathological examination in the intravenous inoculation groups are shown in Tables 9-1 and 9-2 (Appendices 9-1-1 to 9-1-4 and 9-2-1 to 9-2-4), and those in the oral inoculation groups are shown in Tables 10-1 and 10-2 (Appendices 10-1-1, 10-1-2, 10-2-1, and 10-2-2).

<Intravenous inoculation>

In the group treated at 2.4×10^9 CFU/mL, mild granuloma was noted in the liver in 4 of the 5 males and in all 5 females.

No abnormalities were noted in the lungs, liver, spleen, kidneys, or brain in either sex in the other groups.

<Oral inoculation>

No abnormalities were noted in the lungs, liver, spleen, kidneys, or brain in the control group or in the group treated with the *Cryptococcus terrestris* culture.

27. Discussion

To assess the pathogenic potential of *Cryptococcus terrestris*, male and female Slc: ICR mice were inoculated once, either intravenously with *Cryptococcus terrestris* suspensions or orally with a *Cryptococcus terrestris* culture.

No animals died in any of the groups treated intravenously with the *Cryptococcus terrestris* suspensions or in the group treated orally with the *Cryptococcus terrestris* culture, and no abnormalities were noted at necropsy in any of these groups. Decreased locomotor activity was noted in both sexes in the group treated intravenously

with the *Cryptococcus terrestris* suspension at 2.4×10^9 CFU/mL, and inhibited body weight gain accompanying the decreased locomotor activity was noted on Day 1 after inoculation. These changes were transient, and no abnormalities were noted in either clinical signs or body weight changes thereafter. In the other groups treated with the *Cryptococcus terrestris* suspensions and the group treated with the *Cryptococcus terrestris* culture, no abnormalities were noted in either clinical signs or body weight changes in either sex. In the histopathological examination, mild granuloma was noted in the liver in the group treated intravenously with the *Cryptococcus terrestris* suspension at 2.4×10^9 CFU/mL. However, no remaining viable yeast was noted in the brain, lungs, liver, spleen, or kidneys in either sex in any of the intravenous inoculation groups or in either of the oral inoculation groups. Therefore, the mild granuloma formation was attributable to a foreign body (killed yeast) removal reaction to a large amount of the inoculated test yeast; this finding was not judged to be a change suggesting pathogenicity.

From the above results, it was surmised that the *Cryptococcus terrestris* suspensions and the *Cryptococcus terrestris* culture, with which mice had been inoculated once intravenously and orally, respectively, were not infectious under the conditions of the present study, and that the *Cryptococcus terrestris* suspensions and the *Cryptococcus terrestris* culture were not pathogenic.

28. References

1) Agriculture, Forestry and Fisheries Research Council. Development of safety assessment method for animal and microorganism feeds. Research results, 170 (1985).

Table 1-1. Clinical signs in male mice (intravenous inoculation)

Group	CFU/mL	Number of males and clinical signs	Pre	Hours after inoculation			Days after inoculation													
				0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	0	Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Cryptococcus terrestris suspension	2.2×10^{7}	Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	2.5×10^{8}	Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	2.4×10^{9}	Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	2	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Decrease in locomotor activity	0	3	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CFU: Colony forming unit.
Pre: Before inoculation.

Table 1-2. Clinical signs in female mice (intravenous inoculation)

Group	CFU/mL	Number of females and clinical signs	Pre	Hours after inoculation			Days after inoculation													
				0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	0	Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Cryptococcus terrestris suspension	2.2×10^{7}	Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	2.5×10^{8}	Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	2.4×10^{9}	Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	0	0	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Decrease in locomotor activity	0	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CFU: Colony forming unit.
Pre: Before inoculation.

Table 2-1. Clinical signs in male mice (oral inoculation)

Group	CFU/mL	Number of males and clinical signs	Pre	Hours after inoculation			Days after inoculation													
				0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	0	Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Cryptococcus terrestris liquid	2.5×10^{9}	Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

CFU: Colony forming unit.
Pre: Before inoculation.

Table 2-2. Clinical signs in female mice (oral inoculation)

Group	CFU/mL	Number of females and clinical signs	Pre	Hours after inoculation			Days after inoculation													
				0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	0	Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Cryptococcus terrestris liquid	2.5×10^9	Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

CFU: Colony forming unit.
Pre: Before inoculation.

Table 3-1. Body weights of male mice (intravenous inoculation)

Group	Control	*Cryptococcus terrestris* suspension		
CFU/mL	0	2.2×10^{7}	2.5×10^{8}	2.4×10^{9}
Number of males	5	5	5	5
Days after inoculation				
0	33.5 ± 1.1	33.5 ± 1.1	33.4 ± 1.3	33.5 ± 1.0
1	33.7 ± 0.6	33.5 ± 1.0	33.6 ± 1.3	30.7 ± 1.8 **
3	35.0 ± 1.0	35.2 ± 1.0	35.6 ± 1.6	34.1 ± 1.5
7	37.1 ± 1.2	37.2 ± 1.6	38.0 ± 2.3	36.7 ± 1.1
10	38.8 ± 1.1	38.8 ± 2.7	39.7 ± 2.8	39.0 ± 1.3
14	39.8 ± 1.3	39.6 ± 4.0	41.9 ± 3.0	40.4 ± 1.5

Each value shows mean (g) ± S.D.
CFU: Colony forming unit.
Significantly different from the control group (**: $p<0.01$ by Dunnett's test).

Table 3-2. Body weights of female mice (intravenous inoculation)

Group	Control	*Cryptococcus terrestris* suspension		
CFU/mL	0	2.2×10^7	2.5×10^8	2.4×10^9
Number of females	5	5	5	5
Days after inoculation				
0	26.4 ± 0.8	26.5 ± 0.7	26.7 ± 0.9	26.5 ± 0.8
1	26.6 ± 1.0	26.5 ± 0.9	26.5 ± 1.1	24.0 ± 0.8 **
3	26.8 ± 1.3	26.8 ± 1.6	26.5 ± 0.8	26.3 ± 1.1
7	27.7 ± 1.5	27.5 ± 2.3	28.1 ± 1.3	28.4 ± 1.7
10	29.4 ± 2.2	29.2 ± 2.5	29.0 ± 1.9	29.7 ± 1.8
14	30.8 ± 2.7	30.8 ± 2.7	30.7 ± 1.8	31.2 ± 2.3

Each value shows mean (g) ± S.D.
CFU: Colony forming unit.
Significantly different from the control group (**: $p<0.01$ by Dunnett's test).

Table 4-1. Body weights of male mice (oral inoculation)

Group	Control	*Cryptococcus terrestris* liquid
CFU/mL	0	2.5×10^{9}
Number of males	5	5
Days after inoculation		
0 [a)]	33.7 ± 1.2	33.5 ± 1.1
1	34.3 ± 0.9	33.6 ± 1.2
3	35.9 ± 1.1	35.7 ± 1.3
7	38.2 ± 1.2	38.5 ± 1.3
10	39.6 ± 1.4	40.4 ± 1.6
14	41.7 ± 1.1	41.9 ± 2.2

Each value shows mean (g) ± S.D.
CFU: Colony forming unit.
a): Grouping day

Table 4-2. Body weights of female mice (oral inoculation)

Group	Control	*Cryptococcus terrestris* liquid
CFU/mL	0	2.5×10^{9}
Number of females	5	5
Days after inoculation		
0 [a)]	26.8 ± 0.9	26.6 ± 0.9
1	26.6 ± 0.9	26.4 ± 0.7
3	27.5 ± 0.6	27.2 ± 1.1
7	28.4 ± 1.0	28.5 ± 1.5
10	30.3 ± 1.3	29.2 ± 1.5
14	31.4 ± 2.0	30.9 ± 1.6

Each value shows mean (g) ± S.D.
CFU: Colony forming unit.
a): Grouping day

Table 5-1. Necropsy findings in male mice (intravenous inoculation)

Group	Control	*Cryptococcus terrestris* suspension		
CFU/mL	0	2.2×10^{7}	2.5×10^{8}	2.4×10^{9}
Number of males	5	5	5	5
Findings				
Normal	5	5	5	5

CFU: Colony forming unit.

Table 5-2. Necropsy findings in female mice (intravenous inoculation)

Group	Control	*Cryptococcus terrestris* suspension		
CFU/mL	0	2.2×10^{7}	2.5×10^{8}	2.4×10^{9}
Number of females	5	5	5	5
Findings				
Normal	5	5	5	5

CFU: Colony forming unit.

Table 6-1. Necropsy findings in male mice (oral inoculation)

Group	Control	*Cryptococcus terrestris* liquid
CFU/mL	0	2.5×10^{9}
Number of males	5	5
Findings		
Normal	5	5

CFU: Colony forming unit.

Table 6-2. Necropsy findings in female mice (oral inoculation)

Group	Control	*Cryptococcus terrestris* liquid
CFU/mL	0	2.5×10^{9}
Number of females	5	5
Findings		
Normal	5	5

CFU: Colony forming unit.

Table 7-1. Number of remaining viable yeast in male mice (intravenous inoculation)

Group	Control	*Cryptococcus terrestris* suspension		
CFU/mL	0	2.2×10^{7}	2.5×10^{8}	2.4×10^{9}
Number of males	5	5	5	5
Brain	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lung	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Liver	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Spleen	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Kidneys	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Each value shows mean ± S.D.
CFU: Colony forming unit.

Table 7-2. Number of remaining viable yeast in female mice (intravenous inoculation)

Group	Control	*Cryptococcus terrestris* suspension		
CFU/mL	0	2.2×10^{7}	2.5×10^{8}	2.4×10^{9}
Number of females	5	5	5	5
Brain	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lung	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Liver	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Spleen	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Kidneys	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Each value shows mean ± S.D.
CFU: Colony forming unit.

Table 8-1. Number of remaining viable yeast in male mice (oral inoculation)

Group	Control	*Cryptococcus terrestris* liquid
CFU/mL	0	2.5×10^{9}
Number of males	5	5
Brain	0.0 ± 0.0	0.0 ± 0.0
Lung	0.0 ± 0.0	0.0 ± 0.0
Liver	0.0 ± 0.0	0.0 ± 0.0
Spleen	0.0 ± 0.0	0.0 ± 0.0
Kidneys	0.0 ± 0.0	0.0 ± 0.0

Each value shows mean ± S.D.
CFU: Colony forming unit.

Table 8-2. Number of remaining viable yeast in female mice (oral inoculation)

Group	Control	*Cryptococcus terrestris* liquid
CFU/mL	0	2.5×10^{9}
Number of females	5	5
Brain	0.0 ± 0.0	0.0 ± 0.0
Lung	0.0 ± 0.0	0.0 ± 0.0
Liver	0.0 ± 0.0	0.0 ± 0.0
Spleen	0.0 ± 0.0	0.0 ± 0.0
Kidneys	0.0 ± 0.0	0.0 ± 0.0

Each value shows mean ± S.D.
CFU: Colony forming unit.

Table 9-1. Histopathological findings in male mice (intravenous inoculation)

Group	Control					*Cryptococcus terrestris* suspension														
CFU/mL	0					2.2×10^{7}					2.5×10^{8}					2.4×10^{9}				
Number of males	5					5					5					5				
Grade	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+
Findings																				
Liver																				
Granuloma	5	0	0	0	0	5	0	0	0	0	5	0	0	0	0	1	0	4	0	0

Grade of histopathological findings: -: none, ±: slight, +: mild, 2+: moderate, 3+: marked.
Examined organs/tissues were the lung, liver, spleen, kidney, and brain.
CFU: Colony forming unit.

Table 9-2. Histopathological findings in female mice (intravenous inoculation)

Group	Control					*Cryptococcus terrestris* suspension														
CFU/mL	0					2.2×10^7					2.5×10^8					2.4×10^9				
Number of females	5					5					5					5				
Grade	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+
Findings																				
Liver																				
Granuloma	5	0	0	0	0	5	0	0	0	0	5	0	0	0	0	0	0	5	0	0

Grade of histopathological findings: -: none, ±: slight, +: mild, 2+: moderate, 3+: marked.
Examined organs/tissues were the lung, liver, spleen, kidney, and brain.
CFU: Colony forming unit.

Table 10-1. Histopathological findings in male mice (oral inoculation)

Group	Control	*Cryptococcus terrestris* liquid
CFU/mL	0	2.5×10^{9}
Number of males	5	5
Findings		
All organs and tissues		
No abnormality detected	5	5

Examined organs/tissues were the lung, liver, spleen, kidney, and brain.
CFU: Colony forming unit.

Table 10-2. Histopathological findings in female mice (oral inoculation)

Group	Control	*Cryptococcus terrestris* liquid
CFU/mL	0	2.5×10^{9}
Number of femals	5	5
Findings		
All organs and tissues		
No abnormality detected	5	5

Examined organs/tissues were the lung, liver, spleen, kidney, and brain.
CFU: Colony forming unit.

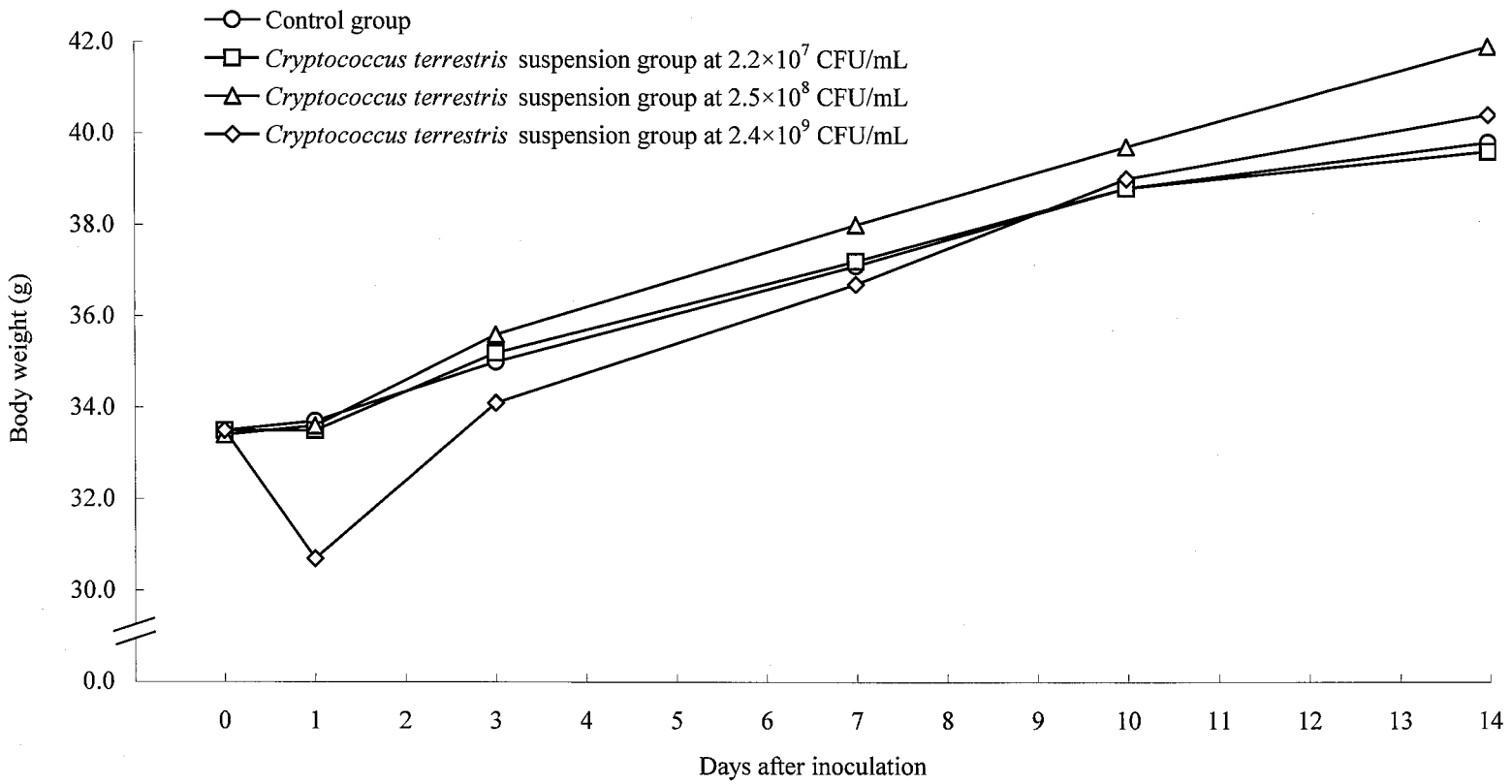

Fig. 1-1. Body weights of male mice (intravenous inoculation).
CFU: Colony forming unit.

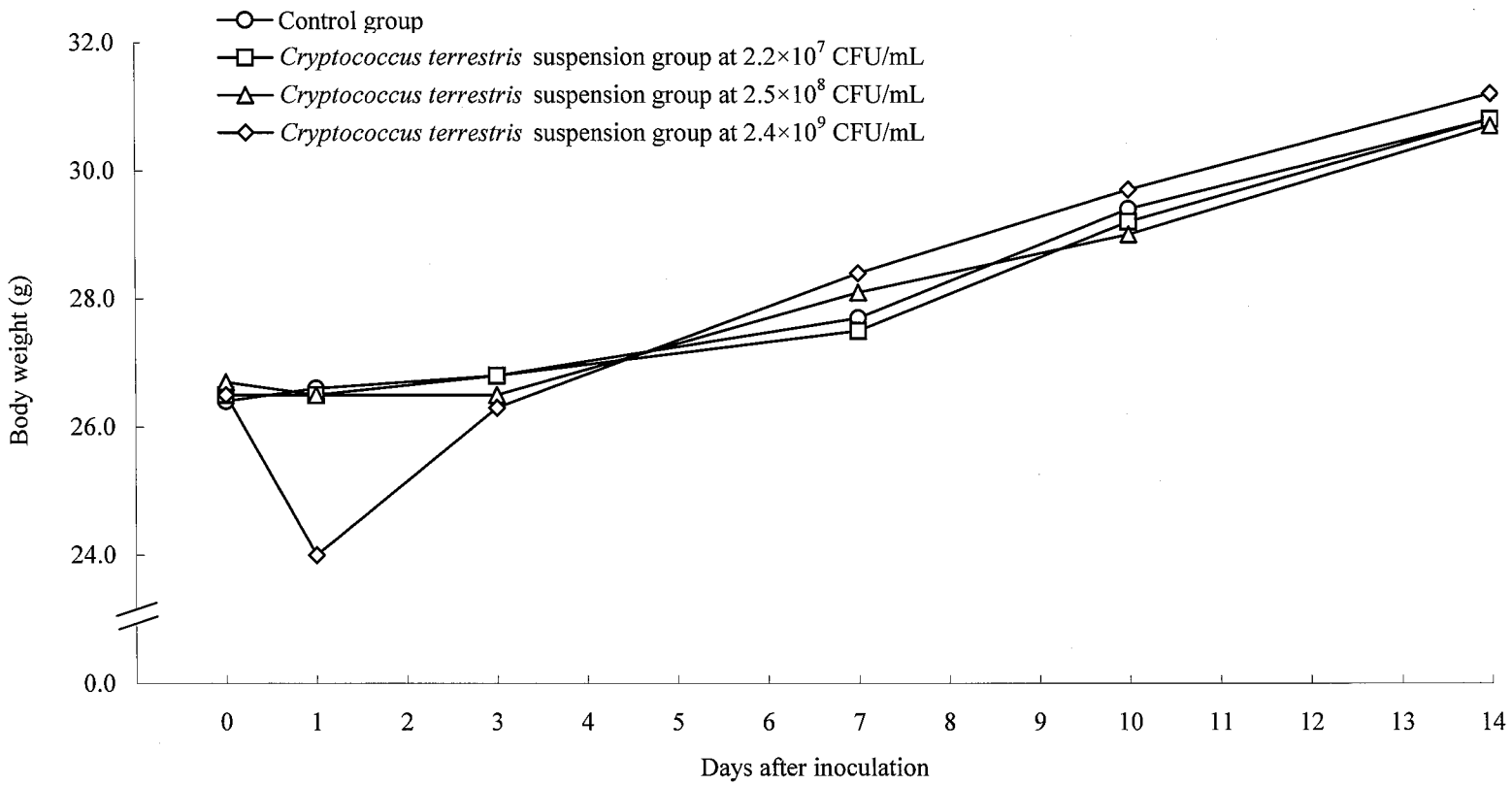

Fig. 1-2. Body weights of female mice (intravenous inoculation).
CFU: Colony forming unit.

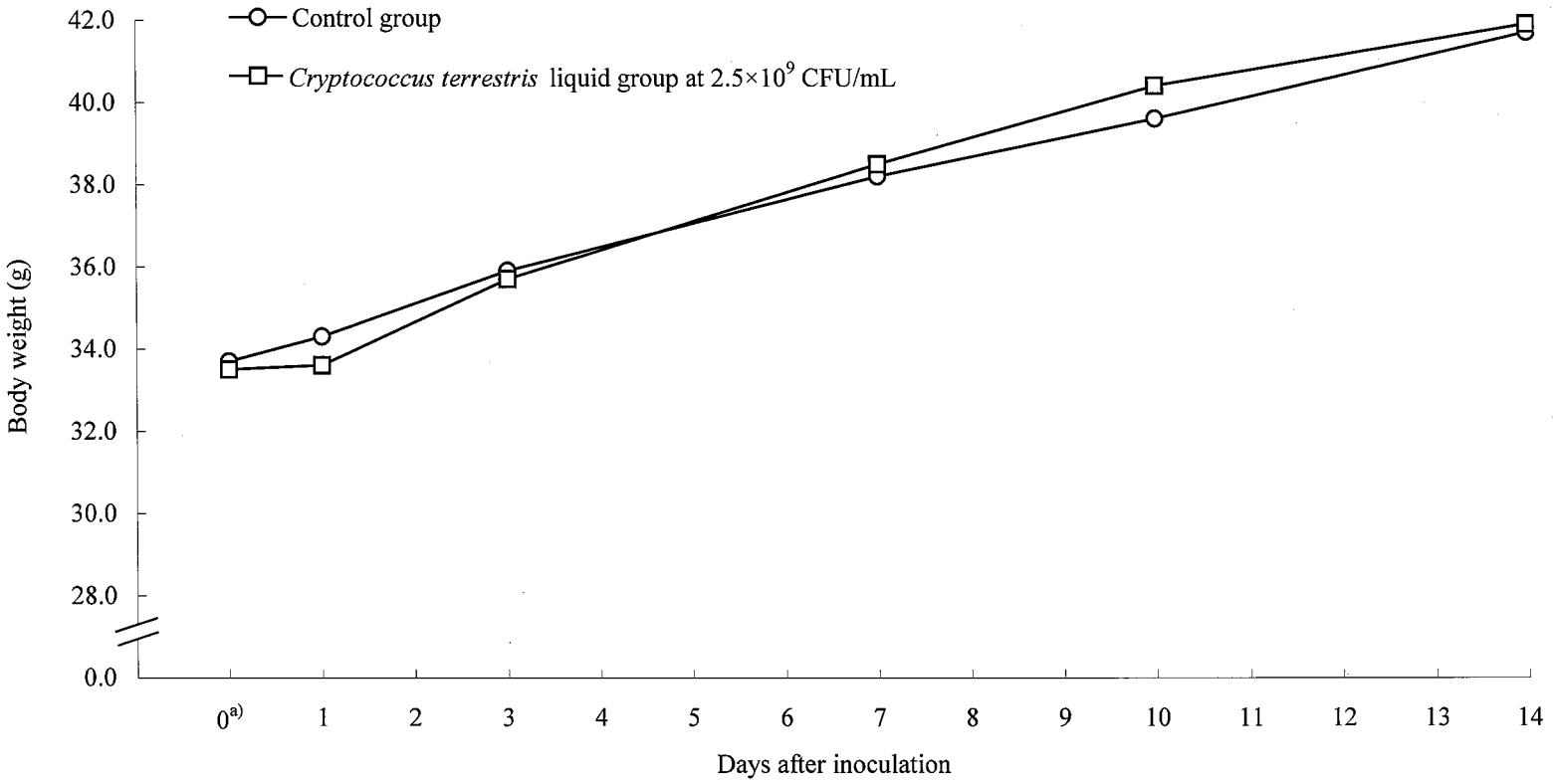

Fig. 2-1. Body weights of male mice (oral inoculation).
CFU: Colony forming unit.
a): Grouping day

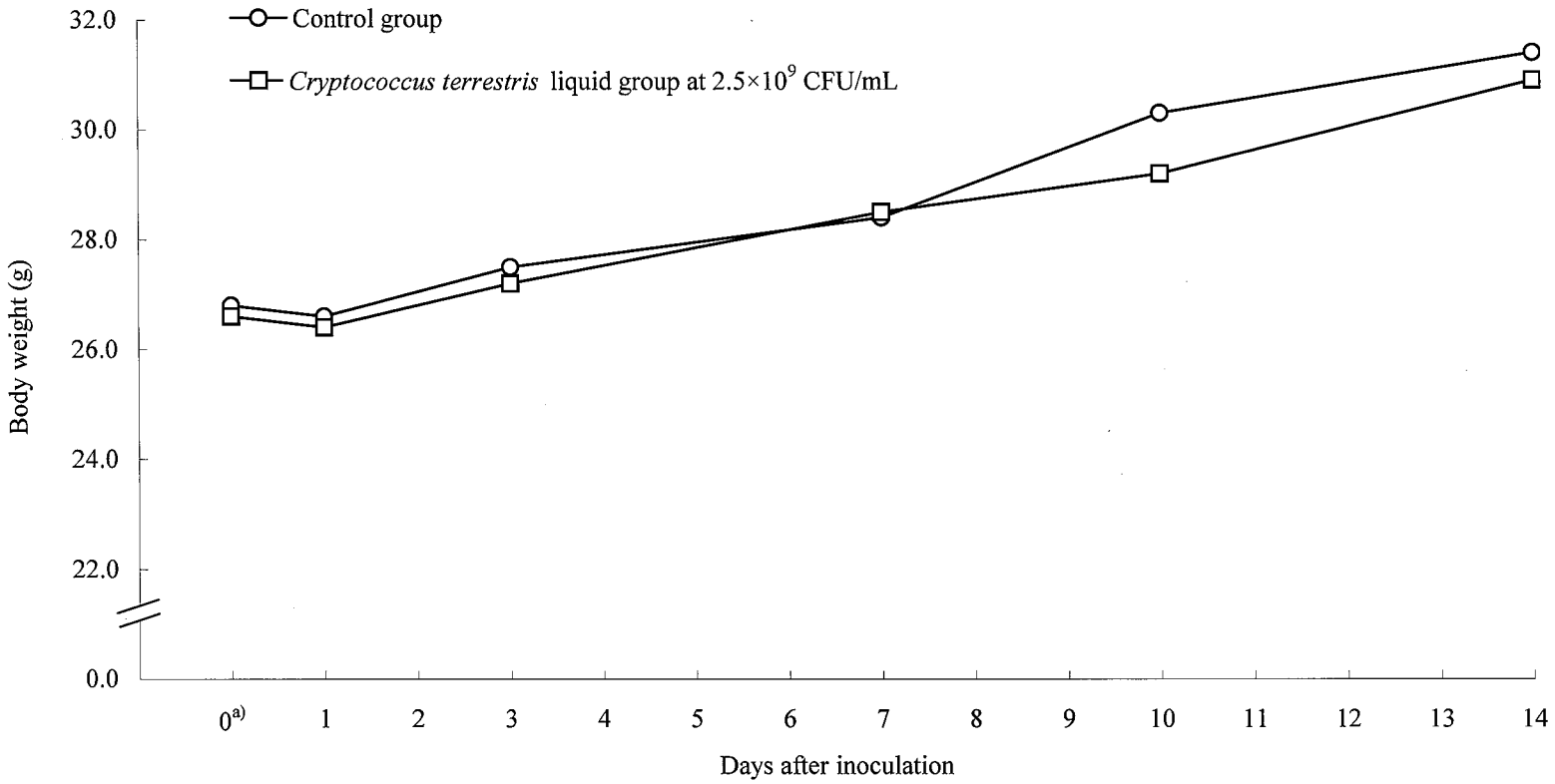

Fig. 2-2. Body weights of female mice (oral inoculation).
CFU: Colony forming unit.
a): Grouping day

Appendix 1-1-1. Individual clinical signs in male mice (intravenous inoculation)

Control group

Male No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M01101	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M01102	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M01103	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M01104	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M01105	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Pre: Before inoculation.
N: Normal.

Appendix 1-1-2. Individual clinical signs in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Male No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M02201	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M02202	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M02203	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M02204	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M02205	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

CFU: Colony forming unit.
Pre: Before inoculation.
N: Normal.

Appendix 1-1-3. Individual clinical signs in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Male No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M03301	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M03302	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M03303	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M03304	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M03305	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

CFU: Colony forming unit.
Pre: Before inoculation.
N: Normal.

Appendix 1-1-4. Individual clinical signs in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Male No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M04401	N	N	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M04402	N	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M04403	N	N	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M04404	N	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M04405	N	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	2	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
A	0	3	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CFU: Colony forming unit.
Pre: Before inoculation.
N: Normal.
A: Decrease in locomotor activity.

Appendix 1-2-1. Individual clinical signs in female mice (intravenous inoculation)

Control group

Female No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
F01151	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F01152	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F01153	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F01154	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F01155	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Pre: Before inoculation.
N: Normal.

Appendix 1-2-2. Individual clinical signs in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Female No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
F02251	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F02252	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F02253	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F02254	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F02255	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

CFU: Colony forming unit.
Pre: Before inoculation.
N: Normal.

Appendix 1-2-3. Individual clinical signs in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Female No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
F03351	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F03352	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F03353	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F03354	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F03355	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

CFU: Colony forming unit.
Pre: Before inoculation.
N: Normal.

Appendix 1-2-4. Individual clinical signs in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Female No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
F04451	N	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F04452	N	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F04453	N	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F04454	N	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F04455	N	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	0	0	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5
A	0	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CFU: Colony forming unit.
Pre: Before inoculation.
N: Normal.
A: Decrease in locomotor activity.

Appendix 2-1-1. Individual clinical signs in male mice (oral inoculation)

Control group

Male No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M05501	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M05502	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M05503	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M05504	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M05505	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Pre: Before inoculation.
N: Normal.

Appendix 2-1-2. Individual clinical signs in male mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Male No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M06601	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M06602	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M06603	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M06604	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
M06605	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

CFU: Colony forming unit.
Pre: Before inoculation.
N: Normal.

Appendix 2-2-1. Individual clinical signs in female mice (oral inoculation)

Control group

Female No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
F05551	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F05552	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F05553	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F05554	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F05555	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Pre: Before inoculation.
N: Normal.

Appendix 2-2-2. Individual clinical signs in female mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Female No.	Pre	Hours after inoculation			Days after inoculation													
		0-1	1-2	2-4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
F06651	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F06652	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F06653	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F06654	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
F06655	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

CFU: Colony forming unit.
Pre: Before inoculation.
N: Normal.

Appendix 3-1-1. Individual body weights of male mice (intravenous inoculation)

Control group

Male No.	Days after inoculation					
	0	1	3	7	10	14
M01101	34.8	33.3	36.1	38.2	39.2	41.4
M01102	34.0	34.0	35.4	37.7	39.1	40.4
M01103	33.8	34.6	35.3	37.8	39.8	40.3
M01104	32.2	33.2	34.3	36.5	38.9	38.5
M01105	32.5	33.2	33.7	35.4	36.9	38.4
Number of males	5	5	5	5	5	5
Mean	33.5	33.7	35.0	37.1	38.8	39.8
S.D.	1.1	0.6	1.0	1.2	1.1	1.3

Unit: g.

Appendix 3-1-2. Individual body weights of male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Male No.	Days after inoculation					
	0	1	3	7	10	14
M02201	34.6	34.6	36.5	37.2	39.8	41.3
M02202	33.8	33.4	35.3	37.3	38.3	38.6
M02203	33.4	33.3	35.0	37.5	39.5	41.4
M02204	31.7	32.0	33.7	34.7	34.5	33.3
M02205	33.9	34.1	35.5	39.1	41.8	43.6
Number of males	5	5	5	5	5	5
Mean	33.5	33.5	35.2	37.2	38.8	39.6
S.D.	1.1	1.0	1.0	1.6	2.7	4.0
Significance	NS	NS	NS	NS	NS	NS
Statistical method	DU	DU	DU	DU	DU	DU

Unit: g.
CFU: Colony forming unit.
NS: Not significantly different from the control group.
DU: Analysis by Dunnett's test.

Appendix 3-1-3. Individual body weights of male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Male No.	Days after inoculation					
	0	1	3	7	10	14
M03301	34.9	34.9	37.3	40.8	43.5	45.6
M03302	33.8	33.8	36.2	39.4	41.1	44.2
M03303	33.1	33.6	35.2	37.4	38.6	39.5
M03304	31.5	31.4	33.1	34.7	35.9	38.8
M03305	33.9	34.3	36.3	37.9	39.3	41.2
Number of males	5	5	5	5	5	5
Mean	33.4	33.6	35.6	38.0	39.7	41.9
S.D.	1.3	1.3	1.6	2.3	2.8	3.0
Significance	NS	NS	NS	NS	NS	NS
Statistical method	DU	DU	DU	DU	DU	DU

Unit: g.
CFU: Colony forming unit.
NS: Not significantly different from the control group.
DU: Analysis by Dunnett's test.

Appendix 3-1-4. Individual body weights of male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Male No.	Days after inoculation					
	0	1	3	7	10	14
M04401	33.6	32.9	35.3	37.8	40.2	41.0
M04402	32.4	28.3	32.6	35.8	37.5	38.4
M04403	34.8	31.9	35.6	37.5	40.1	42.3
M04404	34.0	31.0	34.4	37.1	39.3	40.6
M04405	32.6	29.6	32.4	35.3	37.7	39.6
Number of males	5	5	5	5	5	5
Mean	33.5	30.7	34.1	36.7	39.0	40.4
S.D.	1.0	1.8	1.5	1.1	1.3	1.5
Significance	NS	**	NS	NS	NS	NS
Statistical method	DU	DU	DU	DU	DU	DU

Unit: g.
CFU: Colony forming unit.
Significantly different from the control group (**: $p<0.01$ by Dunnett's test).
NS: Not significantly different from the control group.
DU: Analysis by Dunnett's test.

Appendix 3-2-1. Individual body weights of female mice (intravenous inoculation)

Control group

Female No.	Days after inoculation					
	0	1	3	7	10	14
F01151	27.3	27.0	26.9	27.7	30.2	30.6
F01152	26.5	26.8	26.5	27.6	28.8	30.0
F01153	27.1	27.3	27.8	29.6	32.6	35.2
F01154	25.9	26.9	28.2	28.1	28.3	30.5
F01155	25.3	24.8	24.8	25.3	26.9	27.8
Number of females	5	5	5	5	5	5
Mean	26.4	26.6	26.8	27.7	29.4	30.8
S.D.	0.8	1.0	1.3	1.5	2.2	2.7

Unit: g.

Appendix 3-2-2. Individual body weights of female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Female No.	Days after inoculation					
	0	1	3	7	10	14
F02251	27.2	27.6	29.4	30.5	32.6	33.1
F02252	26.9	26.3	27.1	28.1	29.0	30.2
F02253	26.8	27.2	25.7	26.3	27.9	28.2
F02254	26.0	26.2	25.9	28.4	30.6	34.2
F02255	25.6	25.2	25.7	24.3	26.1	28.4
Number of females	5	5	5	5	5	5
Mean	26.5	26.5	26.8	27.5	29.2	30.8
S.D.	0.7	0.9	1.6	2.3	2.5	2.7
Significance	NS	NS	NS	NS	NS	NS
Statistical method	DU	DU	DU	DU	DU	DU

Unit: g.
CFU: Colony forming unit.
NS: Not significantly different from the control group.
DU: Analysis by Dunnett's test.

Appendix 3-2-3. Individual body weights of female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Female No.	Days after inoculation					
	0	1	3	7	10	14
F03351	26.1	25.6	25.8	26.6	27.3	28.2
F03352	25.7	25.5	26.1	28.0	28.9	31.9
F03353	26.5	26.2	26.0	27.1	28.8	31.9
F03354	27.2	27.9	27.4	30.0	32.3	32.0
F03355	28.1	27.4	27.3	28.7	27.9	29.3
Number of females	5	5	5	5	5	5
Mean	26.7	26.5	26.5	28.1	29.0	30.7
S.D.	0.9	1.1	0.8	1.3	1.9	1.8
Significance	NS	NS	NS	NS	NS	NS
Statistical method	DU	DU	DU	DU	DU	DU

Unit: g.
CFU: Colony forming unit.
NS: Not significantly different from the control group.
DU: Analysis by Dunnett's test.

Appendix 3-2-4. Individual body weights of female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Female No.	Days after inoculation					
	0	1	3	7	10	14
F04451	25.4	23.1	26.0	28.4	28.8	31.5
F04452	27.3	24.1	27.6	29.6	32.3	33.0
F04453	26.0	23.3	25.2	26.4	27.9	29.1
F04454	26.8	24.8	27.2	30.5	30.6	33.7
F04455	26.9	24.9	25.5	27.2	28.9	28.6
Number of females	5	5	5	5	5	5
Mean	26.5	24.0	26.3	28.4	29.7	31.2
S.D.	0.8	0.8	1.1	1.7	1.8	2.3
Significance	NS	**	NS	NS	NS	NS
Statistical method	DU	DU	DU	DU	DU	DU

Unit: g.
CFU: Colony forming unit.
Significantly different from the control group (**: $p<0.01$ by Dunnett's test).
NS: Not significantly different from the control group.
DU: Analysis by Dunnett's test.

Appendix 4-1-1. Individual body weights of male mice (oral inoculation)

Control group

Male No.	Days after inoculation					
	0[a)]	1	3	7	10	14
M05501	32.6	34.3	35.1	37.4	38.4	40.6
M05502	33.8	33.3	35.4	37.6	39.4	41.9
M05503	34.3	34.6	36.9	39.6	40.7	42.3
M05504	32.4	33.8	34.8	37.0	38.2	40.5
M05505	35.2	35.7	37.2	39.2	41.3	43.1
Number of males	5	5	5	5	5	5
Mean	33.7	34.3	35.9	38.2	39.6	41.7
S.D.	1.2	0.9	1.1	1.2	1.4	1.1

Unit: g.

a): Grouping day

Appendix 4-1-2. Individual body weights of male mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Male No.	Days after inoculation					
	0[a)]	1	3	7	10	14
M06601	34.8	34.3	36.6	39.1	41.1	42.5
M06602	32.6	32.2	34.1	36.9	38.5	38.9
M06603	33.9	34.3	36.0	39.4	40.5	42.0
M06604	34.2	34.8	37.0	39.9	42.5	44.9
M06605	32.2	32.4	34.6	37.3	39.2	41.0
Number of males	5	5	5	5	5	5
Mean	33.5	33.6	35.7	38.5	40.4	41.9
S.D.	1.1	1.2	1.3	1.3	1.6	2.2
Significance	NS	NS	NS	NS	NS	NS
Statistical method	TT	TT	TT	TT	TT	TT

Unit: g.
CFU: Colony forming unit.
NS: Not significantly different from the control group.
TT: Analysis by Student's t-test.
a): Grouping day

Appendix 4-2-1. Individual body weights of female mice (oral inoculation)

Control group

Female No.	Days after inoculation					
	0[a)]	1	3	7	10	14
F05551	28.1	27.3	27.4	28.3	30.0	32.4
F05552	25.8	26.7	26.6	27.3	29.2	28.8
F05553	27.1	27.7	28.2	29.1	31.0	32.2
F05554	26.4	25.6	27.6	27.7	29.1	30.0
F05555	26.5	25.9	27.8	29.6	32.1	33.7
Number of females	5	5	5	5	5	5
Mean	26.8	26.6	27.5	28.4	30.3	31.4
S.D.	0.9	0.9	0.6	1.0	1.3	2.0

Unit: g.

a): Grouping day

Appendix 4-2-2. Individual body weights of female mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Female No.	Days after inoculation					
	0[a)]	1	3	7	10	14
F06651	26.6	27.2	28.1	28.9	30.2	32.4
F06652	25.5	25.6	26.9	29.0	29.1	30.1
F06653	27.0	25.8	25.5	26.2	27.3	28.8
F06654	26.1	26.5	27.1	28.1	28.4	30.6
F06655	27.8	26.9	28.2	30.4	31.1	32.5
Number of females	5	5	5	5	5	5
Mean	26.6	26.4	27.2	28.5	29.2	30.9
S.D.	0.9	0.7	1.1	1.5	1.5	1.6
Significance	NS	NS	NS	NS	NS	NS
Statistical method	TT	TT	TT	TT	TT	TT

Unit: g.
CFU: Colony forming unit.
NS: Not significantly different from the control group.
TT: Analysis by Student's t-test.
a): Grouping day

Appendix 5-1-1. Individual necropsy findings in male mice (intravenous inoculation)

Control group

Male No.		Finding
M01101	All organs and tissues	Normal
M01102	All organs and tissues	Normal
M01103	All organs and tissues	Normal
M01104	All organs and tissues	Normal
M01105	All organs and tissues	Normal

Appendix 5-1-2. Individual necropsy findings in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Male No.		Finding
M02201	All organs and tissues	Normal
M02202	All organs and tissues	Normal
M02203	All organs and tissues	Normal
M02204	All organs and tissues	Normal
M02205	All organs and tissues	Normal

CFU: Colony forming unit.

Appendix 5-1-3. Individual necropsy findings in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Male No.		Finding
M03301	All organs and tissues	Normal
M03302	All organs and tissues	Normal
M03303	All organs and tissues	Normal
M03304	All organs and tissues	Normal
M03305	All organs and tissues	Normal

CFU: Colony forming unit.

Appendix 5-1-4. Individual necropsy findings in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Male No.	Finding	
M04401	All organs and tissues	Normal
M04402	All organs and tissues	Normal
M04403	All organs and tissues	Normal
M04404	All organs and tissues	Normal
M04405	All organs and tissues	Normal

CFU: Colony forming unit.

Appendix 5-2-1. Individual necropsy findings in female mice (intravenous inoculation)

Control group

Female No.		Finding
F01151	All organs and tissues	Normal
F01152	All organs and tissues	Normal
F01153	All organs and tissues	Normal
F01154	All organs and tissues	Normal
F01155	All organs and tissues	Normal

Appendix 5-2-2. Individual necropsy findings in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Female No.		Finding
F02251	All organs and tissues	Normal
F02252	All organs and tissues	Normal
F02253	All organs and tissues	Normal
F02254	All organs and tissues	Normal
F02255	All organs and tissues	Normal

CFU: Colony forming unit.

Appendix 5-2-3. Individual necropsy findings in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Female No.		Finding
F03351	All organs and tissues	Normal
F03352	All organs and tissues	Normal
F03353	All organs and tissues	Normal
F03354	All organs and tissues	Normal
F03355	All organs and tissues	Normal

CFU: Colony forming unit.

Appendix 5-2-4. Individual necropsy findings in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Female No.		Finding
F04451	All organs and tissues	Normal
F04452	All organs and tissues	Normal
F04453	All organs and tissues	Normal
F04454	All organs and tissues	Normal
F04455	All organs and tissues	Normal

CFU: Colony forming unit.

Appendix 6-1-1. Individual necropsy findings in male mice (oral inoculation)

Control group

Male No.		Finding
M05501	All organs and tissues	Normal
M05502	All organs and tissues	Normal
M05503	All organs and tissues	Normal
M05504	All organs and tissues	Normal
M05505	All organs and tissues	Normal

Appendix 6-1-2. Individual necropsy findings in male mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Male No.	Finding	
M06601	All organs and tissues	Normal
M06602	All organs and tissues	Normal
M06603	All organs and tissues	Normal
M06604	All organs and tissues	Normal
M06605	All organs and tissues	Normal

CFU: Colony forming unit.

Appendix 6-2-1. Individual necropsy findings in female mice (oral inoculation)

Control group

Female No.		Finding
F05551	All organs and tissues	Normal
F05552	All organs and tissues	Normal
F05553	All organs and tissues	Normal
F05554	All organs and tissues	Normal
F05555	All organs and tissues	Normal

Appendix 6-2-2. Individual necropsy findings in female mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Female No.		Finding
F06651	All organs and tissues	Normal
F06652	All organs and tissues	Normal
F06653	All organs and tissues	Normal
F06654	All organs and tissues	Normal
F06655	All organs and tissues	Normal

CFU: Colony forming unit.

Appendix 7-1-1. Individual number of remaining viable yeast in male mice (intravenous inoculation)

Control group

Male No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
M01101	0	0	0	0	0
M01102	0	0	0	0	0
M01103	0	0	0	0	0
M01104	0	0	0	0	0
M01105	0	0	0	0	0
Number of males	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0

Appendix 7-1-2. Individual number of remaining viable yeast in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Male No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
M02201	0	0	0	0	0
M02202	0	0	0	0	0
M02203	0	0	0	0	0
M02204	0	0	0	0	0
M02205	0	0	0	0	0
Number of males	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0
Significance	NS	NS	NS	NS	NS
Statistical method	JO	JO	JO	JO	JO

CFU: Colony forming unit.
NS: Not significantly different from the control group.
JO: Analysis by Jonckheere's test.

Appendix 7-1-3. Individual number of remaining viable yeast in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Male No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
M03301	0	0	0	0	0
M03302	0	0	0	0	0
M03303	0	0	0	0	0
M03304	0	0	0	0	0
M03305	0	0	0	0	0
Number of males	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0
Significance	NS	NS	NS	NS	NS
Statistical method	JO	JO	JO	JO	JO

CFU: Colony forming unit.
NS: Not significantly different from the control group.
JO: Analysis by Jonckheere's test.

Appendix 7-1-4. Individual number of remaining viable yeast in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Male No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
M04401	0	0	0	0	0
M04402	0	0	0	0	0
M04403	0	0	0	0	0
M04404	0	0	0	0	0
M04405	0	0	0	0	0
Number of males	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0
Significance	NS	NS	NS	NS	NS
Statistical method	JO	JO	JO	JO	JO

CFU: Colony forming unit.
NS: Not significantly different from the control group.
JO: Analysis by Jonckheere's test.

Appendix 7-2-1. Individual number of remaining viable yeast in female mice (intravenous inoculation)

Control group

Female No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
F01151	0	0	0	0	0
F01152	0	0	0	0	0
F01153	0	0	0	0	0
F01154	0	0	0	0	0
F01155	0	0	0	0	0
Number of females	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0

Appendix 7-2-2. Individual number of remaining viable yeast in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Female No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
F02251	0	0	0	0	0
F02252	0	0	0	0	0
F02253	0	0	0	0	0
F02254	0	0	0	0	0
F02255	0	0	0	0	0
Number of females	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0
Significance	NS	NS	NS	NS	NS
Statistical method	JO	JO	JO	JO	JO

CFU: Colony forming unit.
NS: Not significantly different from the control group.
JO: Analysis by Jonckheere's test.

Appendix 7-2-3. Individual number of remaining viable yeast in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Female No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
F03351	0	0	0	0	0
F03352	0	0	0	0	0
F03353	0	0	0	0	0
F03354	0	0	0	0	0
F03355	0	0	0	0	0
Number of females	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0
Significance	NS	NS	NS	NS	NS
Statistical method	JO	JO	JO	JO	JO

CFU: Colony forming unit.
NS: Not significantly different from the control group.
JO: Analysis by Jonckheere's test.

Appendix 7-2-4. Individual number of remaining viable yeast in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Female No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
F04451	0	0	0	0	0
F04452	0	0	0	0	0
F04453	0	0	0	0	0
F04454	0	0	0	0	0
F04455	0	0	0	0	0
Number of females	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0
Significance	NS	NS	NS	NS	NS
Statistical method	JO	JO	JO	JO	JO

CFU: Colony forming unit.
NS: Not significantly different from the control group.
JO: Analysis by Jonckheere's test.

Appendix 8-1-1. Individual number of remaining viable yeast in male mice (oral inoculation)

Control group

Male No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
M05501	0	0	0	0	0
M05502	0	0	0	0	0
M05503	0	0	0	0	0
M05504	0	0	0	0	0
M05505	0	0	0	0	0
Number of males	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0

Appendix 8-1-2. Individual number of remaining viable yeast in male mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Male No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
M06601	0	0	0	0	0
M06602	0	0	0	0	0
M06603	0	0	0	0	0
M06604	0	0	0	0	0
M06605	0	0	0	0	0
Number of males	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0
Significance	NS	NS	NS	NS	NS
Statistical method	TT	TT	TT	TT	TT

CFU: Colony forming unit.
NS: Not significantly different from the control group.
TT: Analysis by Student's t-test.

Appendix 8-2-1. Individual number of remaining viable yeast in female mice (oral inoculation)

Control group

Female No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
F05551	0	0	0	0	0
F05552	0	0	0	0	0
F05553	0	0	0	0	0
F05554	0	0	0	0	0
F05555	0	0	0	0	0
Number of females	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0

Appendix 8-2-2. Individual number of remaining viable yeast in female mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Female No.	Number of remaining viable yeast				
	Brain	Lung	Liver	Spleen	Kidneys
F06651	0	0	0	0	0
F06652	0	0	0	0	0
F06653	0	0	0	0	0
F06654	0	0	0	0	0
F06655	0	0	0	0	0
Number of females	5	5	5	5	5
Mean	0.0	0.0	0.0	0.0	0.0
S.D.	0.0	0.0	0.0	0.0	0.0
Significance	NS	NS	NS	NS	NS
Statistical method	TT	TT	TT	TT	TT

CFU: Colony forming unit.
NS: Not significantly different from the control group.
TT: Analysis by Student's t-test.

Appendix 9-1-1. Individual histopathological findings in male mice (intravenous inoculation)

Control group

Male No.	Organ/Tissue	Finding
M01101	All organs and tissues	No abnormality detected
M01102	All organs and tissues	No abnormality detected
M01103	All organs and tissues	No abnormality detected
M01104	All organs and tissues	No abnormality detected
M01105	All organs and tissues	No abnormality detected

Appendix 9-1-2. Individual histopathological findings in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Male No.	Organ/Tissue	Finding
M02201	All organs and tissues	No abnormality detected
M02202	All organs and tissues	No abnormality detected
M02203	All organs and tissues	No abnormality detected
M02204	All organs and tissues	No abnormality detected
M02205	All organs and tissues	No abnormality detected

CFU: Colony forming unit.

Appendix 9-1-3. Individual histopathological findings in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Male No.	Organ/Tissue	Finding
M03301	All organs and tissues	No abnormality detected
M03302	All organs and tissues	No abnormality detected
M03303	All organs and tissues	No abnormality detected
M03304	All organs and tissues	No abnormality detected
M03305	All organs and tissues	No abnormality detected

CFU: Colony forming unit.

Appendix 9-1-4. Individual histopathological findings in male mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Male No.	Organ/Tissue	Finding
M04401	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected
M04402	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected
M04403	All organs and tissues	No abnormality detected
M04404	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected
M04405	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected

Grade of histopathological findings: ±: slight, +: mild, 2+: moderate, 3+: marked.
CFU: Colony forming unit.

Appendix 9-2-1. Individual histopathological findings in female mice (intravenous inoculation)

Control group

Female No.	Organ/Tissue	Finding
F01151	All organs and tissues	No abnormality detected
F01152	All organs and tissues	No abnormality detected
F01153	All organs and tissues	No abnormality detected
F01154	All organs and tissues	No abnormality detected
F01155	All organs and tissues	No abnormality detected

Appendix 9-2-2. Individual histopathological findings in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.2×10^7 CFU/mL

Female No.	Organ/Tissue	Finding
F02251	All organs and tissues	No abnormality detected
F02252	All organs and tissues	No abnormality detected
F02253	All organs and tissues	No abnormality detected
F02254	All organs and tissues	No abnormality detected
F02255	All organs and tissues	No abnormality detected

CFU: Colony forming unit.

Appendix 9-2-3. Individual histopathological findings in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.5×10^8 CFU/mL

Female No.	Organ/Tissue	Finding
F03351	All organs and tissues	No abnormality detected
F03352	All organs and tissues	No abnormality detected
F03353	All organs and tissues	No abnormality detected
F03354	All organs and tissues	No abnormality detected
F03355	All organs and tissues	No abnormality detected

CFU: Colony forming unit.

Appendix 9-2-4. Individual histopathological findings in female mice (intravenous inoculation)

Cryptococcus terrestris suspension group at 2.4×10^9 CFU/mL

Female No.	Organ/Tissue	Finding
F04451	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected
F04452	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected
F04453	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected
F04454	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected
F04455	Liver	Granuloma: +
	Other organs and tissues	No abnormality detected

Grade of histopathological findings: ±: slight, +: mild, 2+: moderate, 3+: marked.
CFU: Colony forming unit.

Appendix 10-1-1. Individual histopathological findings in male mice (oral inoculation)

Control group

Male No.	Organ/Tissue	Finding
M05501	All organs and tissues	No abnormality detected
M05502	All organs and tissues	No abnormality detected
M05503	All organs and tissues	No abnormality detected
M05504	All organs and tissues	No abnormality detected
M05505	All organs and tissues	No abnormality detected

Appendix 10-1-2. Individual histopathological findings in male mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^9 CFU/mL

Male No.	Organ/Tissue	Finding
M06601	All organs and tissues	No abnormality detected
M06602	All organs and tissues	No abnormality detected
M06603	All organs and tissues	No abnormality detected
M06604	All organs and tissues	No abnormality detected
M06605	All organs and tissues	No abnormality detected

CFU: Colony forming unit.

Appendix 10-2-1. Individual histopathological findings in female mice (oral inoculation)

Control group

Female No.	Organ/Tissue	Finding
F05551	All organs and tissues	No abnormality detected
F05552	All organs and tissues	No abnormality detected
F05553	All organs and tissues	No abnormality detected
F05554	All organs and tissues	No abnormality detected
F05555	All organs and tissues	No abnormality detected

Appendix 10-2-2. Individual histopathological findings in female mice (oral inoculation)

Cryptococcus terrestris liquid group at 2.5×10^{9} CFU/mL

Female No.	Organ/Tissue	Finding
F06651	All organs and tissues	No abnormality detected
F06652	All organs and tissues	No abnormality detected
F06653	All organs and tissues	No abnormality detected
F06654	All organs and tissues	No abnormality detected
F06655	All organs and tissues	No abnormality detected

CFU: Colony forming unit.

Attachment 1.

Amano Enzyme Inc.
Gifu R&D Center

Tel:058-379-1223 fax:058-379-1228

23. May 2016

Report of Counting of Viable Yeast

Study No.: 360030

Title: Single Intravenous Inoculation of *Cryptococcus terrestris* Suspensions and Single Oral Inoculation of *Cryptococcus terrestris* Culture

Result:

	Lot. No.	Before Inoculation[1] CFU[2]/ mL	After Inoculation[1] CFU[2]/ mL
Cryptococcus terrestris Suspensions	20160516-1	1.3×10^7	1.0×10^7
	20160516-2	1.3×10^8	1.0×10^8
	20160516-3	1.3×10^9	1.0×10^9
Cryptococcus terrestris Culture	20160517-1	1.2×10^9	1.0×10^9

1) Count of the number of viable yeast on the agar medium incubated at 27 degrees Celsius for 2 days

2) Colony forming unit

Conclusion:

It was confirmed that the each lot has almost same viable yeast between before and after inoculation.

Food & Industrial Enzyme Division

Signature (b) (6)

Yukiko Hoshi

Statement of Quality Assurance Unit

Study No.: 360030

Title: Single Intravenous Inoculation of *Cryptococcus terrestris* Suspensions and Single Oral Inoculation of *Cryptococcus terrestris* Culture

This statement certifies that this report accurately describes the methods of the study, and the reported results accurately reflect the raw data obtained during the course of the study.

Details of the inspections are shown below. Findings of the inspections were reported to the Management and Study Director.

Item inspected	Date of inspection	Date on which findings were reported to the Management and Study Director
1. Raw data	August 2, 2016	August 3, 2016
2. Draft report (1st draft)	August 3, 2016	August 3, 2016
3 Raw data (2nd inspection)	August 17, 2016	August 17, 2016
4. Draft report (1st draft) (2nd inspection)	August 17, 2016	August 17, 2016
5. Final report	August 22, 2016	August 22, 2016

August 22, 2016

Hashima Laboratory, Nihon Bioresearch Inc.

Manager of Quality Assurance Unit: Yoshiaki Hongo (seal)

APPENDIX 4

Final Report

A BACTERIAL REVERSE MUTATION TEST OF β-GALACTOSIDASE CONCENTRATE

Study Number: T-2095

Study Period: May 19, 2016 to July 6, 2016

Test Facility

Tokyo Laboratory, BoZo Research Center Inc.
1-3-11, Hanegi, Setagaya-ku, Tokyo 156-0042, Japan

Sponsor

Amano Enzyme Inc.
1-2-7, Nishiki, Naka-ku, Nagoya 460-8630 Japan

Contractor

BoZo Research Center Inc.
36-7, Oyama-cho, Shibuya-ku, Tokyo 151-0065, Japan

1. GLP Statement

Study Number: T-2095
Study Title: A bacterial reverse mutation test of β-galactosidase concentrate

I, the undersigned, hereby declare that this study was conducted in compliance with the following GLP regulation.

- "The Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs", Ordinance No. 21 of the Ministry of Health and Welfare, Japan, March 26, 1997
- "OECD Principles of Good Laboratory Practice" (OECD Council: November 26, 1997)

(Sealed in the original) March 9, 2016

Kazuyuki Minegawa Date
Study Director
Tokyo Laboratory, BoZo Research Center Inc.

2. Table of Contents

Tables

Figures

Attached Data

3. Outline of Study

3.1 Study Number

T-2095

3.2 Study Title

A bacterial reverse mutation test of β-galactosidase concentrate

3.3 Purpose of Study

A bacterial reverse mutation test was conducted to evaluate the presence or absence of gene mutation inducibility of β-galactosidase concentrate.

3.4 Sponsor

Amano Enzyme Inc.

2-7, Nishiki 1-chome, Naka-ku, Nagoya-shi, Aichi-ken 460-8630, Japan

3.5 Contractor

BoZo Research Center Inc.

36-7 Oyama-cho, Shibuya-ku, Tokyo 151-0065, Japan

3.6 Testing Facility

Tokyo Laboratory, BoZo Research Center Inc.

1-3-11 Hanegi, Setagaya-ku, Tokyo 156-0042, Japan

3.7 Study Schedule

Start of Study:	May 19, 2016
Start of Dose-finding Test:	May 19, 2016
End of Dose-finding Test:	May 23, 2016
Start of First Main Test:	May 26, 2016
End of First Main Test:	May 30, 2016
Start of Second Main Test:	June 2, 2016
End of Second Main Test:	June 6, 2016
End of Study:	July 6, 2016

3.8 Study Director

Kazuyuki Minegawa

The First Laboratory for Safety Evaluation, Tokyo Laboratory, BoZo Research Center Inc.

3.9 Study Contributors

The First Laboratory for Safety Evaluation, Tokyo Laboratory, BoZo Research Center Inc.

Person Responsible for Test Article Archiving:
Kaori Nakamura

Study Contributors: Kaori Nakamura, Noriko Yamaguchi, Ryosuke Sato

3.10 Unforeseeable Circumstances that May Have Affected the Reliability of the Study and Deviations from the Protocol

There were not unforeseeable circumstances that might have affected the reliability of the study. As the deviation from the protocol, 0.20 μm filter was used, but the protocol was originally described that syringe filter for sterilization when used to prepare test article was 20 μm in pore size. But this description is misprint, it was judged no influence with this study because it was conducted the operation that was being planned.

3.11 Archives

The original protocol, written records, raw data and reports (including the original of the final report) will be retained in the archive of Gotemba Laboratory, BoZo Research Center Inc. for a period of 5 years after submission of the Final Report. At the end of this period, Amano Enzyme Inc. and BoZo Research Center Inc. will discuss and determine the disposition of the said materials.

3.12 Signature of Study Director and Date

(Sealed in the original) July 6, 2016

Kazuyuki Minegawa Date

Study Director

Tokyo Laboratory, BoZo Research Center Inc.

4. Summary

In order to examine the gene mutation inducibility of β-galactosidase concentrate, a reverse mutation assay was conducted in *Salmonella typhimurium* (hereinafter referred to as *S. typhimurium*) TA100, TA1535, TA98 and TA1537, and *Escherichia coli* (hereinafter referred to as *E. coli*) WP2 *uvrA* irrespective of the presence/absence of metabolic activation by the pre-incubation method. Distilled water was used as the vehicle for the test article.

A dose-finding test was conducted at dose levels between 19.5 and 5000 μg/plate. From the result of the dose-finding test, 5000μg/plate which showed growth inhibition was selected as maximum dose for main test, which was conducted at 6 dose levels between 156 and 5000 μg/plate for all strains in the absence of metabolic activation. In the case of all strains in the presence of metabolic activation, the main test was conducted at 5 dose levels between 313 and 5000 μg/plate because no growth inhibition was observed. The main test was conducted twice at the same dose levels.

1) Precipitation and Coloration by Test Article

Precipitation and coloration by the test article on the plate was not observed irrespective of the presence/absence of metabolic activation.

2) Growth Inhibition

In the observation of bacterial background lawn using a stereoscopic microscope, growth inhibition was observed at 2500 μg/plate or more for all strains irrespective of the presence/absence of metabolic activation.

3) Number of Revertant Colonies

In the two main tests, there was neither increase in the number of revertant colonies of two-fold or more in comparison with that of the negative control group nor dose-response in any strains irrespective of the presence/absence of metabolic activation.

In conclusion, β-galactosidase concentrate was judged to have no gene mutation inducibility (negative) under the conditions of this study.

5. Introduction

This study was conducted at BoZo Research Center Inc. on behalf of Amano Enzyme Inc. in compliance with the standards and guidelines listed below.

1) GLP

- "The Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs", Ordinance No. 21 of the Ministry of Health and Welfare, Japan, March 26, 1997
- "OECD Principles of Good Laboratory Practice" (OECD Council: November 26, 1997)

2) Guidelines

- "Guidance for Genotoxicity studies of Pharmaceuticals and Interpretation" Notification No. 0920-2 of the Head of Evaluation and Licensing Division, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare
- "Guidelines on Genotoxicity Testing of Pharmaceuticals", Notification No. 1604 of the Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare, Japan, November 1, 1999
- "Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives", Notification No. 29 of the Environmental Health Bureau, Ministry of Health and Welfare, Japan, March 22, 1996
- "OECD Guidelines for Testing of Chemicals 471" (OECD Council: July 21, 1997)

6. Test Article and Preparation of Test Solutions

6.1 Test Article and Vehicle

6.1.1 Test Article

Supplier: Amano Enzyme Inc.

The following test article information is based on the results of non-GLP studies conducted by the study sponsor of this study (Attached Data 1).

Amount Received:	20 g
Date of Receipt:	May 10, 2016
Name:	β-Galactosidase concentrate
Another Name:	Lactase concentrate
Lot Number:	GFE68-001@K
CAS No.:	9031-11-2
Purity:	100 wt%
Outline of Manufacturing Method:	The producing microorganism is cultured, extracted, condensed, subjected to alcohol precipitation and then vacuum-dried to obtain powder.
Characteristics at Ordinary Temperature:	Light yellowish brown powder
Enzyme Activity Value:	2,550 u/g (β-Galactosidase activity, Lactose substrate method)
Loss on Drying:	4.9% (Drying method, 100 g, 105°C)
Total Ash:	5.1% (JSFA method)
Lead:	Not mor than 1 μg/g (JSFA method)
Total Number of Live Aerobic Bacteria:	1.8×10^2 cfu/g (SCD agar plate method)
Stability:	No reaction to light; no effects from the air.
Solubility:	Water: dissolved
Stability in Solvent:	It was confirmed that the 50, 25 and 0.2 mg/mL was stable and homogeneous for at least 5 hours at room temperature and under cooling with ice (Attached Data 2).
Storage Conditions:	In a refrigerator
Date of Manufacturing:	February 13, 2016
Expiration:	February 13, 2017

Storage Place:	Test article storage, Tokyo Laboratory
Storage Temperature:	Measured temperature during the storage period From May 10, 2016 to June 3, 2016: 4.6 to 5.5°C.
Treatment of Remainder:	All the test article remaining after the end of experiment was returned to the supplier.

The solubility in water is information obtained in the preparation of the test solution in BoZo Research Center Inc.

6.1.2 Vehicle

Name:	Distilled water
Manufacturer:	Otsuka Pharmaceutical Factory, Inc.
Lot Number:	K6A80
Specification:	Japanese Pharmacopoeia
Storage Conditions:	Room temperature
Storage Place:	The test article preparation room at Tokyo Laboratory

6.1.3 Reason for Selection of Vehicle

According to the information from the Study Sponsor, the test article is soluble in water and stable for at least 5 hours in water at room temperature (Attached Data 2). Therefore, distilled water was used as the solvent in this study.

6.2 Preparation Methods of Test Solutions

6.2.1 Preparation Methods of Dose-finding Test Solutions

The test article (250.0 mg) was weighed using an electronic balance (GR-120, A&D Co., Ltd.) in a sterilized 5-mL measuring flask for preparation, and distilled water was added to dissolve, and diluted to the specified volume to prepare the 50 mg/mL test article solution. And then, this test article solution was sterilized using syringe filter (IWAKI 0.20 μm, Lot No. 26114004), and was diluted 4 times using a common ratio of 4 to prepare the test article solution at a total of 5 concentrations: 50, 12.5, 3.13, 0.781and 0.195 mg/mL. The test article solutions were prepared at the time of use under fluorescent lamps with ultraviolet-absorbing films, and there were neither exothermic reactions nor generation of gasses during the preparation.

6.2.2 Preparation Methods of First and Second Main Test Solutions

The test article (500.0 mg) was weighed using an electronic balance (GR-120, A&D Co., Ltd.) in a sterilized 10-mL measuring flask for preparation, and distilled water was added to dissolve,

and diluted to the specified volume to prepare the 50 mg/mL test article solution. And then, this test article solution was sterilized using syringe filter (IWAKI 0.20 μm, Lot No. 26114004), and diluted 5 times using a common ratio of 2 to prepare the test article solution at a total of 6 concentrations: 50, 25, 12.5, 6.25, 3.13 and 1.56 mg/mL. The test article solutions were prepared at the time of use under fluorescent lamps with ultraviolet-absorbing films, and there were neither exothermic reactions nor generation of gasses during the preparation.

7. Materials and Methods

7.1 Tester Strains

7.1.1 Type of Strains

The following 5 strains were used.

Base-pair substitution type:

S. typhimurium TA100

S. typhimurium TA1535

E. coli WP2 *uvrA*

Frame-shift type:

S. typhimurium TA98

S. typhimurium TA1537

S. typhimurium TA strains were obtained from the Division of Genetics and Mutagenesis, National Institute of Health Sciences by Gotemba Laboratory, BoZo Research Center Inc. on October 9, 1997. Batches of the strains were separated from those stored by Gotemba Laboratory, BoZo Research Center Inc. and transferred to Tokyo Laboratory, BoZo Research Center Inc. on July 21, 2005. *E. coli* WP2 *uvrA* was obtained from the National Institute of Technology and Evaluation on October 20, 2011.

7.1.2 Reason for Selection of Tester Strains

These strains have high sensitivity to mutagens and are used widely in bacterial mutagenicity studies.

7.1.3 Storage and Thawing of Tester Strains

The frozen stock bacterial strains subcultured from the original tester strains were cultured and 0.7 mL of dimethylsulfoxide (hereinafter referred to as DMSO: Wako Pure Chemical Industries, Ltd., JIS Reagent Special Grade, Lot No. ECE6658) was added to 8.0 mL of each

bacterial suspension. These tester strain suspensions were placed in sterilized tubes (0.3 mL each), then were rapidly frozen with dry ice-acetone and stored in a deep freezer (SANYO Electric Biomedical Co., Ltd.: MDF-192) set at −70°C or lower (measured temperature during the storage period from February 18, 2016 to June 2, 2016: −86.2 to −76.5°C). They were thawed at room temperature at the time of use and the residue was discarded after culturing.

	Date of frozen storage
S. typhimurium TA98	February 18, 2016
S. typhimurium TA100	February 18, 2016
S. typhimurium TA1535	February 18, 2016
S. typhimurium TA1537	February 18, 2016
E. coli WP2 *uvrA*	February 18, 2016

7.1.4 Characteristics Tests of Tester Strains

At the time of preparation of frozen stock bacterial strains as described in 7.1.3, the tester strains were examined for the following characteristics: amino acid requirement, *rfa* mutation, drug resistance factor (R-factor) plasmid, UV sensitivity, growth rate property, negative control value, positive control values, etc. The tester strains were used in the study after verifying that they maintained specific characteristics.

	Date of Characteristics Tests
S. typhimurium TA98	February 18, 2016 to February 22, 2016
S. typhimurium TA100	February 18, 2016 to February 22, 2016
S. typhimurium TA1535	February 18, 2016 to February 22, 2016
S. typhimurium TA1537	February 18, 2016 to February 22, 2016
E. coli WP2 *uvrA*	February 18, 2016 to February 22, 2016

7.2 Control Articles

7.2.1 Negative Control Article

Distilled water, which was used for preparation of the test solution, was selected as the negative control article.

7.2.2 Positive Control Articles

The following mutagens were used as positive control articles.

Text Table 1 List of Positive Control Articles

Positive Control Articles (abbr.)	Lot Number	Purity (%)	Storage Conditions	Manufacturer
2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2)	STQ3987	99.7%	Room temperature, protected from light	Wako Pure Chemical Industries, Ltd.
Sodium azide (SAZ)	YSF7467	99.9%	Room temperature, protected from light	Wako Pure Chemical Industries, Ltd.
2-Methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine·2HCl (ICR-191)	562079		Room temperature, protected from light	Polysciences, Inc.
2-Aminoanthracene (2AA)	CTK0326	96.7%	Room temperature, protected from light	Wako Pure Chemical Industries, Ltd.
Benzo[*a*]pyrene (B[*a*]P)	KPK3371	99.8%	Refrigerator, protected from light	Wako Pure Chemical Industries, Ltd.

Storage Place: The bacterial study room at Tokyo Laboratory

7.2.3 Methods of Preparation

AF-2, ICR-191, 2AA and B[*a*]P were dissolved in DMSO (JIS Reagent Special Grade, Wako Pure Chemical Industries, Ltd., Lot No.: ECE6658), while SAZ was dissolved in distilled water (Japanese Pharmacopoeia, Otsuka Pharmaceutical Factory, Inc., Lot No.: K5F99). Each solution was divided into about 1-mL aliquots and stored at −20°C or lower. They were thawed at the time of use. The prepared concentrations are shown in Text Table 2.

Text Table 2 Concentrations of Positive Controls

Strains	Without metabolic activation		With metabolic activation	
	Positive controls	Concentration (μg/mL)	Positive controls	Concentration (μg/mL)
S. typhimurium TA100	AF-2	0.1 (0.01)	B[*a*]P	50 (5.0)
S. typhimurium TA1535	SAZ	5 (0.5)	2AA	20 (2.0)
E. coli WP2 *uvrA*	AF-2	0.1 (0.01)	2AA	100 (10.0)
S. typhimurium TA98	AF-2	1 (0.1)	B[*a*]P	50 (5.0)
S. typhimurium TA1537	ICR-191	10 (1.0)	B[*a*]P	50 (5.0)

Figures in parentheses indicate dose levels (μg/plate) when plates are prepared.

7.3 Reagents

7.3.1 Preparation of S9 Mix

After one vial of Cofactor-I was dissolved completely in 9.0 mL of sterile purified water, the solution was filter-sterilized (NALGENE 0.45 μm, Lot Nos. 1160775). To the vial of Cofactor-I, 1.0 mL of S9 was added to make S9 Mix. After preparation, it was stored in a refrigerator until the time of use, and the residue remaining after use was discarded.

1) S9

Name:	S9
Manufacturer:	Kikkoman Biochemifa Company
Lot Number:	RAA201603A
Date of Manufacture:	March 18, 2016
Date of Purchase:	April 7, 2016
Species/Strain:	Rat/SD strain
Age/Sex:	7 weeks of age/male
Body Weight:	202–2548 g
Inducers:	Phenobarbital (PB) and 5,6-benzoflavone (BF)
Route of Administration:	Intraperitoneal injection
Duration of Administration and Dose Levels:	
	PB for 4 days, 30+60+60+60 (mg/kg body weight)
	BF on Day 3 of PB, 80 (mg/kg body weight)
Storage Conditions:	Frozen (−70°C or lower)
Storage Place:	In a deep freezer (SANYO Electric Biomedical Co., Ltd.: MDF-192), test article preparation room at Tokyo

Laboratory

Measured Temperature During the Storage Period:

From April 7, 2016 to June 3, 2016: −86.2 to −79.1°C

1) Cofactor

Name:	Cofactor-I
Manufacturer:	Oriental Yeast Co., Ltd.
Lot Number:	999601
Expiration:	January 8, 2017
Date of Purchase:	April 5, 2016 and May 19, 2016
Storage Conditions:	Refrigerator
Storage Place:	In a refrigerator (SANYO Electric Biomedical Co., Ltd.: Pharmaceutical refrigerator with freezer MPR-411FR), bacterial study room at Tokyo Laboratory

Measured Temperature During the Storage Period:

From April 5, 2016 to June 3, 2016: 3.8 to 4.8°C

2) Constituents of S9 Mix (in 1 mL)

Water:	0.9 mL
S9:	0.1 mL
$MgCl_2$:	8 μmol/mL
KCl:	33 μmol/mL
Glucose-6-phosphate:	5 μmol/mL

Reduced nicotinamide adenine dinucleotide phosphate (NADPH):

4 μmol/mL

Reduced nicotinamide adenine dinucleotide (NADH):

4 μmol/mL

Sodium-phosphate buffer (pH 7.4):

100 μmol/mL

7.3.2 Medium

1) Minimal Glucose Agar Plate Medium

Name:	Vital Media AMT-O medium
Manufacturer:	Kyokuto Pharmaceutical Industrial Co., Ltd.
Lot Number:	DZLH3802
Date of Manufacture:	March 8, 2016
Date of Purchase:	May 19, 2016

Storage Conditions:	Room temperature
Storage Place:	The agar medium storage room at Tokyo Laboratory

2) Agar used

Name:	OXOID AGAR No.1
Manufacturer:	OXOID LTD.
Lot Number:	1309432

7.3.3 Nutrient Broth No. 2 Solution

Nutrient Broth No. 2 was dissolved in purified water to make 2.5 wt% and the solution was sterilized in an autoclave (121°C, 20 minutes). After preparation, it was stored in a refrigerator until the time of use.

Name:	Nutrient Broth No. 2
Lot Number:	1239615
Manufacturer:	OXOID LTD.
Storage Conditions:	Room temperature
Storage Place:	The bacterial study room at Tokyo Laboratory

7.3.4 0.1 mol/L Phosphate Buffer (pH 7.4)

Three packs of phosphate buffer powder were dissolved in 2 L of purified water. The buffer solution was sterilized in an autoclave (121°C, 20 minutes). After preparation, it was stored in a refrigerator until the time of use.

Name:	Phosphate Buffer Powder (1/15 mol/l, pH 7.4)
Manufacturer:	Wako Pure Chemical Industries, Ltd.
Lot Number:	SAM0022
Storage Conditions:	Room temperature
Storage Place:	The bacterial study room at Tokyo Laboratory

7.3.5 Top Agar

Soft agar (0.6 wt% Agar, 0.6 wt% NaCl) was prepared using the agar specified below and sterilized in an autoclave (121°C, 20 minutes). A 1/10 volume of 0.5 mmol/L D-biotin – 0.5 mmol/L L-histidine – 0.5 mmol/L L-tryptophan solution was added to the soft agar to prepare top agar for *S. typhimurium* TA strains and *E. coli* strain. After preparation, top agars were stored at room temperature, and after being warmed in a microwave at the time of use, they were kept in a thermostatic chamber set at 45°C to prevent fixation.

1) Agar

Name:	Bacto Agar
Manufacturer:	Becton, Dickinson and Company
Lot Number:	5054861
Storage Conditions:	Room temperature
Storage Place:	The bacterial study room at Tokyo Laboratory

2) NaCl

Manufacturer:	Wako Pure Chemical Industries, Ltd.
Lot Number:	ECJ2666
Storage Conditions:	Room temperature
Storage Place:	The bacterial study room at Tokyo Laboratory

3) D-Biotin

Manufacturer:	Wako Pure Chemical Industries, Ltd.
Lot Number:	SAL6212
Storage Conditions:	Refrigerator, protected from light
Storage Place:	The bacterial study room at Tokyo Laboratory

4) L-Histidine hydrochloride monohydrate

Manufacturer:	Wako Pure Chemical Industries, Ltd.
Lot Number:	CTK0488
Storage Conditions:	Room temperature, protected from light
Storage Place:	The bacterial study room at Tokyo Laboratory

5) L-Tryptophan

Manufacturer:	Wako Pure Chemical Industries, Ltd.
Lot Number:	CTH2695
Storage Conditions:	Room temperature, protected from light
Storage Place:	The bacterial study room at Tokyo Laboratory

7.4 Test Methods

7.4.1 Pre-culturing

1) After the frozen bacterial strains were thawed, the bacterial suspensions, 20 μL of each *S. typhimurium* TA strain or 10 μL of *E. coli* strain bacteria, were seeded to a sterilized L-shaped tube (48-mL capacity) containing 10 mL of Nutrient Broth No. 2. The culture was set in a thermostatically controlled bath shaker (ML-10 Cool Bath Shaker with PU-6, Taitec Corporation). The bacterial suspensions remaining after use were discarded.
2) The culture remained program-controlled at 4°C until the start of pre-culturing (for 6 hours and 30 minutes), and was then pre-cultured while shaking (100 rpm) for 9 hours at 37°C.

3) At the end of pre-culturing, absorbance was measured by a spectrophotometer (Mini photo 518R, Taitec Corporation) and the level of acceptance for the viable cell count of bacteria was set at 1×10^9 cells/mL or higher. The bacterial suspensions were allowed to stand at room temperature until use. Each viable cell count of bacteria is shown in Text Table 3.

Text Table 3 Viable Cell Count of Bacteria

Strains	Cell Count of Bacteria (cells/mL)		
	Dose-finding Test	First Main Test	Second Main Test
S. typhimurium TA100	4.44×10^9	4.88×10^9	4.50×10^9
S. typhimurium TA1535	4.80×10^9	4.50×10^9	4.66×10^9
E. coli WP2 *uvrA*	8.66×10^9	8.97×10^9	8.46×10^9
S. typhimurium TA98	6.04×10^9	7.34×10^9	6.15×10^9
S. typhimurium TA1537	3.70×10^9	3.53×10^9	3.68×10^9

7.4.2 Number of Plates

For the test article treatment group, negative control group and positive control group, the number of plates used at each dose level was 2 in the dose-finding test, while it was 3 in the two main tests.

7.4.3 Test Procedures (Pre-incubation Method)

1) Test solution, vehicle or positive control article (0.1 mL of each) was placed into a sterilized small test tube, 0.5 mL of 0.1 mol/L phosphate buffer (pH 7.4) for the system without metabolic activation or 0.5 mL of S9 Mix for the system with metabolic activation was added, and then 0.1 mL of bacterial solution was added to each tube.
2) Each mixture was pre-incubated while shaking (80 rpm) at 37°C for 20 minutes immediately after stirring.
3) After pre-incubation, 2.0 mL of top agar which was dissolved using an electronic oven and kept at 45°C in a unit incubator was added to each tube, and this mixture was shaken and overlaid uniformly on the minimal glucose agar plate medium.
4) For a sterility test, 0.1 mL of the test solution at the highest dose or 0.5 mL of S9 Mix was measured in a small test tube, and after 2.0 mL of top agar was added, it was overlaid uniformly on the minimal glucose agar plate medium. These processes, 1) to 4), were performed under fluorescent lamps with ultraviolet-absorbing film.

5) In the observation for the solidification of the overlaid top agar and precipitation and coloration by the test article on the plate, no such changes were observed irrespective of the presence/absence of metabolic activation. After observation for precipitation and coloration, the minimal glucose agar plate medium was put upside down in an incubator and incubated at 37°C for 48 hours.
6) After incubation, the culture was observed for the presence or absence of precipitation of the test article and coloration of the test article on the plate. Since precipitation and coloration of the test article was observed on the plates, the number of revertant colonies was counted by visual observation. Since measurement was not affected, the numbers of revertant colonies of positive controls were counted with an automatic colony counter (Colony Analyzer CA-11D systems, System Science Co., Ltd.) (Area correction, correction factor: 1.21) since there were no effects on the counting using the instruments. The presence or absence of growth inhibition of the bacteria was observed using a stereoscopic microscope.

7.5 Judging Criteria

If a two-fold or more increase in the number of revertant colonies compared to that of spontaneous revertant colonies (the negative control value) and dose-response and reproducibility were noted, or even if no clear dose-response was observed but there was at least two-fold increase in comparison with the number of spontaneous revertant colonies and reproducibility was observed in the two main tests, the test article was judged to be positive. For the results of the main tests, the mean ± S.D. was also recorded.

8. Results

The results of the dose-finding test are shown in Table 1. Results of the first main and the second main tests are shown in Tables 2 and 3, respectively. Figures 1 to 10 were prepared on the basis of Table 2.

8.1 Observations of Dose-finding Test and Selection of Dose Levels for Main Tests

To set the dose levels for the main tests, the test article undiluted solution was diluted 4 times using a common ratio of 4 and a total of 5 dose levels were selected (19.5, 78.1, 313, 1250 and 5000 μg/plate) in the dose-finding test.

In the results, neither precipitation nor coloration of the test article on the plate was observed at any dose concentration at the time of stratification on the plate at any dose level irrespective of the presence/absence of metabolic activation. In the observation of bacterial background lawn using a stereoscopic microscope, growth inhibition was observed at 5000 μg/plate for all strains in the absence of metabolic activation.

There was neither increase in the number of revertant colonies of two-fold or more in comparison with that of the negative control group nor dose-response for any strains irrespective of the presence/absence of metabolic activation.

Therefore, the maximum dose for the main test was set at 5000μg/plate which showed growth inhibition was selected as maximum dose for main test, which was conducted at 6 dose levels between 156 and 5000 μg/plate for all strains in the absence of metabolic activation. In the case of all strains in the presence of metabolic activation, the main test was conducted at 5 dose levels between 313 and 5000 μg/plate because no growth inhibition was observed. The main test was conducted twice at the same dose levels.

8.2 Observations of Two Main Tests

Neither precipitation nor coloration of the test article on the plate was observed at any dose concentration at the time of stratification on the plate at any dose level irrespective of the presence/absence of metabolic activation. In the observation of bacterial background lawn using a stereoscopic microscope, growth inhibition was observed at 2500 μg/plate or more for all strains of the absence of metabolic activation.

There was neither increase in the number of revertant colonies of two-fold or more in comparison with that of the negative control group nor dose-response for any strains irrespective of the presence/absence of metabolic activation.

8.3 Acceptance Criteria of Test System

It was judged that this test was conducted appropriately since two-fold or more increase in the number of revertant colonies in comparison with the negative control was observed in the plate treated with the positive control article for each tester strain, the number of revertant colonies in the plates in the negative control group and positive control group were within the range of the

control limit of background data (Attached Data 3), and no contaminants such as other bacteria were seen in this test system.

9. Discussion

In the two main tests, there was neither increase in the number of revertant colonies of two-fold or more in comparison with that of the negative control group nor dose-response for any strains irrespective of the presence/absence of metabolic activation.

Since two-fold or more increase in the number of revertant colonies in comparison with the negative control group was observed in the positive control group for each tester strain, it was judged that the reactions of the bacterial strains to the mutagenic agents were suitable and thus the study was conducted appropriately.

In conclusion, β-Galactosidase concentrate was judged to have no gene mutation inducibility (negative) under the conditions of this study.

10. References

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(Table 1)

Study Results (Dose-finding Test)

Name of test article: β-galactosidase concentrate

No. T-2095

Term		From May 19, 2016 to May 23, 2016				
With (+) or Without (-) S9Mix	Test article dose (μg/plate)	Number of revertants (number of colonies / plate)				
		Base - pair substitution type			Frame - shift type	
		TA100	TA1535	WP2*uvrA*	TA98	TA1537
S9Mix (-)	Negative control (Water)	136 124 (130)	7 11 (9)	30 26 (28)	22 23 (23)	10 8 (9)
	19.5	120 106 (113)	7 8 (8)	21 35 (28)	18 13 (16)	7 5 (6)
	78.1	149 125 (137)	9 12 (11)	26 36 (31)	19 16 (18)	8 10 (9)
	313	100 108 (104)	11 12 (12)	35 30 (33)	23 19 (21)	6 6 (6)
	1250	139 104 (122)	10 9 (10)	32 34 (33)	25 26 (26)	9 11 (10)
	5000	118 * 133 *(126)	7 * 9 *(8)	32 * 30 *(31)	18 * 17 *(18)	6 * 9 *(8)
S9Mix (+)	Negative control (Water)	157 148 (153)	10 7 (9)	32 27 (30)	41 33 (37)	12 7 (10)
	19.5	156 137 (147)	12 13 (13)	31 30 (31)	30 44 (37)	9 9 (9)
	78.1	131 140 (136)	13 6 (10)	29 36 (33)	39 33 (36)	9 12 (11)
	313	145 151 (148)	12 12 (12)	34 41 (38)	36 42 (39)	8 8 (8)
	1250	139 122 (131)	7 8 (8)	41 38 (40)	29 30 (30)	6 8 (7)
	5000	144 145 (145)	8 9 (9)	35 34 (35)	35 37 (36)	7 7 (7)
Positive control S9Mix (-)	Name	AF-2	SAZ	AF-2	AF-2	ICR-191
	Dose(μg/plate)	0.01	0.5	0.01	0.1	1.0
	Number of colonies/plate	622 598 (610)	284 300 (292)	82 76 (79)	339 370 (355)	1249 1015 (1132)
Positive control S9Mix (+)	Name	B[*a*]P	2AA	2AA	B[*a*]P	B[*a*]P
	Dose(μg/plate)	5.0	2.0	10.0	5.0	5.0
	Number of colonies/plate	863 892 (878)	215 254 (235)	718 805 (762)	414 397 (406)	120 101 (111)

(Note)

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

SAZ : Sodium azide

ICR-191 : 2-Methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine·2HCl

B[*a*]P : Benzo[*a*]pyrene

2AA : 2-Aminoanthracene

* : Growth inhibition of tester strains was observed.

Average of counted colony number of two plates are shown in parenthesis.

(Table 2)

Study Results (First Main Test)

Name of test article: β-ガラクトシダーゼ原末 No. T-2095

Term		From May 26, 2016 to May 30, 2016				
With (+) or Without (-) S9Mix	Test article dose (μg/plate)	Number of revertants (number of colonies / plate)				
		Base-pair substitution type			Frame-shift type	
		TA100	TA1535	WP2*uvrA*	TA98	TA1537
S9Mix (-)	Negative control (Water)	125 117 128 (123 ± 5.7)	8 10 10 (9 ± 1.2)	30 31 30 (30 ± 0.6)	22 16 22 (20 ± 3.5)	8 7 8 (8 ± 0.6)
	156	107 134 124 (122 ± 13.7)	7 13 11 (10 ± 3.1)	42 41 31 (38 ± 6.1)	19 24 25 (23 ± 3.2)	8 14 8 (10 ± 3.5)
	313	88 119 143 (117 ± 27.6)	7 13 8 (9 ± 3.2)	32 30 36 (33 ± 3.1)	14 24 19 (19 ± 5.0)	13 11 7 (10 ± 3.1)
	625	137 116 125 (126 ± 10.5)	10 8 8 (9 ± 1.2)	29 33 41 (34 ± 6.1)	19 14 13 (15 ± 3.2)	8 8 11 (9 ± 1.7)
	1250	120 131 80 (110 ± 26.8)	12 10 6 (9 ± 3.1)	42 33 29 (35 ± 6.7)	14 20 22 (19 ± 4.2)	8 8 11 (9 ± 1.7)
	2500	91 * 90 * 80 * (87 ± 6.1)	13 * 9 * 8 * (10 ± 2.6)	26 * 34 * 35 * (32 ± 4.9)	18 * 12 * 22 * (17 ± 5.0)	6 * 7 * 7 * (7 ± 0.6)
	5000	97 * 82 * 83 * (87 ± 8.4)	8 * 7 * 10 * (8 ± 1.5)	31 * 41 * 29 * (34 ± 6.4)	30 * 21 * 20 * (24 ± 5.5)	8 * 11 * 11 * (10 ± 1.7)
S9Mix (+)	Negative control (Water)	138 154 169 (154 ± 15.5)	9 11 13 (11 ± 2.0)	36 29 35 (33 ± 3.8)	39 42 41 (41 ± 1.5)	8 10 7 (8 ± 1.5)
	313	167 108 119 (131 ± 31.4)	8 16 18 (14 ± 5.3)	34 31 36 (34 ± 2.5)	36 39 29 (35 ± 5.1)	11 10 6 (9 ± 2.6)
	625	119 124 120 (121 ± 2.6)	14 10 11 (12 ± 2.1)	42 28 41 (37 ± 7.8)	32 38 39 (36 ± 3.8)	11 5 4 (7 ± 3.8)
	1250	112 168 137 (139 ± 28.1)	9 16 10 (12 ± 3.8)	36 36 47 (40 ± 6.4)	33 36 29 (33 ± 3.5)	7 7 7 (7 ± 0.0)
	2500	133 122 107 (121 ± 13.1)	12 12 13 (12 ± 0.6)	41 44 41 (42 ± 1.7)	41 27 27 (32 ± 8.1)	7 10 6 (8 ± 2.1)
	5000	139 125 136 (133 ± 7.4)	15 17 11 (14 ± 3.1)	48 38 36 (41 ± 6.4)	35 41 41 (39 ± 3.5)	13 14 12 (13 ± 1.0)
Positive control S9Mix (-)	Name	AF-2	SAZ	AF-2	AF-2	ICR-191
	Dose(μg/plate)	0.01	0.5	0.01	0.1	1.0
	Number of colonies/plate	617 536 543 (565 ± 44.9)	211 265 213 (230 ± 30.6)	70 77 94 (80 ± 12.3)	299 299 257 (285 ± 24.2)	1077 1223 1289 (1196 ± ####)
Positive control S9Mix (+)	Name	B[*a*]P	2AA	2AA	B[*a*]P	B[*a*]P
	Dose(μg/plate)	5.0	2.0	10.0	5.0	5.0
	Number of colonies/plate	1136 902 1033 (1024 ± ####)	246 287 203 (245 ± 42.0)	803 726 927 (819 ± 101.4)	486 386 378 (417 ± 60.2)	149 102 102 (118 ± 27.1)

(Note)

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
SAZ : Sodium azide
ICR-191 : 2-Methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine·2HCl
2AA : 2-Aminoanthracene
B[*a*]P : Benzo[*a*]pyrene

* : Growth inhibition of tester strains was observed.
Average and standard deviation of counted colony numbers of three plates are shown in parenthesis.

(Table 3)

Study Results (Second Main Test)

Name of test article: β-galactosidase concentrate

No. T-2095

Term		From June 2, 2016 to June 6, 2016				
With (+) or Without (-) S9Mix	Test article dose (μg/plate)	Number of revertants (number of colonies / plate)				
		Base-pair substitution type			Frame-shift type	
		TA100	TA1535	WP2*uvrA*	TA98	TA1537
S9Mix (-)	Negative control (Water)	111 121 107 (113 ± 7.2)	8 10 7 (8 ± 1.5)	22 23 18 (21 ± 2.6)	15 16 21 (17 ± 3.2)	7 9 8 (8 ± 1.0)
	156	88 98 108 (98 ± 10.0)	9 5 5 (6 ± 2.3)	16 25 20 (20 ± 4.5)	15 12 15 (14 ± 1.7)	9 10 4 (8 ± 3.2)
	313	96 87 100 (94 ± 6.7)	10 6 6 (7 ± 2.3)	25 23 16 (21 ± 4.7)	17 20 16 (18 ± 2.1)	10 4 5 (6 ± 3.2)
	625	105 88 98 (97 ± 8.5)	5 4 4 (4 ± 0.6)	16 18 22 (19 ± 3.1)	18 19 18 (18 ± 0.6)	8 5 3 (5 ± 2.5)
	1250	103 90 96 (96 ± 6.5)	6 7 7 (7 ± 0.6)	20 21 19 (20 ± 1.0)	21 18 16 (18 ± 2.5)	6 8 7 (7 ± 1.0)
	2500	111 * 121 * 102 * (111 ± 9.5)	4 * 8 * 8 * (7 ± 2.3)	22 * 23 * 24 * (23 ± 1.0)	16 * 15 * 14 * (15 ± 1.0)	4 * 7 * 6 * (6 ± 1.5)
	5000	102 * 100 * 88 * (97 ± 7.6)	7 * 5 * 5 * (6 ± 1.2)	21 * 16 * 16 * (18 ± 2.9)	14 * 13 * 17 * (15 ± 2.1)	5 * 6 * 2 * (4 ± 2.1)
S9Mix (+)	Negative control (Water)	103 123 111 (112 ± 10.1)	10 7 8 (8 ± 1.5)	20 23 22 (22 ± 1.5)	32 28 24 (28 ± 4.0)	10 8 9 (9 ± 1.0)
	313	121 145 123 (130 ± 13.3)	8 5 10 (8 ± 2.5)	24 22 23 (23 ± 1.0)	28 25 23 (25 ± 2.5)	8 6 12 (9 ± 3.1)
	625	108 123 118 (116 ± 7.6)	9 6 6 (7 ± 1.7)	22 23 21 (22 ± 1.0)	25 26 30 (27 ± 2.6)	7 4 7 (6 ± 1.7)
	1250	118 133 103 (118 ± 15.0)	4 3 8 (5 ± 2.6)	28 23 22 (24 ± 3.2)	30 27 25 (27 ± 2.5)	6 5 5 (5 ± 0.6)
	2500	121 118 127 (122 ± 4.6)	5 4 6 (5 ± 1.0)	23 19 20 (21 ± 2.1)	26 23 25 (25 ± 1.5)	5 7 6 (6 ± 1.0)
	5000	111 114 106 (110 ± 4.0)	6 5 7 (6 ± 1.0)	17 24 16 (19 ± 4.4)	24 20 23 (22 ± 2.1)	6 3 7 (5 ± 2.1)
Positive control S9Mix (-)	Name	AF-2	SAZ	AF-2	AF-2	ICR-191
	Dose(μg/plate)	0.01	0.5	0.01	0.1	1.0
	Number of colonies/plate	555 567 657 (593 ± 55.7)	255 234 264 (251 ± 15.4)	65 71 61 (66 ± 5.0)	322 302 355 (326 ± 26.8)	1120 1027 1008 (1052 ± 59.9)
Positive control S9Mix (+)	Name	B[*a*]P	2AA	2AA	B[*a*]P	B[*a*]P
	Dose(μg/plate)	5.0	2.0	10.0	5.0	5.0
	Number of colonies/plate	990 1020 987 (999 ± 18.2)	258 264 246 (256 ± 9.2)	778 778 779 (778 ± 0.6)	432 405 381 (406 ± 25.5)	112 102 113 (109 ± 6.1)

(Note)

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

SAZ : Sodium azide

ICR-191 : 2-Methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine·2HCl

2AA : 2-Aminoanthracene

B[*a*]P : Benzo[*a*]pyrene

* : Growth inhibition of tester strains was observed.

Average and standard deviation of counted colony numbers of three plates are shown in parenthesis.

Figure 1

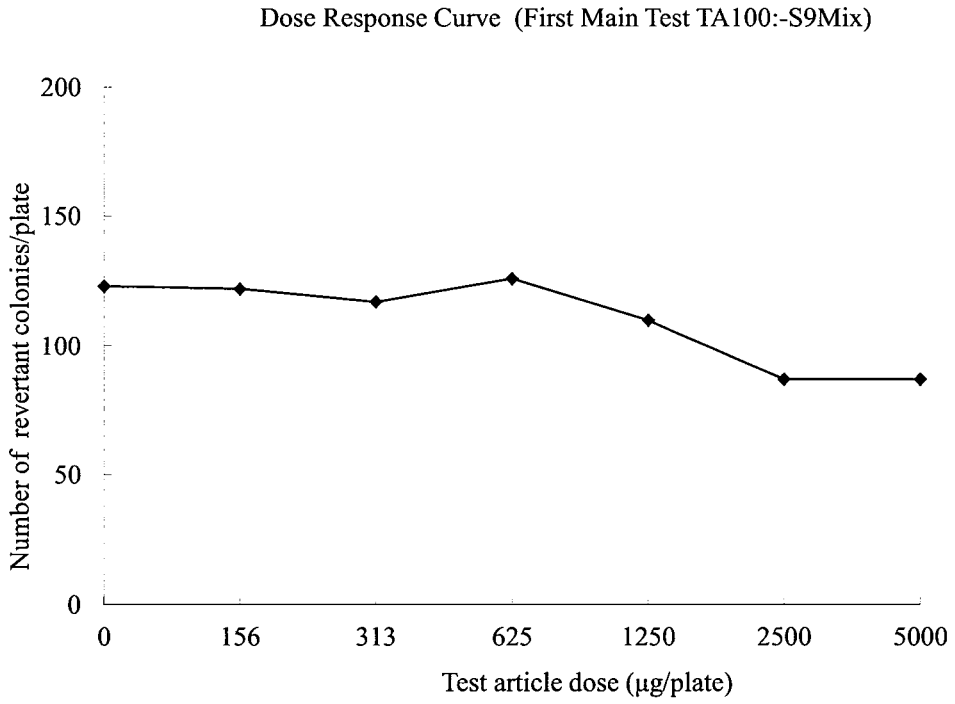


Figure 2

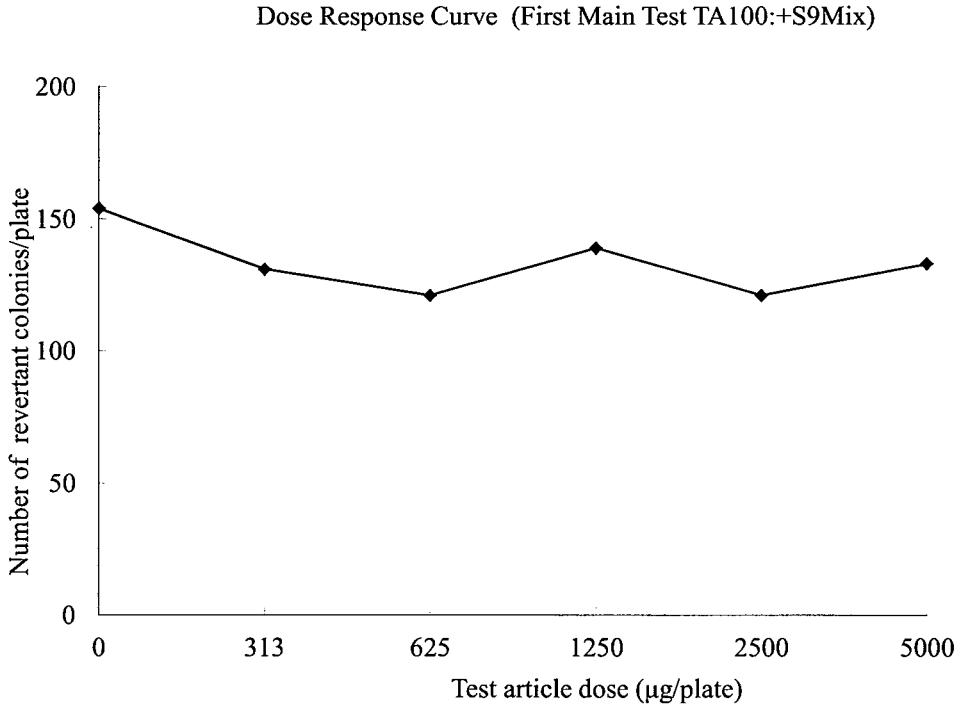


Figure 3

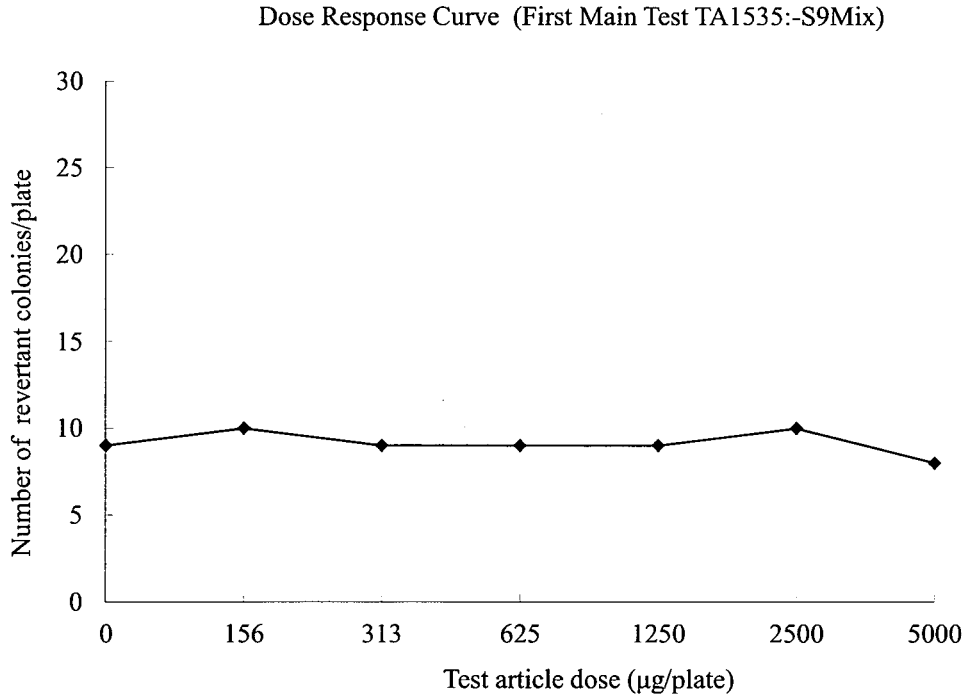


Figure 4

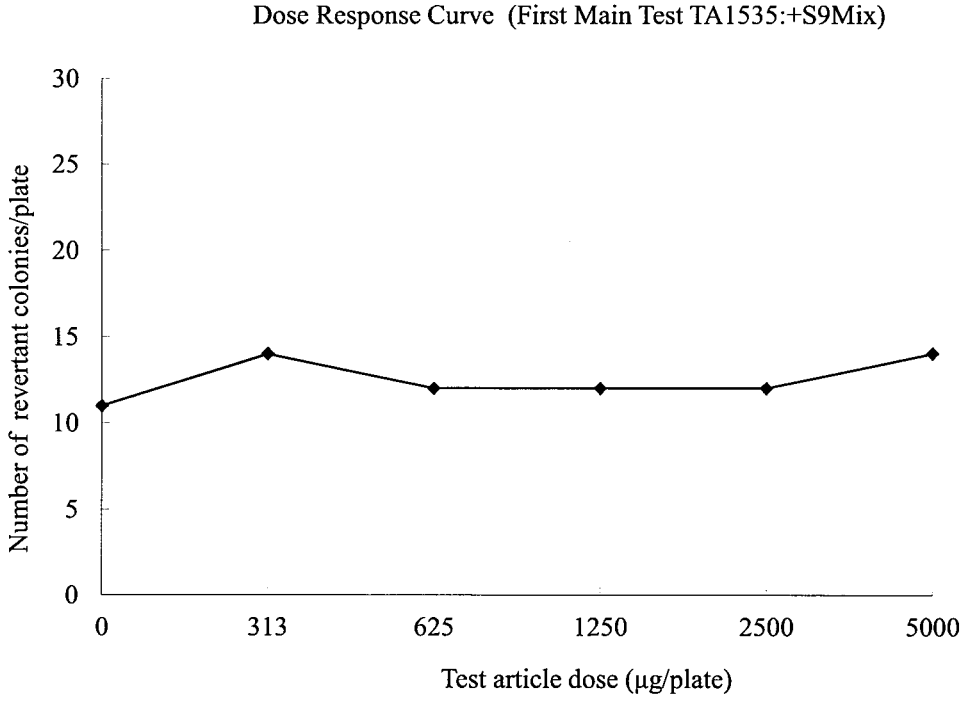


Figure 5

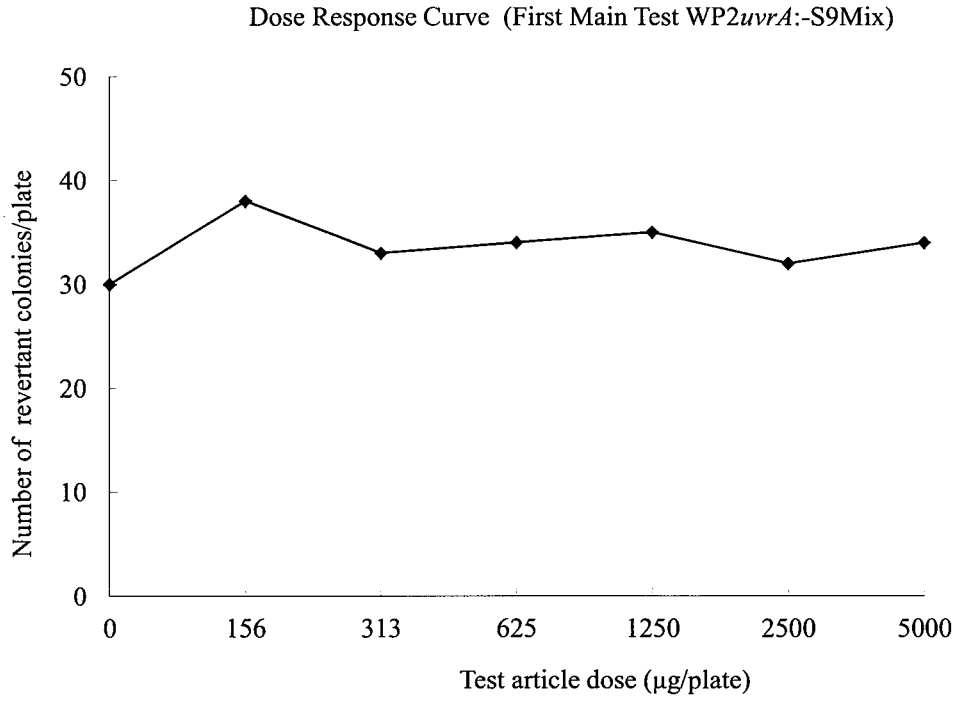


Figure 6

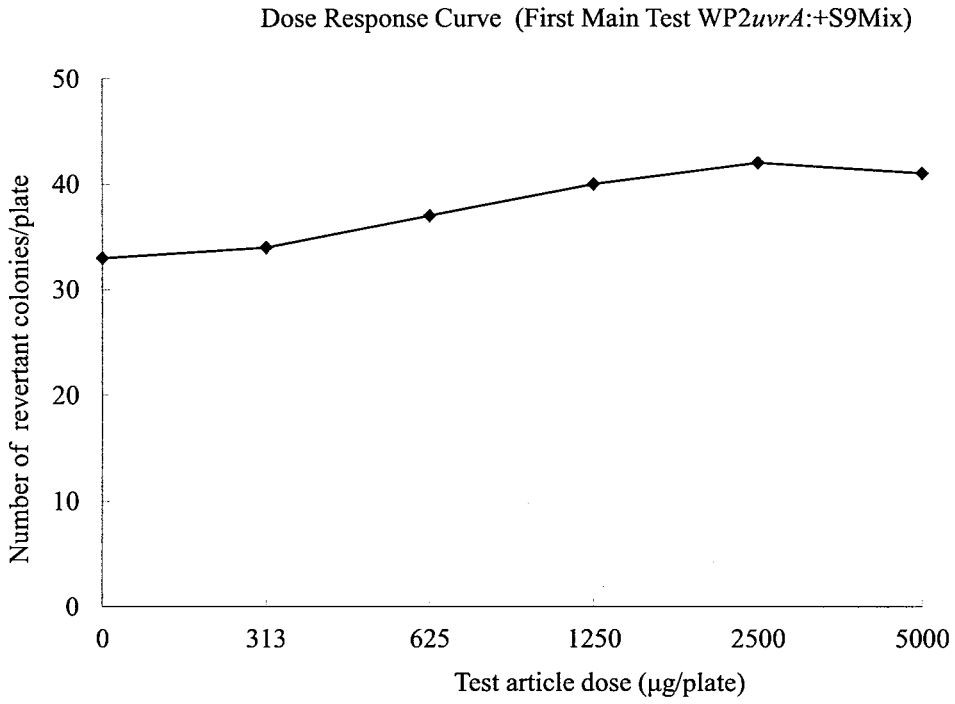


Figure 7

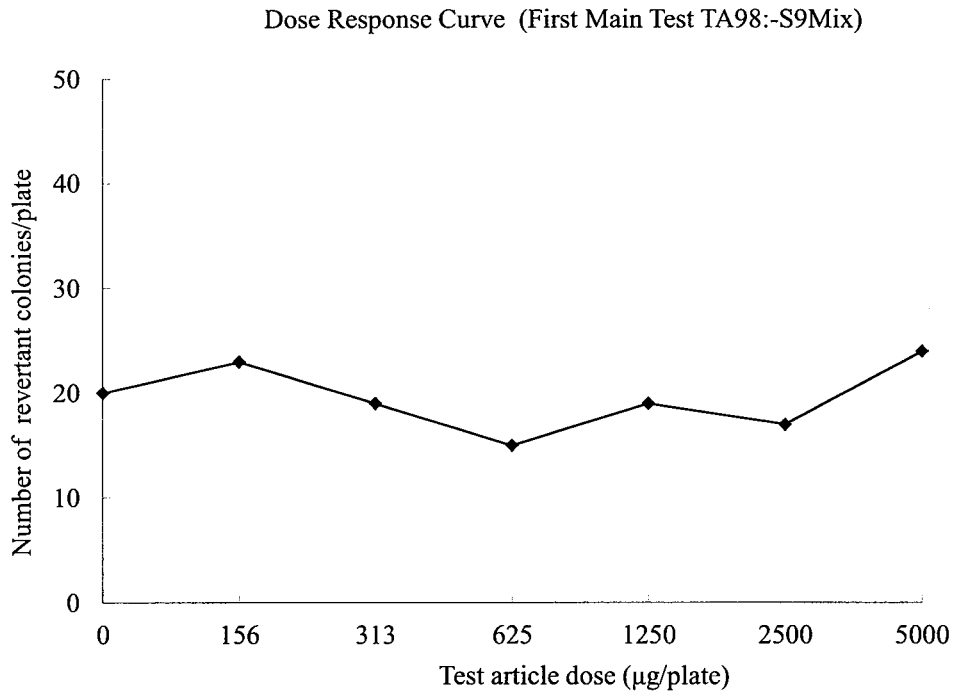


Figure 8

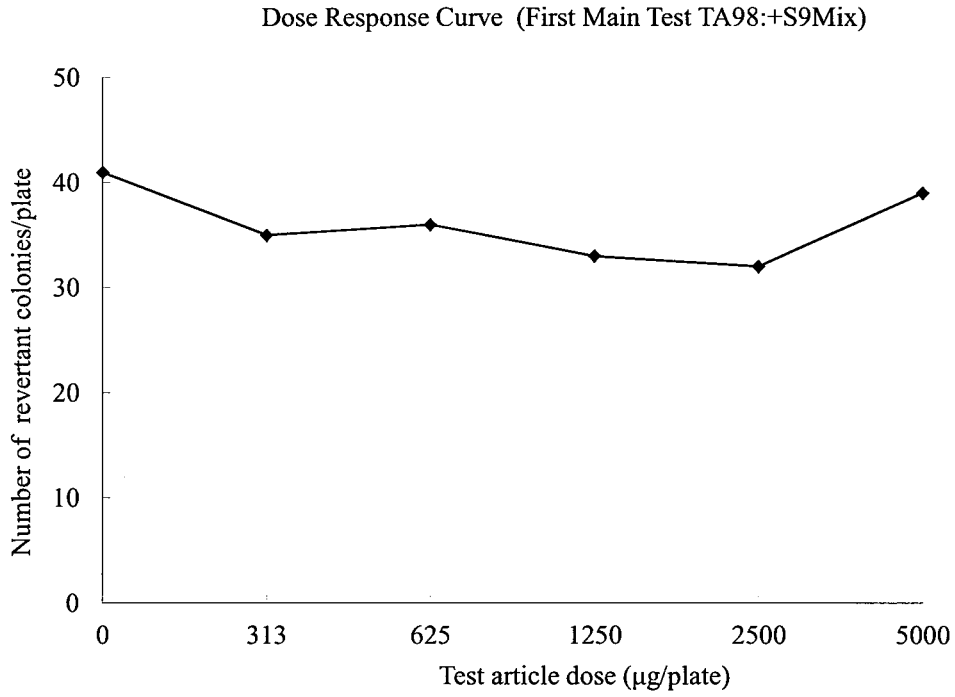


Figure 9

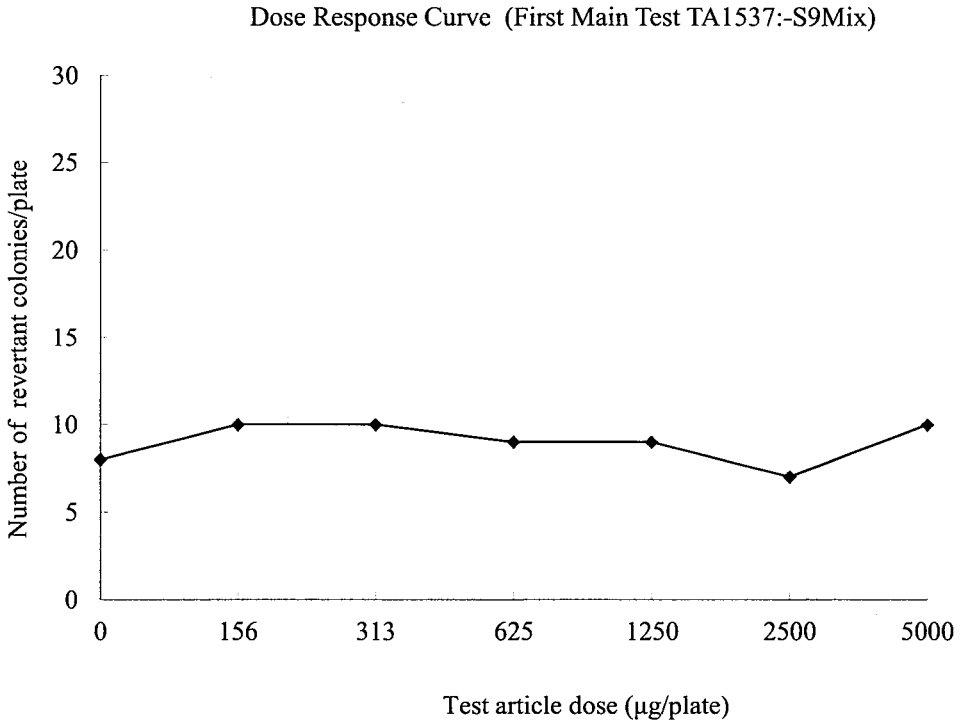
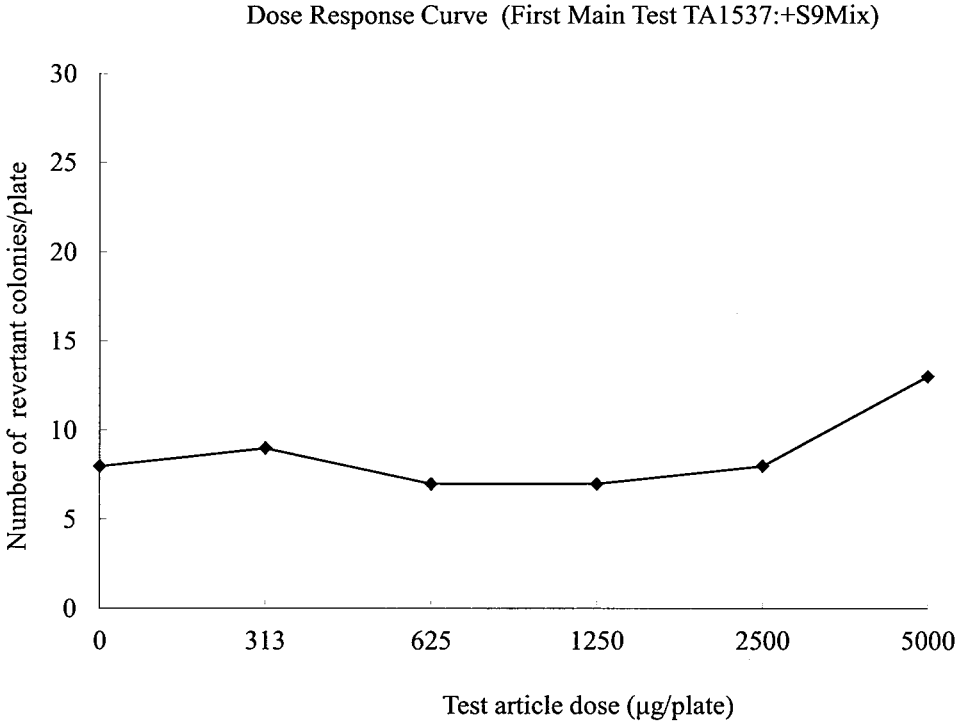


Figure 10



T-2095
Attached Data 1

Test Material Data Sheet

Identity	:	β-Galactosidase concentrate
Specification	:	β-Galactosidase
Origin	:	*Cryptococcus terrestris*
Lot No.	:	GFE68-001@K
Appearance	:	Pale yellowish brown powder
Activity	:	2,550 u/g (β-Galactosidase activity, Lactose substrate method)
Loss on drying	:	4.9 % (Drying method, 100g, 105°C)
Ash	:	5.1 % (JSFA* method)
Lead	:	Not more than 1 μg/g (FCC method)
Total viable aerobic count	:	1.8×10^2 cfu/g (SCD Agar plate method)
Coliforms	:	< 3.0 CFU/g (FDA BAM)
Escherichia coli	:	Negative/25g (FDA BAM)
Salmonella	:	Negative/25g (FDA BAM)
Antibiotic activity	:	Negative (JECFA method)
Storage conditions	:	Refrigeration
Date of production	:	February 13, 2016
Expiration date	:	February 12, 2017

*: Japanese Standards of Food Additives

(b) (6)

Atsushi Mizutani April 8, 2016

Quality Assurance Dept., Amano Enzyme Inc.

Stability and Homogeneity of Test Material

Test material : β-Galactosidase concentrate
Lot No. : GFE68-001@K
Assay Parameter : β-Galactosidase activity, Lactose substrate method
Assayer : Masamichi Okada / R & D Dept.
Date of assay : March 4, 2016

Results :

	Room temperature						Ice cold					
Concentration	0hrs		3hrs		5hrs		0hrs		3hrs		5hrs	
0.2mg/mL	1	4.9	1	5.1	1	5.3	1	4.9	1	5.3	1	5.2
	2	5.2	2	5.2	2	5.1	2	5.2	2	5.3	2	5.1
	3	5.1	3	5.3	3	5.0	3	5.1	3	5.2	3	5.2
	Average	5.1	Average	5.2	Average	5.1	Average	5.1	Average	5.3	Average	5.2
	S.D.	0.2	S.D.	0.1	S.D.	0.2	S.D.	0.2	S.D.	0.1	S.D.	0.1
	R. A.*	100	R. A.	103	R. A.	101	R. A.	100	R. A.	104	R. A.	102
	C.V.**	3.0	C.V.	1.9	C.V.	3.0	C.V.	3.0	C.V.	1.1	C.V.	1.1
25mg/mL	1	59	1	62	1	63	1	59	1	61	1	62
	2	61	2	63	2	63	2	61	2	61	2	62
	3	60	3	61	3	62	3	60	3	61	3	61
	Average	60	Average	62	Average	63	Average	60	Average	61	Average	62
	S.D.	1.0	S.D.	1.0	S.D.	0.6	S.D.	1.0	S.D.	0.0	S.D.	0.6
	R. A.	100	R. A.	103	R. A.	104	R. A.	100	R. A.	102	R. A.	103
	C.V.	1.7	C.V.	1.6	C.V.	0.9	C.V.	1.7	C.V.	0.0	C.V.	0.9
50mg/mL	1	125	1	121	1	125	1	125	1	121	1	121
	2	124	2	125	2	124	2	124	2	122	2	123
	3	120	3	122	3	125	3	120	3	122	3	122
	Average	123	Average	123	Average	125	Average	123	Average	122	Average	122
	S.D.	2.6	S.D.	2.1	S.D.	0.6	S.D.	2.6	S.D.	0.6	S.D.	1.0
	R. A.	100	R. A.	100	R. A.	101	R. A.	100	R. A.	99	R. A.	99
	C.V.	2.2	C.V.	1.7	C.V.	0.5	C.V.	2.2	C.V.	0.5	C.V.	0.8

* : Percentage of residual activity (vs. 0 hours, Acceptable range: 100±10%)
** : Coefficient of variation (Acceptable range: ≦10%)

Conclusion : Test material was stable and homogenous in each dilutions or storage periods under both room temperature and ice cold.

(b) (6)

Atsushi Mizutani / July 28, 2016
Quality Assurance Dept. Amano Enzyme Inc.

T-2095
Attached Data 3

Background Data of the reverse mutation tests in bacteria at the Tokyo Laboratory of the BoZo Research Center Inc.

CODE No. :151111

(Pre-incubation Method)

Tester Strains	S9 Mix (–) or (+)	Classification	Mean	S.D.	Management ranges		Number of plates
					Lower limit	Upper limit	
TA100	–	Solvent control	103	16.5	56	149	451
		Positive control AF-2(0.01μg/plate)	556	59.0	390	723	451
	+	Solvent control	130	17.0	83	176	451
		Positive control B[*a*]P(5.0μg/plate)	839	91	587	1090	451
TA1535	–	Solvent control	8	2.70	1	15	451
		Positive control SAZ(0.5μg/plate)	251	45.2	115	386	451
	+	Solvent control	10	2.96	1	18	451
		Positive control 2AA(2.0μg/plate)	222	29.3	142	302	451
WP2*uvrA*	–	Solvent control	19	5.37	8	31	451
		Positive control AF-2(0.01μg/plate)	63	8.9	44	82	451
	+	Solvent control	23	5.72	9	36	451
		Positive control 2AA(10.0μg/plate)	651	80	456	846	451
TA98	–	Solvent control	15	3.43	6	24	451
		Positive control AF-2(0.1μg/plate)	345	53.7	190	500	451
	+	Solvent control	29	5.98	11	46	451
		Positive control B[*a*]P(5.0μg/plate)	339	34.3	238	441	451
TA1537	–	Solvent control	7	2.38	1	13	451
		Positive control ICR-191(1.0μg/plate)	921	135	534	1307	451
	+	Solvent control	9	2.87	1	17	451
		Positive control B[*a*]P(5.0μg/plate)	87	13.0	50	123	451

(Notice)

Solvent controls: Water, Dimethylsulfoxide(DMSO), Acetone, *N,N*-dimethylformamide (DMF) and 1,4-Dioxane

Positive controls:
AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
SAZ : Sodium azide
ICR-191 : 2-methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino]acridine·2HCl
B[*a*]P : Benzo[*a*]pyrene
2AA : 2-aminoanthracene

S9Mix:
(–) : without metabolic activation
(+) : with metabolic activation

T-2095

Quality Assurance Statement (1/2)

Study Number: T-2095

Study Title: A Bacterial Reverse Mutation Test of β-galactosidase concentrate

I, the undersigned, hereby declare that this study was conducted in compliance with the following GLP regulations.

- "The Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs", Ordinance No. 21 of the Ministry of Health and Welfare, Japan, March 26, 1997
- "OECD Principles of Good Laboratory Practice" (OECD Council: November 26, 1997)

Inspections were conducted as follows.

(Sealed in the Original)	July 6, 2016
Minoru Izutsu, M.Sc. Manager, Quality Assurance Unit BoZo Research Center Inc.	Date

Study-Based Inspections

Items	Inspectors	Dates of Inspection	Dates of Report to Study Director and Management
Protocol	M. Yoshida	May 19, 2016	May 19, 2016
Preparation / Storage (Test Article) Treatment with Test Article	E. Ko	May 27, 2016	May 27, 2016
Colony Counting	M. Yoshida	May 30, 2016	May 31, 2016
Raw Data	M. Yoshida	June 28, 2016	June 29, 2016
Final Report (Draft) / Figures / Tables	M. Yoshida	June 28, 2016	June 28, 2016
Confirmation of improvement	M. Yoshida	June 30, 2016	June 30, 2016
Final Report	M. Yoshida	July 6, 2016	July 6, 2016

T-2095

Quality Assurance Statement (2/2)

Facility-Based Inspections

Items	Inspectors	Dates of Inspection	Dates of Report to Division Director and Management
Characteristics Test of Bacteria	E. Ko	February 18, 2016	
	M. Yoshida	February 22, 2016	
		February 29, 2016	February 29, 2016
Handling of Positive Control Articles	E. Ko	February 27, 2016	February 29, 2016
	M. Yoshida	March 15, 2016	March 15, 2016
	M. Yoshida	April 13, 2016	April 13, 2016
	M. Yoshida	May 23, 2016	May 23, 2016

T-2095

This English version is an accurate translation of the original Japanese Final Report.

(b) (6)

Kazuyuki Minegawa
Translator / Study Director
BoZo Research Center Inc.

September 6, 2016
Date

APPENDIX 5

APPENDIX 5

T-G212

ORIGINAL

Final Report

CHROMOSOME ABERRATION TEST IN CULTURED MAMMALIAN CELLS TREATED WITH β-GALACTOSIDASE CONCENTRATE

Study Number: T-G212

Study Period: May 18, 2016 to September 15, 2016

Test Facility

Tokyo Laboratory, BoZo Research Center Inc.

1-3-11 Hanegi, Setagaya-ku, Tokyo 156-0042, Japan

Sponsor

Amano Enzyme Inc.

2-7, 1-chome, Nishiki, Naka-ku, Nagoya, Aichi 460-8630, Japan

Contractor

BoZo Research Center Inc.

36-7 Oyama-cho, Shibuya-ku, Tokyo 151-0065, Japan

I certify that this is an accurate copy of the original final report.
BoZo Research Center Inc.
Study Director : (b) (6)
DATE : September 15, 2016

1. GLP Statement

Study Number:	T-G212
Study Title:	Chromosome aberration test in cultured mammalian cells treated with β-Galactosidase concentrate

The above study was conducted in compliance with the following GLP standards:

- "The Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs" (Ordinance No. 21 of the Ministry of Health and Welfare, Japan, March 26, 1997)
- "OECD Principles of Good Laboratory Practice" (OECD Council: November 26, 1997)

(b) (6)

Sho Fujiwara
Study Director
Toxicology Department,
Tokyo Laboratory,
BoZo Research Center Inc.

September 15, 2016
Date

2. Table of Contents

Attached Data

Figures

Tables

Appendices

T-G212

3. Outline of Study

3.1 Study Number

T-G212

3.2 Study Title

Chromosome aberration test in cultured mammalian cells treated with β-Galactosidase concentrate

3.3 Purpose of Study

This study was conducted in order to examine whether β-Galactosidase concentrate induces chromosome aberrations in cultured Chinese hamster (CHL/IU) cells.

3.4 Sponsor

Amano Enzyme Inc.

2-7, 1-chome, Nishiki, Naka-ku, Nagoya, Aichi 460-8630, Japan

[Contact information]

Quality Assurence Division, Nagoya Plant, Amano Enzyme Inc.

27 Hanno, Kunotsubo, Kitanagoya, Aichi 481-8533, Japan

3.5 Contractor

BoZo Research Center Inc.

36-7 Oyama-cho, Shibuya-ku, Tokyo 151-0065, Japan

3.6 Test Facility

Tokyo Laboratory, BoZo Research Center Inc.

1-3-11 Hanegi, Setagaya-ku, Tokyo 156-0042, Japan

3.7 Study Schedule

Start of Study:	May 18, 2016
Receipt of Test Article:	May 10, 2016
Start of Experiment:	May 20, 2016
End of Experiment:	July 20, 2016
End of Study:	September 15, 2016

3.8 Study Director

Sho Fujiwara

Toxicology Department, Tokyo Laboratory, BoZo Research Center Inc.

3.9 Study Contributors

Test Article Archiving:	Kaori Nakamura
Study Contributors:	Michihito Takabe, Maki Nakamura, Ryosuke Sato

3.10 Unexpected Circumstances That Might Have Affected the Reliability of Study and Deviations from the Protocol

There were neither unforeseeable circumstances that might have affected the reliability of study nor deviations from the Protocol.

3.11 Archives

The original Protocol (including protocol amendments), written records, raw data, chromosome specimens and reports (including the original of the Final Report) will be retained in the archive of Gotemba Laboratory, BoZo Research Center Inc. They will be retained for a period of 5 years after submission of the Final Report. At the end of this period, Amano Enzyme Inc. and BoZo Research Center Inc. will discuss and determine the disposition of the said materials.

3.12 Signature of Study Director and Date

(b) (6)

Sho Fujiwara
Toxicology Department, Tokyo Laboratory,
BoZo Research Center Inc.

September 15, 2016
Date

4. Summary

This study was conducted in order to examine whether β-Galactosidase concentrate has the potential to induce chromosome aberrations using cultured Chinese hamster (CHL/IU) cells by the short-term treatment in the presence (+S9 mix) or in the absence (–S9 mix) of metabolic activation and by the continuous treatment in the absence of metabolic activation.

In order to select dose levels for the chromosome aberration test, a cell-growth inhibition test was conducted setting the highest dose level at 5000 μg/mL (vehicle: water for injection), and this dose was diluted using a common ratio of 2 to prepare a total of 8 concentrations. As a result, not less than 50% of cell growth inhibition was not observed in the short-term treatment with or without metabolic activation. On the other hand, not less than 50% of cell growth inhibition was observed at the concentration of 2500 μg/mL and above in the continuous treatment. In accordance with these results and toxicity study guidelines, the chromosome aberration test was conducted setting the highest concentration at 5000 μg/mL for the short-term treatment with or without metabolic activation, at 2000 μg/mL for the continuous treatment. In the short-term treatment without metabolic activation, a total of 4 concentrations diluted using common ratio of 2 from the highest concentration was used. In the short-term treatment with metabolic activation, a total of 3 concentrations diluted using common ratio of 2 from the highest concentration was used. In the continuous treatment, a total of 5 concentrations diluted using equal difference of 400 μg/mL from the highest concentration was used.

In the chromosome aberration test, the incidence of chromosome aberrations excluding gaps (TA value), an index of structural chromosome aberrations, and the incidence of polyploid cells did not show statistically significant increases in any test article treatment group as compared to the negative control group. However, cells forming a ski-pair were observed at a high frequency in the continuous treatment in comparison with the negative control group, and dose dependency was observed. Since delay of cell cycle or cell division inhibition was suspected from the result of this continuous treatment, the confirmation test that the exposure time was set to 48 hours in the continuous treatment was conducted.
As a result, there was no statistically significant increase in TA value and the incidence of polyploid cells between all treated groups and the negative control group. A slight increase in the incidence of a ski-pair was observed, but TA value and the incidence of polyploid cells did not increase under the condition that the cell cycle is probably turning not less than one rotation

(Cell Population Doubling Number (PD) was not less than 1.00). Therefore, the inducibility of structural chromosome aberrations and numerical chromosome aberrations of β-Galactosidase concentrate was judged to be negative.

Three test concentrations and more that meet the acceptability criteria were evaluated in each treatment method, and the data of concurrent negative controls were within the 95% confidence limit. The incidence of cells with chromosome structural abnormalities in the positive control group showed a statistically significant increase when compared to the negative control group. Therefore, it was judged that the test was conducted appropriately.

Based on the results described above, it is concluded that β-Galactosidase concentrate has no potential to induce chromosome aberrations under the conditions of this study.

5. Introduction

In accordance with the request from Amano Enzyme Inc. as a part of safety evaluation of β-Galactosidase concentrate, a chromosome aberration test was conducted using cultured Chinese hamster (CHL/IU) cells and the results are reported here. This study was conducted in compliance with the following regulations and in accordance with the following guidelines:

1) GLPs
- "The Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs" (Ordinance No. 21 of the Ministry of Health and Welfare, Japan, March 26, 1997)
- "OECD Principles of Good Laboratory Practice" (OECD Council: November 26, 1997)

2) Toxicity Study Guidelines
- "OECD Guidelines for Testing of Chemicals TG473" (OECD Council: September 26, 2014)
- "Guidance for Genotoxicity Studies of Pharmaceuticals and Their Interpretation" (Notification No. 0920-2 of the Evaluation and Licensing Division, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, September 20, 2012)
- "Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives" (Notification No. 29 of the Environmental Health Bureau, Ministry of Health and Welfare, Japan, March 22, 1996)

6. Materials and Methods

6.1 Test Article and Vehicle

6.1.1 Test Article

The following information of test article was based on the results of the analysis performed by the Study Sponsor under non-GLP conditions.

Supplier:	Amano Enzyme, Inc.
Name:	β-Galactosidase concentrate
Lot number:	GFE68-001@K
Producing microorganism:	*Cryptococcus terrestris*
Description:	Pale yellowish brown powder
Purity:	100 wt%
Activity value:	2550 u/g [β-Galactosidase activity, Lactose substrate method]
Stability:	One year after manufacture
Expiration date:	February 12, 2017 (Production date: February 13, 2016)
Storage condition:	Refrigeration (actual temperature: 4.3 to 5.5°C, from May 10, 2016 to July 11, 2016)
Storage place:	Refrigerator, Test Article Storage Room, Tokyo Laboratory
Treatment of remaining:	All of the test article remaining after the end of experiment was returned to the sponsor.

6.1.2 Vehicle

1) Information of the vehicle used

Name:	Water for injection
Lot numbers:	5K94N, 6A80N
Manufacturer:	Otsuka Pharmaceutical Factory, Inc.
Specification:	Japanese Pharmacopoeia
Storage condition:	Room temperature
Storage place:	Cultured Cell Test Room, Tokyo Laboratory

2) Reason for selection of vehicle

Water for injection was chosen for the following reasons:

- The test article was dissolved at 50.0 mg/mL in water for injection in the pre-test on the

solubility.

- Test solution is found to be possible to sterilize by filtration from the test article information.
- It is used commonly in chromosome aberration tests.

6.2 Preparation of Test Solutions

6.2.1 Method of Preparation

1) Cell-growth inhibition test

The test article, 0.5000 g, was weighed in a 10-mL measuring flask. The vehicle was added to make the volume to prepare the test solution at the highest concentration of 50.0 mg/mL (the final concentration after addition of 0.500 mL to the plate: 5000 μg/mL), and this solution was sterilized by filtration. Then, the 50.0 mg/mL solution was diluted 7 times using the common ratio of 2 (5 mL of test solution at each concentration with 5 mL of vehicle) to prepare a total of 8 test solutions at the concentrations of 25.0, 12.5, 6.25, 3.13, 1.56, 0.781 and 0.391 mg/mL.

2) Chromosome aberration test

The test article, 0.5000 g, was weighed in a 10-mL measuring flask. The vehicle was added to make the volume to prepare the test solution at the highest concentration of 50.0 mg/mL (the final concentration after addition of 0.500 mL to the plate: 5000 μg/mL), and this solution was sterilized by filtration. Then, the 50.0 mg/mL solution was diluted 3 times using the common ratio of 2 (3 mL of test solution at each concentration with 3 mL of vehicle) to prepare a total of 4 test solutions at the concentrations of 25.0, 12.5 and 6.25 mg/mL. And 0.80, 0.64, 0.48, 0.32 and 0.16 mL of the 50.0 mg/mL solution was added to 1.20, 1.36, 1.52, 1.68 and 1.84 mL of vehicle to make 20.0, 16.0, 12.0, 8.00 and 4.00 mg/mL solutions respectively, a total of 5 concentrations.

3) Confirmation test

The test article, 0.2000 g, was weighed in a 10-mL measuring flask. The vehicle was added to make the volume to prepare the test solution at the highest concentration of 20.0 mg/mL (the final concentration after addition of 0.500 mL to the plate: 2000 μg/mL), and this solution was sterilized by filtration. Then, the 20.0 mg/mL solution was diluted 9 times using the common ratio of 1.25 (8 mL of test solution at each concentration with 2 mL of vehicle) to prepare a total of 10 test solutions at the concentrations of 16.0, 12.8, 10.2, 8.19, 6.55, 5.24, 4.19, 3.36 and 2.68 mg/mL.

4) Confirmation test II

The test article, 0.0300 g, was weighed in a 10-mL measuring flask. The vehicle was added

to make the volume to prepare the test solution at the highest concentration of 3.00 mg/mL (the final concentration after addition of 0.500 mL to the plate: 300 μg/mL), and this solution was sterilized by filtration. Then, the 3.00 mg/mL solution was diluted 7 times using the common ratio of 2 (5 mL of test solution at each concentration with 5 mL of vehicle) to prepare a total of 8 test solutions at the concentrations of 1.50, 0.750, 0.375, 0.188, 0.0938, 0.0469 and 0.0234 mg/mL.

5) Confirmation test III

The test article, 0.0150 g, was weighed in a 20-mL measuring flask. The vehicle was added to make the volume to prepare the test solution at the highest concentration of 0.750 mg/mL (the final concentration after addition of 0.500 mL to the plate: 75.0 μg/mL), and this solution was sterilized by filtration. Then, the 0.750 mg/mL solution was diluted 5 times using the common ratio of 1.5 (4 mL of test solution at each concentration with 2 mL of vehicle) to prepare a total of 6 test solutions at the concentrations of 0.500, 0.333, 0.222, 0.148 and 0.0988 mg/mL.

6.2.2 Frequency of Preparation

Preparation was done at the time of use.

6.2.3 Stability of Test Solutions

It has been confirmed by the study sponsor that the solutions of β-Galactosidase concentrate at concentration of 50, 25 and 0.2 mg/mL is stable and homogeneous for at least 5 hours at room temperature or in a refrigerator under the non-GLP conditions.

6.3 Control Articles

6.3.1 Negative Control

The vehicle (water for injection) was used as the negative control article.

6.3.2 Positive Controls

1) Test system without metabolic activation

Name:	Mitomycin C (MMC)
Lot number:	577AEE
Manufacturer:	Kyowa Hakko Kirin Co., Ltd.
Potency:	2 mg (potency)/bottle
Storage conditions:	Room temperature, protected from light
Storage:	Cultured Cell Test Room, Tokyo Laboratory

2) Test system with metabolic activation

Name:	Cyclophosphamide (CP)
Lot number:	CTN3690
Manufacturer:	Wako Pure Chemical Industries, Ltd.
Purity:	Biochemistry grade (not less than 97.0%)
Storage conditions:	Refrigerated, protected from light
Storage:	Refrigerator, Cultured Cell Test Room, Tokyo Laboratory

3) Method of preparation

All preparations were done at the time of use.

(1) MMC

MMC in a 2-mg vial was dissolved in 2 mL of isotonic sodium chloride solution (Japanese Pharmacopoeia, Otsuka Pharmaceutical Factory, Inc., Lot No. K5J95) (1 mg/mL). This solution was then diluted 20-fold (0.250 mL of the solution with 4.750 mL of isotonic sodium chloride solution) twice to prepare 0.050 and 0.0025 mg/mL solutions (0.150 mL of the 0.0025 mg/mL solution was added to 4.850 mL of culture medium for the short-term treatment method while 0.100 mL of the 0.0025 mg/mL solution was added to 4.900 mL of culture medium for the continuous treatment method, and their final concentrations were 0.075 and 0.050 μg/mL, respectively).

(2) CP

CP (0.0140 g) was weighed in a gamma-ray sterilized plastic centrifuge tube and dissolved in 20 mL of isotonic sodium chloride solution (Japanese Pharmacopoeia, Otsuka Pharmaceutical Factory, Inc., Lot No. K5J95) to prepare 0.70 mg/mL solution (final concentration of CP when 0.100 mL of this solution was added to 4.900 mL of culture medium: 14 μg/mL).

4) Reasons for selection of positive control articles

CP and MMC were used as the positive control articles because they are recommended by the toxicity study guideline and the background data in the test facility are abundant.

6.4 Cultured Cells Used

1) Cultured cells

Chinese hamster lung fibroblast (CHL/IU) cells were used. The cells which were received from the National Institute of Biomedical Innovation, JCRB Cell Bank on April 2, 2014 were preserved in a frozen state. Cells that were confirmed to have appropriate cell characteristics (culture condition, cell doubling time within 15-20 hours, mean number (mode) of

chromosomes of 25, and without contamination with mycoplasma etc.) in the regularly-performed characteristic test were used in the study within the passage number of 30. The passage number of the cells at the time of use was 6 in the cell-growth inhibition test, 9 in the chromosome aberration test, 14 in the confirmation test, 17 in the confirmation test II and 20 in the confirmation test III.

2) Reasons for selection of cells

The cells were selected since the guidelines for genotoxicity studies of drugs recommend their use, the incidence of spontaneous chromosome aberrations is low, the sensitivity to various chemicals is high and they are used widely in chromosome aberration tests using mammalian cells in culture.

3) Culturing conditions

The cells were cultured in a carbon dioxide gas incubator under conditions of 5% CO_2 at 37°C and at high humidity. Subcultivation was carried out every 1 to 4 days.

6.5 Preparation of S9 Mix and Culture Medium

6.5.1 S9 Mix

The S9 and cofactor (S9/cofactor C set, Lot number: C151211101) were mixed to prepare S9 mix. Preparation was done at the time of use.

1) S9

Name:	S9
Manufacturer:	Oriental Yeast Co., Ltd.
Lot number:	15121110
Date of manufacturing:	December 11, 2015
Species/strain:	Rat/SD strain
Age/sex:	7 weeks of age / Male
Inducers:	Phenobarbital (PB) and 5,6-benzoflavone (BF)
Route of administration:	Intraperitoneal
Length of administration period and dose levels:	PB for 4 days, 30+60+60+60 mg/kg body weight BF on day 3 of PB administration, 80 mg/kg body weight
Expiration:	June 10, 2016
Storage condition:	Frozen (at –70°C or below)
Storage:	Ultradeep Freezer, Cultured Cell Test Room, Tokyo Laboratory

2) Cofactor

Name:	Cofactor C
Manufacturer:	Oriental Yeast Co., Ltd.
Lot number:	C15120910
Date of manufacturing:	December 9, 2015
Storage condition:	Frozen (at –70°C or below)
Expiration:	June 8, 2016
Storage:	Ultradeep Freezer, Cultured Cell Test Room, Tokyo Laboratory

3) Composition of S9 mix

S9	2 mL
Cofactor	4.7 mL

20 mmol/L HEPES buffer (pH 7.2)	1.34 mL
50 mmol/L Magnesium chloride	0.67 mL
330 mmol/L Potassium chloride	0.67 mL
50 mmol/L Glucose-6-phosphate	0.67 mL
40 mmol/L Oxidized nicotinamide adenine dinucleotide phosphate (NADP)	0.67 mL
Purified water	0.67 mL

6.5.2 Culture Medium

The culture medium (BS-MEM) which was prepared by supplementation of Minimum Essential Medium (MEM) (GIBCO™, Cat. No. 11095) with 10 v/v% inactivated (56°C, 30 minutes) bovine serum (BS) was used. The BS-MEM prepared was preserved in a refrigerator.

1) Bovine serum

Lot numbers:	1641353, 1610982
Manufacturer:	Life Technologies Corporation
Storage condition:	Frozen (at –20°C or lower)
Storage:	Freezer, Cultured Cell Test Room, Tokyo Laboratory

2) MEM

Lot numbers:	1773562, 1774375
Manufacturer:	Life Technologies Corporation
Storage condition:	Refrigerated (acceptable range: 1 to 10°C)
Storage:	Refrigerator, Cultured Cell Test Room, Tokyo Laboratory

6.6 Test Methods [1)]

The study was conducted in the order of the stages shown below.

1. Cell-growth inhibition test	Short-term treatment	Without metabolic activation, With metabolic activation
	Continuous treatment	24-hour treatment
2. Chromosome aberration test	Short-term treatment	Without metabolic activation, With metabolic activation
	Continuous treatment	24-hour treatment
3. Confirmation test (Chromosome aberration test)	Continuous treatment	48-hour treatment
4. Confirmation test II (Cell-growth inhibition test)	Continuous treatment	48-hour treatment
5. Confirmation test III (Chromosome aberration test)	Continuous treatment	48-hour treatment

6.6.1 Method of Identification

The plates and glass slides were identified by showing the marks or figures indicated in the following table:

Subject	Content	Mark or figure
Plate	Short-term treatment method without metabolic activation	–
	Short-term treatment method with metabolic activation	+
	Continuous treatment method, 24-hour treatment	24-
	Continuous treatment method, 48-hour treatment	48-
	Negative control group	NC
	Test article treatment group	Branch numbers 1, 2, 3, ... n from the highest concentration
	Positive control group	PC
Individual identification within the same treatment group		1, 2
Chromosome specimens	Content of treatment coded randomly by the blind test method	Study No., 2-digit numbers from 00 to 99 which were allotted randomly by a computer and the branch number indicating the number of slides

6.6.2 Selection of Dose Concentrations

1) Cell-growth inhibition test

The highest dose concentration was set at 5000 μg/mL and it was diluted using a common ratio of 2 to prepare a total of 8 dose concentrations: 5000, 2500, 1250, 625, 313, 156, 78.1 and 39.1 μg/mL. In addition, a negative control group was provided.

2) Chromosome aberration test

As a result of the cell-growth inhibition test, not less than 50% of cell growth inhibition was not observed in the short-term treatment with and without metabolic activation, the 50% cell growth inhibitory concentration (the approximate value) was not calculated. On the other hand, not less than 50% of cell growth inhibition was observed at the concentration of 2500 μg/mL

and above in the continuous treatment. The 50% cell growth inhibitory concentration (the approximate value) was calculated to be 1420 μg/mL for the continuous treatment. Based on these results, the doses of each treatment for the chromosome aberration test were set as the table shown below. In addition, a negative and a positive control groups were provided.

Treatment method	Dose (μg/mL)
Short-term treatment method without metabolic activation	5000, 2500, 1250, 625 (common ratio of 2)
Short-term treatment method with metabolic activation	5000, 2500, 1250 (common ratio of 2)
Continuous treatment, 24-hour treatment	2000, 1600, 1200, 800, 400 (equal difference of 400 μg/mL)

3) Confirmation test

As a result of the chromosome aberration test, TA value and the incidence of polyploid cells did not show statistically significant increases in any test article treatment group as compared to the negative control group. However, the incidence of cells forming a ski-pair showed a statistically significant increase in the continuous treatment. Since delay of cell cycle or cell division inhibition was suspected from the result of the continuous treatment, the confirmation test that the exposure time was set to 48 hours in the continuous treatment was conducted. By making reference to RPD (Relative Cell Population Doubling Number) of 24-hour treatment of continuous treatment method, the highest dose concentration was set at 2000 μg/mL and it was diluted using a common ratio of 1.25 to prepare a total of 10 dose concentrations: 2000, 1600, 1280, 1020, 819, 655, 524, 419, 336 and 268 μg/mL. In addition, a negative and a positive control groups were provided.

4) Confirmation test II

As a result of the confirmation test, since RPD was lower than 40% at all dose levels, the confirmation test was stopped. RPD at each concentration in the confirmation test was shown between 20 to 36%. Since it was judged to be difficult to estimate the test doses by which RPD shows 40 to 50% and the test doses which has no cytotoxicity, the cell-growth inhibition test of 48-hour treatment of continuous treatment method had been conducted. By making reference to RPD of the confirmation test, the highest dose concentration was set at 300 μg/mL and it was diluted using a common ratio of 2 to prepare a total of 8 dose concentrations: 300, 150, 75.0, 37.5, 18.8, 9.38, 4.69 and 2.34 μg/mL. In addition, a negative control group was provided.

5) Confirmation test III

As a result of the confirmation test II, not less than 50% of cell growth inhibition was observed at the concentration of 37.5 μg/mL and above. The 50% cell growth inhibitory concentration (the approximate value) was calculated to be 38 μg/mL. Based on these results, the highest dose concentration of the confirmation test III (48-hour treatment of continuous treatment method for the chromosome aberration test) was set at 75.0 μg/mL and it was diluted using a common ratio of 1.5 to prepare a total of 6 dose concentrations: 75.0, 50.0, 33.3, 22.2, 14.8 and

common ratio of 1.5 to prepare a total of 6 dose concentrations: 75.0, 50.0, 33.3, 22.2, 14.8 and 9.88 μg/mL. In addition, a negative and a positive control groups were provided.

6.6.3 Cell-growth Inhibition Test (including the confirmation test II)

This test was conducted as a preliminary test for selection of dose concentrations in the chromosome aberration test. If sterility was necessary in the test procedure described below, the test was conducted under sterile environment by aseptic procedures using sterilized instruments.

1) For each treatment without or with metabolic activation in the short-term treatment and continuous treatment, a negative control group and test article treatment groups were provided. A single plastic plate (60 mm in diameter) was used for each group, and a plate at the start of treatment for the calculation of Cell Population Doubling Number (PD) was provided.
2) For the cells in the logarithmic growth phase, approximately 2×10^4 cells (5.0 mL of culture) were seeded for each plate.
3) After 3 days of cultivation, cells were observed under an inverted phase-contrast microscope and confirmed to have no abnormality. The culture medium was removed and the cells were treated as shown in the table below. In one plate for the measurement at the start of treatment, the cell concentration was measured in accordance with the following methods, and it was considered the cell concentration at the start of treatment.

Treatment	Short-term treatment		Continuous treatment
	Without metabolic activation	With metabolic activation	
Amount of culture medium removed	0.500 mL	1.333 mL	0.500 mL
Amount of S9 mix added		0.833 mL	
Amount of vehicle / test solution added	0.500 mL	0.500 mL	0.500 mL

(1) The medium of the plate was discarded, a proper quantity of Phosphate-Buffered Saline (–) [PBS (–)] was added, and the plate was washed.
(2) The PBS (–) was discarded, and 1 mL of 0.25% trypsin solution was added to the plate, and was left for about 5 minutes.
(3) After the cells were detached from the plate and became single cells by pipetting, 1 mL of 10% BS-MEM medium was added to the cell suspension. The number of cells on the plate was measured using a cell concentration measuring instrument (ADAM-MC, NanoEnTek Inc.).

4) After addition of test solution, each culture was observed macroscopically for color and

They were then cultivated for 6 hours in the short-term treatment while they were cultivated for 24 hours in the continuous treatment, or for 48 hours in the confirmation test II.

5) For the short-term treatments without or with metabolic activation, after cultivation for 6 hours, the plates were observed for the presence/absence of precipitation in the same manner as for 4) and the cell condition was observed under an inverted phase-contrast microscope. Then, the cells were rinsed with isotonic sodium chloride solution containing approximately 2% bovine serum, 5.0 mL of fresh culture medium was added, and the culture was cultivated for further 18 hours.
6) After the end of cultivation, the plates were observed for the presence/absence of precipitation in the same manner as for 4) and the cell condition was observed under the inverted phase-contrast microscope (the results at the end of cultivation were provided as supporting data in short-term treatment).
7) Then, the cell concentration in each plate at the end for treatment was measured in the same manner as for 3).
8) From the cell concentration obtained, the PD was calculated by Formula 1. With the value of the negative control group taken as 100%, RPD in each group was calculated by the cell numbers in accordance with Formula 2.

$$PD = [\log (\text{Post-treatment number of cells} / \text{Initial number of cells})] / \log 2$$

[Formula 1]

$$RPD\ (\%) = \frac{PD \text{ in treated cultures}}{PD \text{ in negative control cultures}} \times 100$$

[Formula 2]

9) The cell-growth inhibition ratio (= 100 – RPD)* in each group was calculated, and calculation of the 50% cell-growth inhibitory concentration (the approximate value) of the test article was attempted using the formula with two concentrations around the predicted concentration of 50% cell-growth inhibition.
*: Values not higher than 0 were regarded as 0 in the calculation.

6.6.4 Chromosome Aberration Test (including the confirmation test and the confirmation test III)

In the test procedure, if sterility was necessary, the test was conducted under sterile environment, by aseptic procedures using sterilized instruments.

1) For the treatments without or with metabolic activation in the short-term treatment and the

1) For the treatments without or with metabolic activation in the short-term treatment and the continuous treatment, a negative control group, test article treatment groups and a positive control group were provided. Plastic plates (60 mm in diameter) were used in duplicate for each group. A plate at the start of treatment for PD was also provided.
2) For the cells in the logarithmic growth phase, approximately 2×10^4 cells (5.0 mL of culture) were seeded for each plate.
3) After 3 days of cultivation, confirming that there was no morphological abnormality under an inverted phase-contrast microscope, the culture medium was removed and the cells were treated as shown in the table below. The plate at the start of treatment was used for measurement of cell concentration in accordance with the cell-growth inhibition test.

Treatment	Short-term treatment		Continuous treatment
	without metabolic activation	with metabolic activation	
Amount of culture removed	0.500 mL (0.150 mL) [a)]	1.333 mL (0.933 mL) [a)]	0.500 mL (0.100 mL) [a)]
Amount of S9 mix added		0.833 mL	
Amount of vehicle / test solution / positive control article added	0.500 mL (MMC: 0.150 mL) [a)]	0.500 mL (CP: 0.100 mL) [a)]	0.500 mL (MMC: 0.100 mL) [a)]

a): The numbers in parentheses indicate the amount of the culture medium removed and the amount of the positive control article added in the positive control group.

4) After addition of test solution, each culture was observed macroscopically for the color and under an inverted phase-contrast microscope for the presence or absence of precipitation. They were then cultivated for 6 hours in the short-term treatment while they were cultivated for 24 hours in the continuous treatment, or for 48 hours in the confirmation test and the confirmation test III.
5) For the short-term treatments without or with metabolic activation, after cultivation for 6 hours, the plates were observed for the presence/absence of precipitation in the same manner as for 4) and the cell condition was observed under an inverted phase-contrast microscope. Then, the cells were rinsed with isotonic sodium chloride solution containing approximately 2% bovine serum, 5.0 mL of fresh culture medium was added, and the cells were cultivated for further 18 hours.
6) At approximately 2 hours before the end of cultivation, the plates were observed for the presence/absence of precipitation in the same manner as for 4) and the cell condition was observed under the inverted phase-contrast microscope (the results at the end of cultivation were provided as supporting data in short-term treatment). Then, 0.1 mL of colcemid (demecolcine solution, 10 μg/mL) was added to 2 plates in each group.
7) After the end of cultivation, the culture medium on the plate was transferred to centrifuge tubes. Then, after cells were detached using 1 mL of 0.25% trypsin solution, 1 mL of 10% BS-MEM medium was added to the cell suspension. At a plate (branch number-2) in each

group, a part of cell suspension was collected and the number of cells on the plate was measured in the same manner as the cell-growth inhibition test, and PD and RPD were calculated. Remaining cell suspension was transferred to each centrifuge tube.

8) Remaining cell suspension from 7) was centrifuged, and the cells that were collected by centrifugation were treated with hypotonic 0.075M potassium chloride solution for approximately 15 minutes, and fixed in methyl alcohol/acetic acid solution (3:1). The fixed cell suspension was dropped onto 2 places per slide. Two chromosome preparations per plate were prepared. After dropping the cell suspension, the slides were air-dried for not less than one day and stained with 2% Giemsa solution for approximately 15 minutes to prepare specimens for observation of chromosomes.

6.6.5 Handling of Numeric Value

1) Cell concentration

(1) For the measurement of cell concentrations at the cells seeding, the mean of the countable number of values in 8 areas in the hemocytometer was indicated in integral values by rounding off (the cell concentration was indicated in the unit of $\times 10^4$ cells/mL).

(2) For the measurement of cell concentrations at the start of treatment and at the end of cultivation, a cell concentration measuring instrument was used, and the cell concentration was expressed as the indicated value of the equipment.

2) PD

PD was calculated using the values indicated in 1) and represented to the second decimal place by rounding off the number in the third decimal place.

3) RPD

RPD was calculated using the values shown in 2) and indicated as integral values by rounding off the first decimal place.

6.6.6 Observation of Chromosome Preparations

6.6.6.1 Observation Process

Chromosome preparations, 150 well-spread metaphases per plate and thus 300 metaphases per group, were observed under a microscope, and the type of structural abnormality and the number of cells with structural aberrations were recorded. At the same time, the incidence of the occurrence of polyploidy was recorded. All slides were observed objectively by the blind method. At the test treatment groups in the confirmation test III, the dose to an observation target was selected before the specimens were stain. Since 40 to 50% of RPD was observed at the concentration of 50.0 μg/mL, the dose concentrations to an observation target were set at

50.0, 33.3, 22.2 and 14.8 μg/mL. After that specimens were stained, and observation of chromosome preparations was conducted in accordance with the above-mentioned.

6.6.6.2 Classification of Chromosome Aberrations

Chromosome aberrations were classified broadly into structural aberrations and numerical aberrations, and the structural aberrations were further classified and defined as follows:

1) Structural aberrations

Gap (g):	For both chromatid (ctg) and chromosome (csg) types, a gap was defined as a clearly unstained region that was not misaligned from the chromatid or chromosome and its width was not larger than that of a chromatid.
Chromatid break (ctb):	A break was defined as an unstained region that was misaligned from the chromatid, or an unstained region that was not misaligned from the chromatid but its width was larger than that of a chromatid.
Chromatid exchange (cte):	Quadriradial interchange etc.
Chromosome break (csb):	A chromosome break was defined as an unstained region that was misaligned from the chromatid showing no centromere, or an unstained region that was not misaligned from the chromosome but its width was larger than that of a chromatid.
Chromosome exchange (cse):	Dicentric chromosome, ring chromosome etc.
Other:	Fragments (frg) and others

2) Numerical aberrations

When the chromosome number was double the inherent number that the cell has (diploid) or more, it was defined as a numerical aberration:

Polyploidy:	Including endoreduplication

6.6.7 Judgment of Results

The test article was evaluated on the basis of the incidence of cells with structural and numerical chromosome aberrations. The total incidence of cells with structural aberrations was calculated in 2 ways, one including gaps (TAG) and the other excluding gaps (TA), and the latter was used for the final evaluation. Statistical analysis was performed and evaluated according to the judging criteria.

6.6.8 Statistical Analysis

For statistical significance of the difference of the incidence of cells with abnormalities, the numbers of the cells with chromosome structural aberrations and numerical aberrations between the negative control group and the test article treatment group were analyzed by Fisher's exact test (one-tailed test, $p<0.05$) [2] and Cochran-Armitage trend test (one-tailed test, $p<0.05$) [3], while the numbers of the cells with chromosome structural aberrations between the negative control group and the positive control group were analyzed by Fisher's exact test (one-tailed test, $p<0.05$) [2].

6.6.9 Judging Criteria

The judgment of results was carried out in accordance with the following criteria:

1) Positive

Cases where significant differences (one-tailed test, $p<0.05$) are observed in the test article group in comparison with the negative control group by Fisher's exact test and by the Cochran-Armitage trend test (one-tailed test, $p<0.05$), and any of the results are outside the 95% probability distribution of the historical negative control data.

2) Negative

Cases where no significant differences (one-tailed test, $p>0.05$) are observed in the test article group in comparison with the negative control group by Fisher's exact test and by the Cochran-Armitage trend test (one-tailed test, $p>0.05$), and any of the results are inside the 95% probability distribution of the historical negative control data.

6.6.10 Criteria for a Valid Test

Since the following items were fulfilled, it was judged that the test article could be evaluated accurately:

1) Number of doses that can be analyzed for the chromosome specimen was above three doses.
2) A frequency of the chromosome aberrations for the negative control group was inside the 95% probability distribution of the historical negative control data.
3) The significant differences were observed a frequency of the chromosome aberrations in the positive control group in comparison with the negative control group by Fisher's exact test.
4) The incidence of chromosome aberration was not remarkably different between the plates in the same dose group.

6.6.11 Confirmation Test

In comparison with the incidence of chromosome aberrations in the negative control group,

there was no statistically significant increase in any test article treatment group. However, the incidence of cells forming a ski-pair showed a statistically significant increase in the continuous treatment. Therefore, the confirmation test that the exposure time was 48 hours in the continuous treatment was conducted.

7. Results

7.1 Cell-growth Inhibition Test

Results are shown in Appendix 1-1, Appendices 2-1 to 2-3 and Appendix 4-1.

1) Presence or absence of precipitation

(1) Immediately after treatment

Precipitation was not observed at any dose for any treatment method.

(2) End of treatment

Precipitation was not observed at any dose for any treatment method.

2) Observation of the color of the culture medium immediately after treatment

Coloration was not observed at any dose for any treatment method.

3) The 50% cell growth inhibitory concentration

Not less than 50% of cell growth inhibition was not observed in the short-term treatment with and without metabolic activation, but not less than 50% of cell growth inhibition was observed at the concentration of 2500 μg/mL and above in the continuous treatment. The 50% cell growth inhibitory concentration (the approximate value) was calculated to be 1420 μg/mL for the continuous treatment.

7.2 Chromosome Aberration Test

Results are shown in Figs. 1 to 3, Tables 1 to 3, Appendices 3-1 to 3-3 and Appendix 5-1.

1) Presence or absence of precipitation

(1) Immediately after treatment

Precipitation was not observed at any dose for any treatment method.

(2) End of treatment

Precipitation was not observed at any dose for any treatment method.

2) Observation of the color of the culture medium immediately after treatment

Coloration was not observed at any dose for any treatment method.

3) Incidence of chromosome aberrations

TA value and the incidence of polyploid cells did not show statistically significant increases in any test article treatment group as compared to the negative control group. However, the incidence of cells forming a ski-pair showed a statistically significant increase only in the continuous treatment.

7.3 Confirmation Test

1) Presence or absence of precipitation

(1) Immediately after treatment

Precipitation was not observed at any dose.

(2) End of treatment

Precipitation was not observed at any dose.

2) Observation of the color of the culture medium immediately after treatment

Coloration was not observed at any dose.

3) Incidence of chromosome aberrations

Since RPD was lower than 40% at all dose levels, the experiment was stopped.

7.4 Confirmation Test II

Results are shown in Appendix 1-2, Appendix 2-4 and Appendix 4-2.

1) Presence or absence of precipitation

(1) Immediately after treatment

Precipitation was not observed at any dose.

(2) End of treatment

Precipitation was not observed at any dose.

2) Observation of the color of the culture medium immediately after treatment

Coloration was not observed at any dose.

3) The 50% cell growth inhibitory concentration

Not less than 50% of cell growth inhibition was observed at the concentration of 37.5 μg/mL and above. The 50% cell growth inhibitory concentration (the approximate value) was calculated to be 38 μg/mL.

7.5 Confirmation Test III

Results are shown in Fig. 4, Table 4, Appendix 3-4 and Appendix 5-2.

1) Presence or absence of precipitation

(1) Immediately after treatment

Precipitation was not observed at any dose.

(2) End of treatment

Precipitation was not observed at any dose.

2) Observation of the color of the culture medium immediately after treatment

Coloration was not observed at any dose.

3) Incidence of chromosome aberrations

TA value and the incidence of polyploid cells did not show statistically significant increases in any test article treatment group as compared to the negative control group. The incidence of cells forming a ski-pair showed a statistically significant increase, but it was not remarkable increase such as observed in 24-hour treatment of continuous treatment method.

8. Discussion

In the chromosome aberration test, TA value and the incidence of polyploid cells did not show statistically significant increases in any test article treatment group as compared to the negative control group. However, the incidence of cells forming a ski-pair showed a statistically significant increase in 24-hour treatment of continuous treatment method ($p<0.05$). Since an increase of a ski-pair suggests delay of cell cycle or cell division inhibition, the confirmation test that the exposure time was set to 48 hours in the continuous treatment was conducted. As a result, there was no statistically significant increase in TA value and the incidence of polyploid cells between all treated groups and the negative control group. A slight increase in the incidence of a ski-pair was observed, but it was not remarkable increase such as observed in 24-hour treatment of continuous treatment method.

Delay of cell cycle and cell division inhibition may lead to false negative for the inducibility of chromosome aberrations because no cell division occur not less than one rotation. However, in the confirmation test, TA value and the incidence of polyploid cells did not increase under the condition that cell cycle is probably turning not less than one rotation (PD was not less than 1.00). Therefore, the inducibility of structural chromosome aberrations and numerical chromosome aberrations of β-Galactosidase concentrate was judged to be negative.

Three test concentrations and more that meet the acceptability criteria were evaluated. The data of the concurrent negative controls were within the 95% probability distribution of the historical negative control data. The incidence of cells with chromosome structural abnormalities in the positive control group showed a statistically significant increase when compared to the negative control group. Therefore, it was judged that the test was conducted appropriately.

Based on the results described above, it is concluded that β-Galactosidase concentrate has no potential to induce structural or numerical chromosome aberrations under the conditions of this study.

9. References

1) Sofuni, T. (Ed) (1999): Chromosomal Aberration Test Data (Revised edition of 1998), pp. 11-23, LIC Inc., Tokyo (in Japanese)
2) Siegel S, Castellan NJ. Jr. Nonparametric statistics for the behavioral sciences. 2nd ed. New York: McGraw-Hill; 1988.
3) Agresti A, Categorical Data Analysis. New Jersey: Wiley InterScience; 2002

T-G212
Attached Data 1 (1/1)

Test Material Data Sheet

Identity	:	β-Galactosidase concentrate
Specification	:	β-Galactosidase
Origin	:	*Cryptococcus terrestris*
Lot No.	:	GFE68-001@K
Appearance	:	Pale yellowish brown powder
Activity	:	2,550 u/g (β-Galactosidase activity, Lactose substrate method)
Loss on drying	:	4.9 % (Drying method, 100g, 105℃)
Ash	:	5.1 % (JSFA* method)
Lead	:	Not more than 1 μg/g (FCC method)
Total viable aerobic count	:	1.8 x 10^2 cfu/g (SCD Agar plate method)
Coliforms	:	< 3.0 CFU/g (FDA BAM)
Escherichia coli	:	Negative/25g (FDA BAM)
Salmonella	:	Negative/25g (FDA BAM)
Antibiotic activity	:	Negative (JECFA method)
Storage conditions	:	Refrigeration
Date of production	:	February 13, 2016
Expiration date	:	February 12, 2017

*: Japanese Standards of Food Additives

(b) (6)

Atsushi Mizutani April 8, 2016

Quality Assurance Dept., Amano Enzyme Inc.

Stability and Homogeneity of Test Material

Test material : β -Galactosidase concentrate
Lot No. : GFE68-001@K
Assay Parameter : β -Galactosidase activity, Lactose substrate method
Assayer : Masamichi Okada / R & D Dept.
Date of assay : March 4, 2016

Results :

	Room temperature						Ice cold					
Concentration	0hrs		3hrs		5hrs		0hrs		3hrs		5hrs	
0.2mg/mL	1	4.9	1	5.1	1	5.3	1	4.9	1	5.3	1	5.2
	2	5.2	2	5.2	2	5.1	2	5.2	2	5.3	2	5.1
	3	5.1	3	5.3	3	5.0	3	5.1	3	5.2	3	5.2
	Average	5.1	Average	5.2	Average	5.1	Average	5.1	Average	5.3	Average	5.2
	S.D.	0.2	S.D.	0.1	S.D.	0.2	S.D.	0.2	S.D.	0.1	S.D.	0.1
	R. A.*	100	R. A.	103	R. A.	101	R. A.	100	R. A.	104	R. A.	102
	C.V.**	3.0	C.V.	1.9	C.V.	3.0	C.V.	3.0	C.V.	1.1	C.V.	1.1
25mg/mL	1	59	1	62	1	63	1	59	1	61	1	62
	2	61	2	63	2	63	2	61	2	61	2	62
	3	60	3	61	3	62	3	60	3	61	3	61
	Average	60	Average	62	Average	63	Average	60	Average	61	Average	62
	S.D.	1.0	S.D.	1.0	S.D.	0.6	S.D.	1.0	S.D.	0.0	S.D.	0.6
	R. A.	100	R. A.	103	R. A.	104	R. A.	100	R. A.	102	R. A.	103
	C.V.	1.7	C.V.	1.6	C.V.	0.9	C.V.	1.7	C.V.	0.0	C.V.	0.9
50mg/mL	1	125	1	121	1	125	1	125	1	121	1	121
	2	124	2	125	2	124	2	124	2	122	2	123
	3	120	3	122	3	125	3	120	3	122	3	122
	Average	123	Average	123	Average	125	Average	123	Average	122	Average	122
	S.D.	2.6	S.D.	2.1	S.D.	0.6	S.D.	2.6	S.D.	0.6	S.D.	1.0
	R. A.	100	R. A.	100	R. A.	101	R. A.	100	R. A.	99	R. A.	99
	C.V.	2.2	C.V.	1.7	C.V.	0.5	C.V.	2.2	C.V.	0.5	C.V.	0.8

* : Percentage of residual activity (vs. 0 hours, Acceptable range: 100±10%)
** : Coefficient of variation (Acceptable range: ≦10%)

Conclusion : Test material was stable and homogenous in each dilutions or storage periods under both room temperature and ice cold.

(b) (6)

Atsushi Mizutani July 28, 2016
Quality Assurance Dept. Amano Enzyme Inc.

T-G212
Attached Data 3 (1/1)

Background Data in the Testing Facility

Cumulative background data of chromosome aberration tests in cultured Chinese hamster cells (line CHL/IU), carried out under the same study conditions at BoZo Research Center Inc.

Period for NC : January-2011-February-2016

	Treatment S9 mix	Time	Cells observed	Poly (%)	TA (%)
NC	+	6-18	(23400)	(68.0)	(82.8)
			Mean	0.3	0.4
			S.D.	0.4	0.4
			UCL	1.1	1.2
	(n=116)		LCL*	0.0	0.0
	-	6-18	(23400)	(38.7)	(86.3)
			Mean	0.2	0.4
			S.D.	0.3	0.5
			UCL	0.8	1.4
	(n=116)		LCL*	0.0	0.0
	-	24-0	(23000)	(29.5)	(52.7)
			Mean	0.1	0.3
			S.D.	0.3	0.4
			UCL	0.7	1.1
	(n=105)		LCL*	0.0	0.0
	-	48-0	(7200)	(8.0)	(13.0)
			Mean	0.1	0.2
			S.D.	0.2	0.4
			UCL	0.5	1.0
	(n=36)		LCL*	0.0	0.0

Period for PC : January-2011-February-2016

	Treatment S9 mix	Time	Cells observed	Poly (%)	TA (%)
PC	+ (CP)	6-18	(23400)	(7.5)	(14279.5)
			Mean	0.0	61.5
			S.D.	0.1	16.9
			UCL	0.2	94.6
	(n=116)		LCL*	0.0	28.4
	- (MMC)	6-18	(21400)	(13.0)	(6073.5)
			Mean	0.1	28.6
			S.D.	0.2	7.2
			UCL	0.5	42.7
	(n=106)		LCL*	0.0	14.5
	- (MMC)	24-0	(19200)	(21.0)	(5964.3)
			Mean	0.1	31.4
			S.D.	0.3	7.7
			UCL	0.7	46.5
	(n=95)		LCL*	0.0	16.3
	- (MMC)	48-0	(7200)	(9.0)	(4118.0)
			Mean	0.1	57.2
			S.D.	0.5	11.9
			UCL	1.1	80.5
	(n=36)		LCL*	0.0	33.9

( ):number of observed.

Negative control (NC) : water for injection, isotonic sodium chloride solution, dimethylsulfoxide, 0.5w/v% sodium carboxymethyl cellulose solution, acetone or culture medium

Positive control (PC) : CP ; Cyclophosphamide, 14 μg/mL
MMC ; Mitomycin C, 0.075 μg/mL (used for the short-term treatment)
MMC : Mitomycin C, 0.050 μg/mL (used for the continuous treatment)

S9 mix : + ; with metabolic activation - ; without metabolic activation

Time : treatment hours - hours of incubation without test article

Poly : polyploide cells

TA : total number of cells with aberrations excluding gaps

n : the number of studies

Mean : average of structural aberration and numerical aberration in cumulative studies

S.D. : standard deviation of structural aberration and numerical aberration in cumulative studies

UCL : 95% control limits(upper control limit)

LCL : 95% control limits(lower control limit)

* : The value was regarded as 0%, when value was 0 and below.

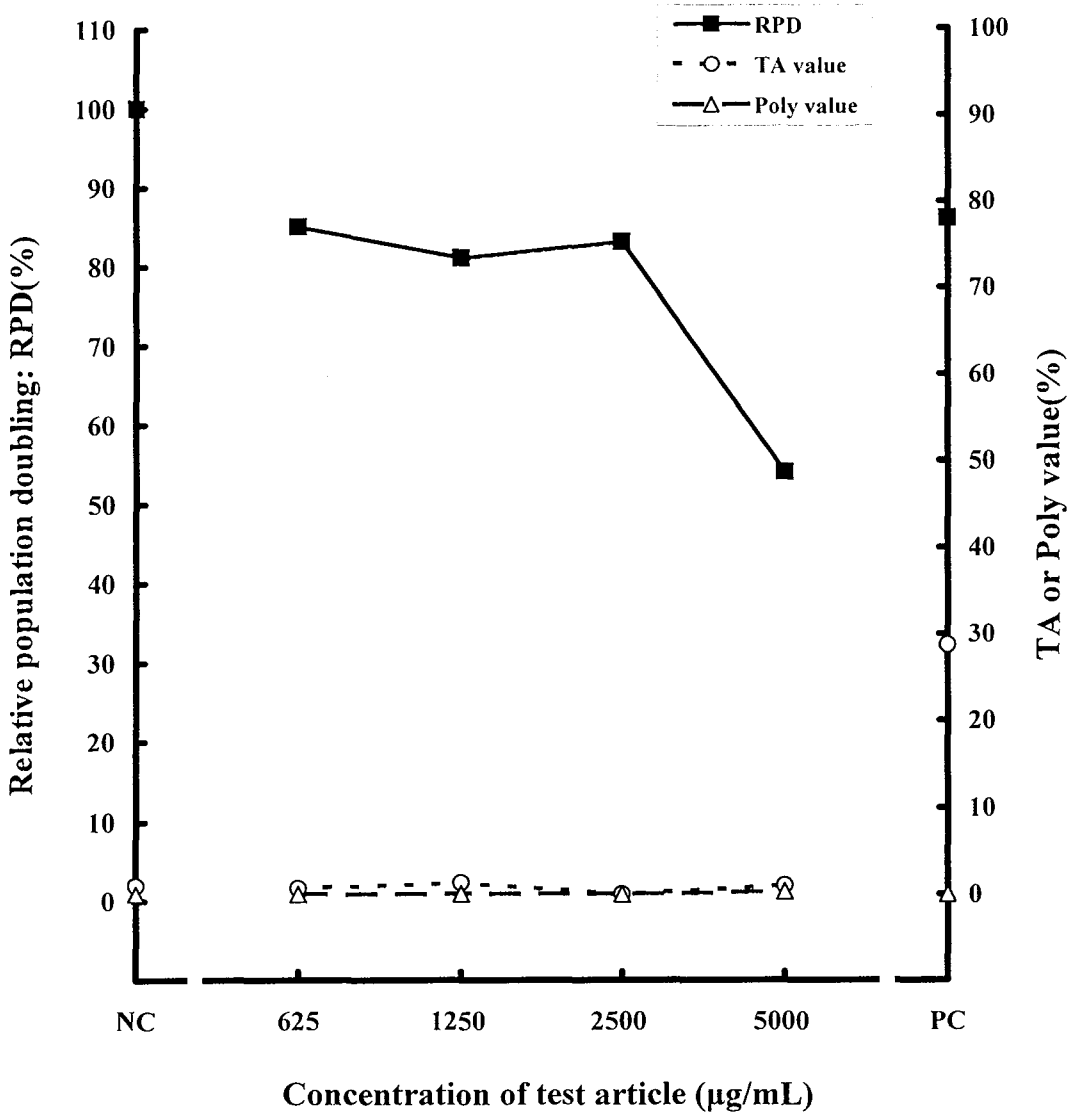


Fig. 1

Results of the chromosome aberration test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Short-term treatment : -S9 mix]

NC : Negative Control (water for injection)

PC : Positive Control (mitomycin C : 0.075 μg/mL)

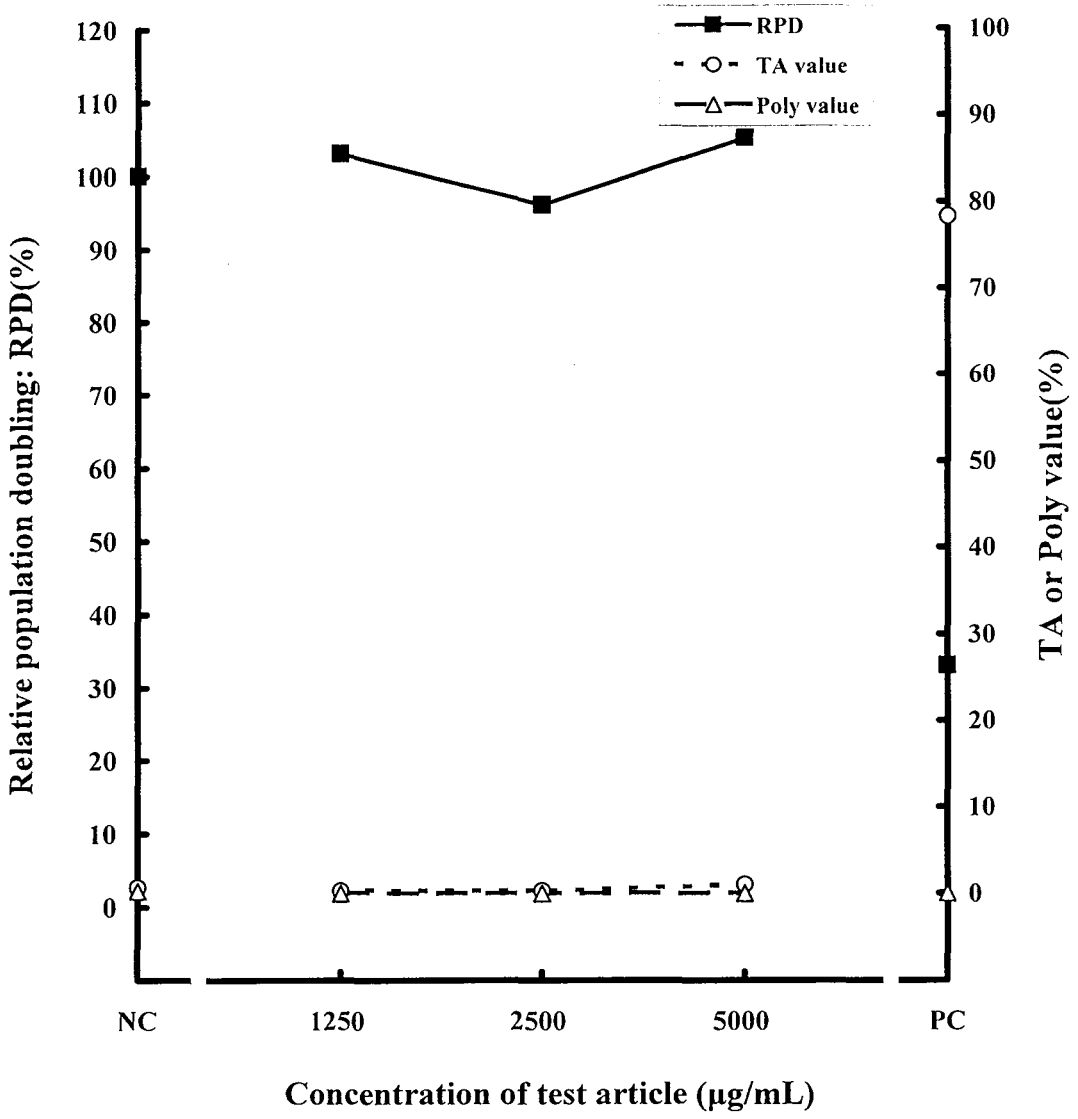


Fig. 2

Results of the chromosome aberration test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Short-term treatment : +S9 mix]

NC : Negative Control (water for injection)

PC : Positive Control (cyclophosphamide : 14 μg/mL)

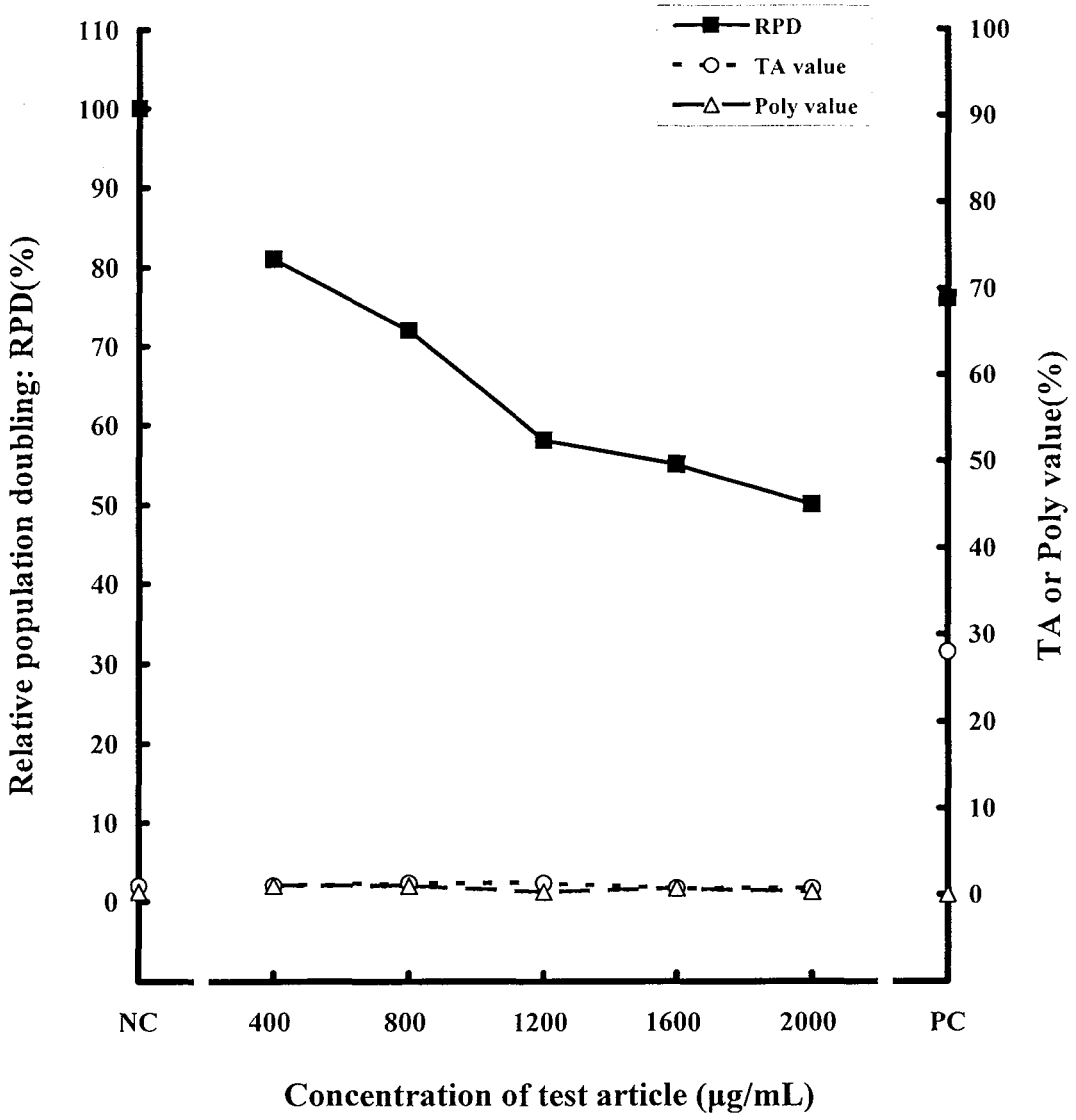


Fig. 3

Results of the chromosome aberration test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Continuous treatment : 24hr]

NC : Negative Control (water for injection)

PC : Positive Control (mitomycin C : 0.050 μg/mL)

T-G212

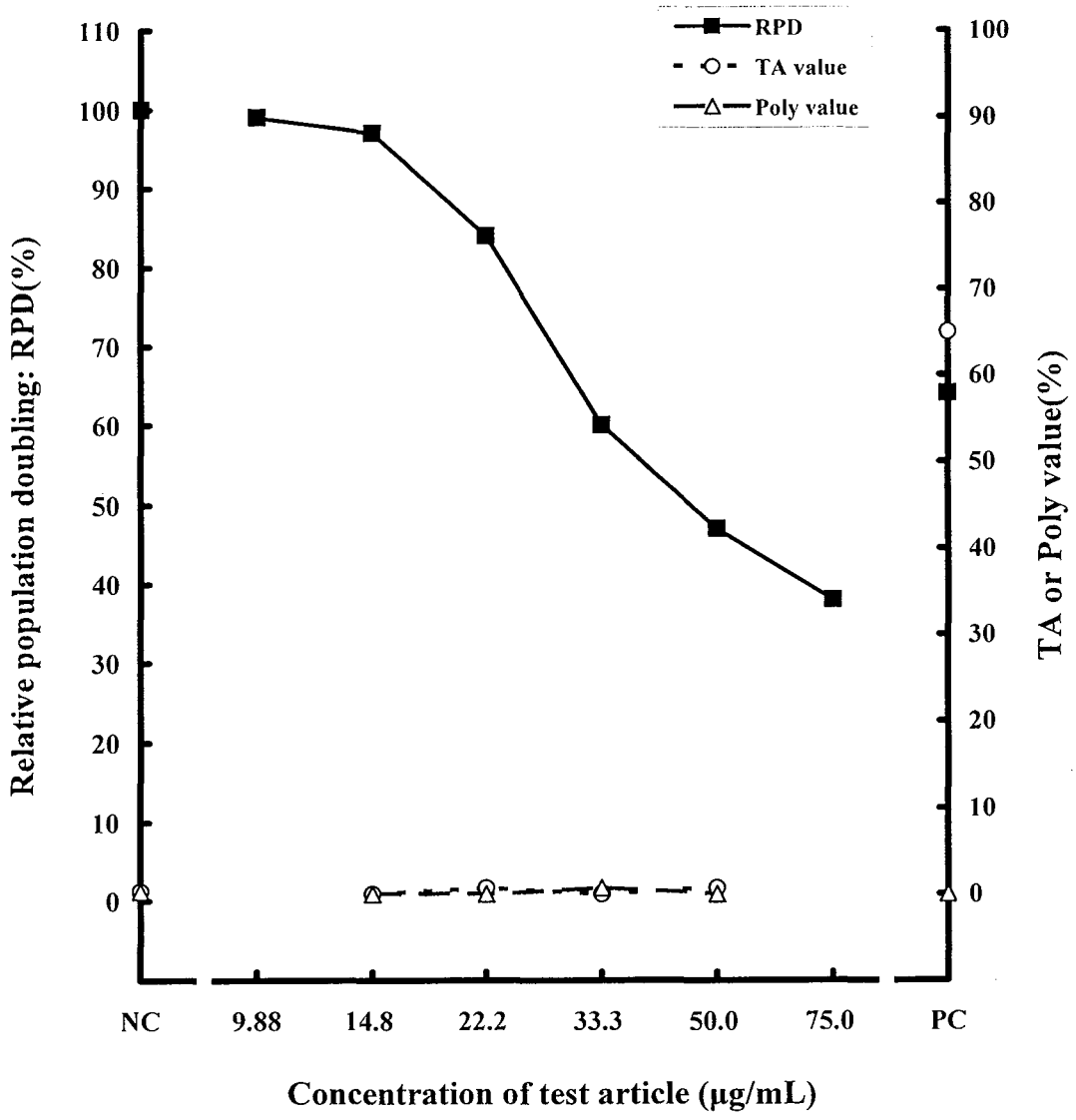


Fig. 4

Results of the confirmation test III in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Continuous treatment : 48hr]

NC : Negative Control (water for injection)

PC : Positive Control (mitomycin C : 0.050 μg/mL)

Table 1 Chromosome aberration in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate
[Short-term treatment:-S9 mix]

Time (h)	S9 mix	Conc. of test article (μg/mL)	Number of cells with structural chromosome aberration (%)										Cell growth Index (%)	Number of cells with numerical chromosome aberration (%)				
			Cells observed	ctb	cte	csb	cse	other	TA(%)	g	TAG(%)	trend test		Cells observed	Polyploid cells	other	Total (%)	trend test
6-18	-	NC	150	2	0	0	0	0	2	0	2			150	0	0	0	
			150	0	1	0	0	0	1	0	1		100	150	0	0	0	
			300	2(0.7)	1(0.3)	0(0.0)	0(0.0)	0(0.0)	3(1.0)	0(0.0)	3(1.0)			300	0(0.0)	0(0.0)	0(0.0)	
		625	150	1	0	0	0	0	1	0	1			150	0	0	0	
			150	1	0	0	0	0	1	0	1		85	150	0	0	0	
			300	2(0.7)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(0.7)	0(0.0)	2(0.7)			300	0(0.0)	0(0.0)	0(0.0)	
		1250	150	1	1	0	0	0	2	0	2			150	0	0	0	
			150	0	2	0	0	0	2	0	2		81	150	0	0	0	
			300	1(0.3)	3(1.0)	0(0.0)	0(0.0)	0(0.0)	4(1.3)	0(0.0)	4(1.3)	N.S.		300	0(0.0)	0(0.0)	0(0.0)	N.S.
		2500	150	0	0	0	0	0	0	0	0			150	0	0	0	
			150	0	0	0	0	0	0	0	0		83	150	0	0	0	
			300	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)			300	0(0.0)	0(0.0)	0(0.0)	
		5000	150	1	1	0	0	0	2	0	2			150	0	0	0	
			150	1	0	0	0	0	1	0	1		54	151	0	1	1	
			300	2(0.7)	1(0.3)	0(0.0)	0(0.0)	0(0.0)	3(1.0)	0(0.0)	3(1.0)			301	0(0.0)	1(0.3)	1(0.3)	
		PC	150	6	39	0	0	0	44	0	44			150	0	0	0	
			150	2	40	0	0	0	42	0	42		86	150	0	0	0	
			300	8(2.7)	79(26.3)	0(0.0)	0(0.0)	0(0.0)	86(28.7) *	0(0.0)	86(28.7)			300	0(0.0)	0(0.0)	0(0.0)	

g: chromatid or chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange,
other: including fragmentation
TA: total number of cells with aberration excluding gap, TAG: total number of cells with aberration including gap.
NC: Negative control (water for injection)
PC: Positive control (mitomycin C, 0.075 μg/mL)
*: Fixher's exact test, $p<0.05$ N.S.: not significant
Cell growth index (%) is shown as the RPD (relative population doubling).

Table 2 Chromosome aberration in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate
[Short-term treatment:+S9 mix]

Time (h)	S9 mix	Conc. of test article (μg/mL)	Number of cells with structural chromosome aberration (%)									Cell growth Index (%)	Number of cells with numerical chromosome aberration (%)				
			Cells observed	ctb	cte	csb	cse	other	TA(%)	g	TAG(%)	trend test		Cells observed	Polyploid cells	other	Total (%)	trend test
6-18	+	NC	150	0	1	0	0	0	1	0	1	N.S.	100	150	0	0	0	N.S.
			150	0	1	0	0	0	1	0	1			151	1	0	1	
			300	0(0.0)	2(0.7)	0(0.0)	0(0.0)	0(0.0)	2(0.7)	0(0.0)	2(0.7)			301	1(0.3)	0(0.0)	1(0.3)	
		1250	150	1	0	0	0	0	1	0	1		103	150	0	0	0	
			150	0	0	0	0	0	0	0	0			150	0	0	0	
			300	1(0.3)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(0.3)	0(0.0)	1(0.3)			300	0(0.0)	0(0.0)	0(0.0)	
		2500	150	0	0	0	0	0	0	0	0		96	150	0	0	0	
			150	0	1	0	0	0	1	0	1			150	0	0	0	
			300	0(0.0)	1(0.3)	0(0.0)	0(0.0)	0(0.0)	1(0.3)	0(0.0)	1(0.3)			300	0(0.0)	0(0.0)	0(0.0)	
		5000	150	0	1	0	0	0	1	0	1		105	150	0	0	0	
			150	1	1	0	0	0	2	0	2			150	0	0	0	
			300	1(0.3)	2(0.7)	0(0.0)	0(0.0)	0(0.0)	3(1.0)	0(0.0)	3(1.0)			300	0(0.0)	0(0.0)	0(0.0)	
		PC	150	8	111	0	0	0	115	0	115		33	150	0	0	0	
			150	1	120	0	0	0	120	0	120			150	0	0	0	
			300	9(3.0)	231(77.0)	0(0.0)	0(0.0)	0(0.0)	235(78.3) *	0(0.0)	235(78.3)			300	0(0.0)	0(0.0)	0(0.0)	

g: chromatid or chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange,
other: including fragmentation
TA: total number of cells with aberration excluding gap, TAG: total number of cells with aberration including gap.
NC: Negative control (water for injection)
PC: Positive control (cyclophosphamide, 14 μg/mL)
*: Fixher's exact test, $p<0.05$ N.S.: not significant
Cell growth index (%) is shown as the RPD (relative population doubling).

Table 3 Chromosome aberration in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate
[Continuous treatment:24hr]

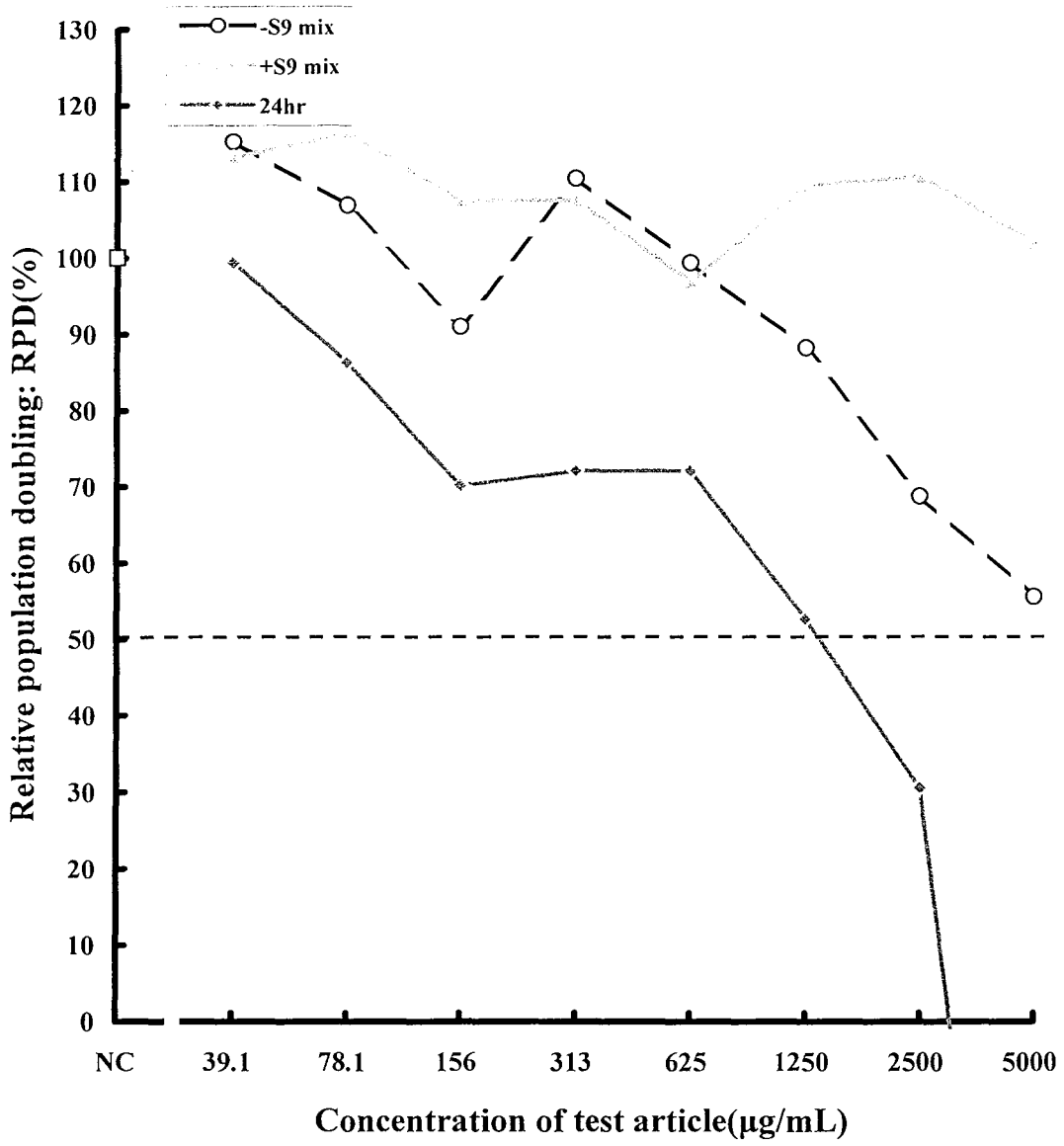
Time (h)	S9 mix	Conc. of test article (μg/mL)	Number of cells with structural chromosome aberration (%)									trend test	Cell growth Index (%)	Number of cells with numerical chromosome aberration (%)				trend test
			Cells observed	ctb	cte	csb	cse	other	TA(%)	g	TAG(%)			Cells observed	Polyploid cells	other	Total (%)	
24-0	-	NC	150	1	0	0	0	0	1	0	1	N.S.	100	151	1	0	1	N.S.
			150	1	1	0	0	0	2	0	2			150	0	0	0	
			300	2(0.7)	1(0.3)	0(0.0)	0(0.0)	0(0.0)	3(1.0)	0(0.0)	3(1.0)			301	1(0.3)	0(0.0)	1(0.3)	
		400	150	1	0	0	0	0	1	0	1		81	151	1	0	1	
			150	1	1	0	0	0	2	0	2			152	2	0	2	
			300	2(0.7)	1(0.3)	0(0.0)	0(0.0)	0(0.0)	3(1.0)	0(0.0)	3(1.0)			303	3(1.0)	0(0.0)	3(1.0)	
		800	150	1	2	0	0	0	3	0	3		72	152	2	0	2	
			150	0	1	0	0	0	1	0	1			151	1	0	1	
			300	1(0.3)	3(1.0)	0(0.0)	0(0.0)	0(0.0)	4(1.3)	0(0.0)	4(1.3)			303	3(1.0)	0(0.0)	3(1.0)	
		1200	150	2	0	0	0	0	2	0	2		58	150	0	0	0	
			150	2	0	0	0	0	2	0	2			151	1	0	1	
			300	4(1.3)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4(1.3)	0(0.0)	4(1.3)			301	1(0.3)	0(0.0)	1(0.3)	
		1600	150	2	0	0	0	0	2	0	2		55	151	1	0	1	
			150	0	0	0	0	0	0	0	0			151	1	0	1	
			300	2(0.7)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(0.7)	0(0.0)	2(0.7)			302	2(0.7)	0(0.0)	2(0.7)	
		2000	150	1	1	0	0	0	2	0	2		50	151	1	0	1	
			150	0	0	0	0	0	0	0	0			150	0	0	0	
			300	1(0.3)	1(0.3)	0(0.0)	0(0.0)	0(0.0)	2(0.7)	0(0.0)	2(0.7)			301	1(0.3)	0(0.0)	1(0.3)	
		PC	150	7	39	0	0	0	45	0	45		76	150	0	0	0	
			150	6	34	0	0	0	39	0	39			150	0	0	0	
			300	13(4.3)	73(24.3)	0(0.0)	0(0.0)	0(0.0)	84(28.0) *	0(0.0)	84(28.0)			300	0(0.0)	0(0.0)	0(0.0)	

g: chromatid or chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange,
other: including fragmentation
TA: total number of cells with aberration excluding gap, TAG: total number of cells with aberration including gap.
NC: Negative control (water for injection)
PC: Positive control (mitomycin C, 0.050 μg/mL)
*: Fixher's exact test, $p<0.05$ N.S.: not significant
Cell growth index (%) is shown as the RPD (relative population doubling).

Table 4 Chromosome aberration in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate
[Continuous treatment:48hr]

Time (h)	S9 mix	Conc. of test article (μg/mL)	Number of cells with structural chromosome aberration (%)									Cell growth Index (%)	Number of cells with numerical chromosome aberration (%)				
			Cells observed	ctb	cte	csb	cse	other	TA(%)	g	TAG(%)	trend test		Cells observed	Polyploid cells	other	Total (%)	trend test
48-0	-	NC	150	1	0	0	0	0	1	0	1	N.S.	100	151	1	0	1	N.S.
			150	0	0	0	0	0	0	0	0			150	0	0	0	
			300	1(0.3)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(0.3)	0(0.0)	1(0.3)			301	1(0.3)	0(0.0)	1(0.3)	
		14.8	150	0	0	0	0	0	0	0	0		97	150	0	0	0	
			150	0	0	0	0	0	0	0	0			150	0	0	0	
			300	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)			300	0(0.0)	0(0.0)	0(0.0)	
		22.2	150	0	0	0	0	0	0	0	0		84	150	0	0	0	
			150	0	2	0	0	0	2	0	2			150	0	0	0	
			300	0(0.0)	2(0.7)	0(0.0)	0(0.0)	0(0.0)	2(0.7)	0(0.0)	2(0.7)			300	0(0.0)	0(0.0)	0(0.0)	
		33.3	150	0	0	0	0	0	0	0	0		60	151	1	0	1	
			150	0	0	0	0	0	0	0	0			151	1	0	1	
			300	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)			302	2(0.7)	0(0.0)	2(0.7)	
		50.0	150	0	1	0	0	0	1	1	2		47	150	0	0	0	
			150	0	1	0	0	0	1	0	1			150	0	0	0	
			300	0(0.0)	2(0.7)	0(0.0)	0(0.0)	0(0.0)	2(0.7)	1(0.3)	3(1.0)			300	0(0.0)	0(0.0)	0(0.0)	
		PC	150	4	92	0	0	0	94	0	94		64	150	0	0	0	
			150	4	97	0	0	0	101	0	101			150	0	0	0	
			300	8(2.7)	189(63.0)	0(0.0)	0(0.0)	0(0.0)	195(65.0) *	0(0.0)	195(65.0)			300	0(0.0)	0(0.0)	0(0.0)	

g: chromatid or chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange,
other: including fragmentation
TA: total number of cells with aberration excluding gap, TAG: total number of cells with aberration including gap.
NC: Negative control (water for injection)
PC: Positive control (mitomycin C, 0.050 μg/mL)
*: Fixher's exact test, $p<0.05$ N.S.: not significant
Cell growth index (%) is shown as the RPD (relative population doubling).

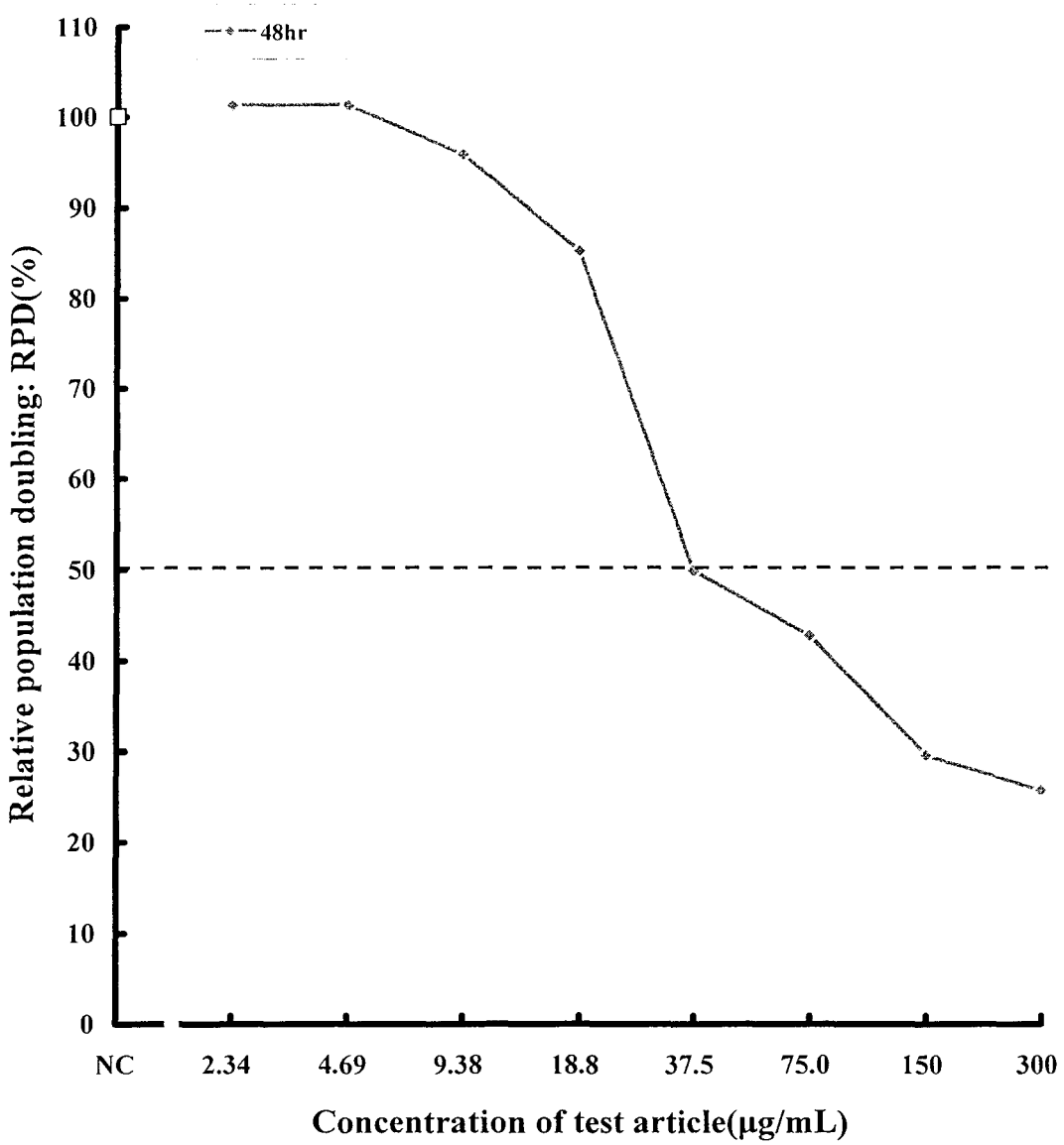


Appendix 1-1

Results of the cell-growth inhibition test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

NC : Negative Control (water for injection)

At the concentration of 5000 μg/mL in the continuous treatment, RPD was not shown, because the RPD was less than 0%.



Appendix 1-2

Results of the confirmation test II in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

NC : Negative Control (water for injection)

Appendix 2-1

Cell-growth ratio in the cell-growth inhibition test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Short-term treatment : -S9 mix]

Cell-growth inhibition test								
Study type		Treatment and Concentration (μg/mL)		RPD[a] (%)	Cell-growth inhibition ratio(%)[b]	Observation[c]		
S9 mix	time (hr)					Condition of cells[d]	Color of medium[e]	Precipitates/Crystals[f] 1)	Precipitates/Crystals[f] 2)
-	6-18	0 (NC)		100	0	-	-	-	-
		Test article	39.1	115	0	-	-	-	-
			78.1	107	0	-	-	-	-
			156	91	9	-	-	-	-
			313	110	0	-	-	-	-
			625	99	1	-	-	-	-
			1250	88	12	+	-	-	-
			2500	69	31	++	-	-	-
			5000	56	44	+++	-	-	-
Concentration of 50% cell-growth inhibition : above **5000** μg/mL									

NC : Negative Control (water for injection)

a) The plate in the negative control group was regarded as a 100% growth.

b) Cell-growth inhibition ratio was shown as 100 - RPD. The value was regarded as 0%, when value was less than or equal to zero.

c) Condition of cells was observed at the end of treatment. Color of medium was observed immediately after addition of the test solutions. Precipitates/crystals were observed [1)]immediately after addition of the test solutions and [2)]at the end of treatment.

d) - : Most of the cells were attached to the surface of plates and grew as a monolayer. Their shape was normal.
+ : A small number of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
++ : Approximately half of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
+++ : Most of the cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.

e) - : No changes of color

f) - : Absence of precipitates

All calculations were carried out using Excel 2010

Appendix 2-2

Cell-growth ratio in the cell-growth inhibition test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Short-term treatment : +S9 mix]

Cell-growth inhibition test									
Study type		Treatment and Concentration (μg/mL)		RPD[a] (%)	Cell-growth inhibition ratio(%)[b]	Observation[c]			
S9 mix	time (hr)					Condition of cells[d]	Color of medium[e]	Precipitates/Crystals[f]	
								1)	2)
+	6-18	0 (NC)		100	0	-	-	-	-
		Test article	39.1	113	0	-	-	-	-
			78.1	116	0	-	-	-	-
			156	108	0	-	-	-	-
			313	108	0	-	-	-	-
			625	97	3	-	-	-	-
			1250	109	0	-	-	-	-
			2500	111	0	-	-	-	-
			5000	102	0	+	-	-	-
Concentration of 50% cell-growth inhibition : above **5000** μg/mL									

NC : Negative Control (water for injection)

a) The plate in the negative control group was regarded as a 100% growth.

b) Cell-growth inhibition ratio was shown as 100 - RPD. The value was regarded as 0%, when value was less than or equal to zero.

c) Condition of cells was observed at the end of treatment. Color of medium was observed immediately after addition of the test solutions. Precipitates/crystals were observed [1)]immediately after addition of the test solutions and [2)]at the end of treatment.

d) - : Most of the cells were attached to the surface of plates and grew as a monolayer. Their shape was normal.
+ : A small number of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.

e) - : No changes of color

f) - : Absence of precipitates

All calculations were carried out using Excel 2010

Appendix 2-3

Cell-growth ratio in the cell-growth inhibition test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Continuous treatment : 24hr]

Cell-growth inhibition test									
Study type		Treatment and Concentration (μg/mL)		RPD[a] (%)	Cell-growth inhibition ratio(%)[b]	Observation[c]			
S9 mix	time (hr)					Condition of cells[d]	Color of medium[e]	Precipitates/Crystals[f]	
								1)	2)
-	24-0	0 (NC)		100	0	-	-	-	-
		Test article	39.1	99	1	-	-	-	-
			78.1	86	14	+	-	-	-
			156	70	30	+	-	-	-
			313	72	28	+	-	-	-
			625	72	28	+	-	-	-
			1250	53	47	+++	-	-	-
			2500	31	69	+++	-	-	-
			5000	-86	186	+++	-	-	-
Concentration of 50% cell-growth inhibition : **1420** μg/mL									

NC : Negative Control (water for injection)

a) The plate in the negative control group was regarded as a 100% growth.

b) Cell-growth inhibition ratio was shown as 100 - RPD. The value was regarded as 0%, when value was less than or equal to zero.

c) Condition of cells was observed at the end of treatment. Color of medium was observed immediately after addition of the test solutions. Precipitates/crystals were observed [1]immediately after addition of the test solutions and [2]at the end of treatment.

d) - : Most of the cells were attached to the surface of plates and grew as a monolayer. Their shape was normal.
+ : A small number of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
+++ : Most of the cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.

e) - : No changes of color

f) - : Absence of precipitates

All calculations were carried out using Excel 2010

Appendix 2-4

Cell-growth ratio in the confirmation test II in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Continuous treatment : 48hr]

Cell-growth inhibition test									
Study type		Treatment and Concentration (μg/mL)		RPD[a] (%)	Cell-growth inhibition ratio(%)[b]	Observation[c]			
S9 mix	time (hr)					Condition of cells[d]	Color of medium[e]	Precipitates/Crystals[f]	
								1)	2)
-	48-0	0 (NC)		100	0	-	-	-	-
		Test article	2.34	101	0	-	-	-	-
			4.69	101	0	-	-	-	-
			9.38	96	4	+	-	-	-
			18.8	85	15	+	-	-	-
			37.5	50	50	++	-	-	-
			75.0	43	57	++	-	-	-
			150	30	70	++	-	-	-
			300	26	74	++	-	-	-
Concentration of 50% cell-growth inhibition : **38** μg/mL									

NC : Negative Control (water for injection)

a) The plate in the negative control group was regarded as a 100% growth.

b) Cell-growth inhibition ratio was shown as 100 - RPD. The value was regarded as 0%, when value was less than or equal to zero.

c) Condition of cells was observed at the end of treatment. Color of medium was observed immediately after addition of the test solutions. Precipitates/crystals were observed [1]immediately after addition of the test solutions and [2]at the end of treatment.

d) - : Most of the cells were attached to the surface of plates and grew as a monolayer. Their shape was normal.
+ : A small number of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
++ : Approximately half of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.

e) - : No changes of color

f) - : Absence of precipitates

All calculations were carried out using Excel 2010

Appendix 3-1

Results of observation in the chromosome aberration test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Short-term treatment : -S9 mix]

Chromosome aberration test							
Study type		Treatment and Concentration (μg/mL)		Observation[a]			
S9 mix	time (hr)			Condition of cells[b]	Color of medium[c]	Precipitates/Crystals[d]	
						1)	2)
-	6-18	0 (NC)		-	-	-	-
		Test article	625	-	-	-	-
			1250	+	-	-	-
			2500	++	-	-	-
			5000	+++	-	-	-
		PC		-	-	-	-

NC : Negative Control (water for injection)

PC : Positive Control (mitomycin C : 0.075 μg/mL)

a) Condition of cells was observed at the end of treatment. Color of medium was observed immediately after addition of the test solutions. Precipitates/crystals were observed [1)]immediately after addition of the test solutions and [2)]at the end of treatment.

b) - : Most of the cells were attached to the surface of plates and grew as a monolayer. Their shape was normal.
+ : A small number of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
++ : Approximately half of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
+++ : Most of the cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.

c) - : No changes of color

d) - : Absence of precipitates

Appendix 3-2

Results of observation in the chromosome aberration test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate
[Short-term treatment : +S9 mix]

Chromosome aberration test							
Study type		Treatment and Concentration (μg/mL)		Observation[a]			
S9 mix	time (hr)			Condition of cells[b]	Color of medium[c]	Precipitates/Crystals[d]	
						1)	2)
+	6-18	0 (NC)		-	-	-	-
		Test article	313	-	-	-	-
			625	-	-	-	-
			1250	-	-	-	-
			2500	-	-	-	-
			5000	+	-	-	-
		PC		-	-	-	-

NC : Negative Control (water for injection)
PC : Positive Control (cyclophosphamide : 14 μg/mL)

a) Condition of cells was observed at the end of treatment. Color of medium was observed immediately after addition of the test solutions. Precipitates/crystals were observed [1]immediately after addition of the test solutions and [2]at the end of treatment.

b) - : Most of the cells were attached to the surface of plates and grew as a monolayer. Their shape was normal.
+ : A small number of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.

c) - : No changes of color

d) - : Absence of precipitates

Appendix 3-3

Results of observation in the chromosome aberration test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Continuous treatment : 24hr]

Chromosome aberration test						
Study type		Treatment and Concentration (μg/mL)		Observation[a)]		
S9 mix	time (hr)			Condition of cells[b)]	Color of medium[c)]	Precipitates/Crystals[d)]
						1) / 2)
-	24-0	0 (NC)		-	-	- / -
		Test article	400	+	-	- / -
			800	++	-	- / -
			1200	++	-	- / -
			1600	+++	-	- / -
			2000	+++	-	- / -
		PC		-	-	- / -

NC : Negative Control (water for injection)

PC : Positive Control (mitomycin C : 0.050 μg/mL)

a) Condition of cells was observed at the end of treatment. Color of medium was observed immediately after addition of the test solutions. Precipitates/crystals were observed [1)]immediately after addition of the test solutions and [2)]at the end of treatment.

b) - : Most of the cells were attached to the surface of plates and grew as a monolayer. Their shape was normal.
+ : A small number of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
++ : Approximately half of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
+++ : Most of the cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.

c) - : No changes of color

d) - : Absence of precipitates

Appendix 3-4

Results of observation in the confirmation test III in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Continuous treatment : 48hr]

Chromosome aberration test						
Study type		Treatment and Concentration (μg/mL)		Observation[a]		
S9 mix	time (hr)			Condition of cells[b]	Color of medium[c]	Precipitates/Crystals[d]
						1) / 2)
-	48-0	0 (NC)		-	-	- / -
		Test article	9.88	+	-	- / -
			14.8	+	-	- / -
			22.2	++	-	- / -
			33.3	++	-	- / -
			50.0	+++	-	- / -
			75.0	+++	-	- / -
		PC		-	-	- / -

NC : Negative Control (water for injection)

PC : Positive Control (mitomycin C : 0.050 μg/mL)

a) Condition of cells was observed at the end of treatment. Color of medium was observed immediately after addition of the test solutions. Precipitates/crystals were observed [1)]immediately after addition of the test solutions and [2)]at the end of treatment.

b) - : Most of the cells were attached to the surface of plates and grew as a monolayer. Their shape was normal.
+ : A small number of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
++ : Approximately half of cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.
+++ : Most of the cells were detached from the surface of the plate and floated in the culture medium. The shape of attached cells was also altered.

c) - : No changes of color

d) - : Absence of precipitates

Appendix 4-1

Cell concentration and population doubling in the cell-growth inhibition test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Short-term treatment : -S9 mix]

Study type		Treatment and Concentration (μg/mL)		Cell counts [a] ($\times 10^6$ cells/mL)	Cell counts [b] ($\times 10^6$ cells/mL)	PD
S9 mix	time (hr)					
-	6-18	0 (NC)		0.108	0.293	1.44
		Test article	39.1		0.341	1.66
			78.1		0.314	1.54
			156		0.267	1.31
			313		0.325	1.59
			625		0.292	1.43
			1250		0.260	1.27
			2500		0.215	0.99
			5000		0.188	0.80

[Short-term treatment : +S9 mix]

Study type		Treatment and Concentration (μg/mL)		Cell counts [a] ($\times 10^6$ cells/mL)	Cell counts [b] ($\times 10^6$ cells/mL)	PD
S9 mix	time (hr)					
+	6-18	0 (NC)		0.108	0.326	1.59
		Test article	39.1		0.375	1.80
			78.1		0.388	1.85
			156		0.354	1.71
			313		0.354	1.71
			625		0.314	1.54
			1250		0.360	1.74
			2500		0.366	1.76
			5000		0.333	1.62

[Continuous treatment : 24hr]

Study type		Treatment and Concentration (μg/mL)		Cell counts [a] ($\times 10^6$ cells/mL)	Cell counts [b] ($\times 10^6$ cells/mL)	PD
S9 mix	time (hr)					
-	24-0	0 (NC)		0.108	0.315	1.54
		Test article	39.1		0.311	1.53
			78.1		0.271	1.33
			156		0.228	1.08
			313		0.233	1.11
			625		0.233	1.11
			1250		0.190	0.81
			2500		0.150	0.47
			5000		0.043	-1.32

NC : Negative Control (water for injection)

The number of cells on the plate of each dose was measured using the auto cell counter at the time of start [a] and end [b] of treatment.

PD : Population Doubling was determined as;
[log (Post-treatment number of cells / Initial number of cells)] / log 2

All calculations were carried out using Excel 2010

Appendix 4-2

Cell concentration and population doubling in the confirmation test II in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Continuous treatment : 48hr]

Study type		Treatment and Concentration (μg/mL)		Cell counts [a] ($\times 10^6$ cells/mL)	Cell counts [b] ($\times 10^6$ cells/mL)	PD
S9 mix	time (hr)					
-	48-0	0 (NC)		0.134	1.160	3.11
		Test article	2.34		1.190	3.15
			4.69		1.190	3.15
			9.38		1.060	2.98
			18.8		0.839	2.65
			37.5		0.393	1.55
			75.0		0.336	1.33
			150		0.253	0.92
			300		0.233	0.80

NC : Negative Control (water for injection)

The number of cells on the plate of each dose was measured using the auto cell counter at the time of start [a] and end [b] of treatment.

PD : Population Doubling was determined as;
[log (Post-treatment number of cells / Initial number of cells)] / log 2

All calculations were carried out using Excel 2010

Appendix 5-1

Cell concentration and population doubling in the chromosome aberration test in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Short-term treatment : -S9 mix]

Study type		Treatment and Concentration (μg/mL)		Cell counts [a] ($\times 10^6$ cells/mL)	Cell counts [b] ($\times 10^6$ cells/mL)	PD
S9 mix	time (hr)					
-	6-18	0 (NC)		0.126	0.264	1.07
		Test article	625		0.236	0.91
			1250		0.230	0.87
			2500		0.233	0.89
			5000		0.188	0.58
		PC			0.239	0.92

[Short-term treatment : +S9 mix]

Study type		Treatment and Concentration (μg/mL)		Cell counts [a] ($\times 10^6$ cells/mL)	Cell counts [b] ($\times 10^6$ cells/mL)	PD
S9 mix	time (hr)					
+	6-18	0 (NC)		0.126	0.289	1.20
		Test article	1250		0.297	1.24
			2500		0.280	1.15
			5000		0.302	1.26
		PC			0.166	0.40

[Continuous treatment : 24hr]

Study type		Treatment and Concentration (μg/mL)		Cell counts [a] ($\times 10^6$ cells/mL)	Cell counts [b] ($\times 10^6$ cells/mL)	PD
S9 mix	time (hr)					
-	24-0	0 (NC)		0.126	0.284	1.17
		Test article	400		0.243	0.95
			800		0.225	0.84
			1200		0.202	0.68
			1600		0.197	0.64
			2000		0.190	0.59
		PC			0.234	0.89

NC : Negative Control (water for injection)

PC : Positive Control (mitomycin C or cyclophosphamide)

The number of cells on the plate of each dose was measured using the auto cell counter at the time of start [a] and end [b] of treatment.

PD : Population Doubling was determined as;
[log (Post-treatment number of cells / Initial number of cells)] / log 2

All calculations were carried out using Excel 2010

Appendix 5-2

Cell concentration and population doubling in the confirmation test III in cultured Chinese hamster (CHL/IU) cells treated with β-Galactosidase concentrate

[Continuous treatment : 48hr]

Study type		Treatment and Concentration (%)		Cell counts [a] ($\times 10^6$ cells/mL)	Cell counts [b] ($\times 10^6$ cells/mL)	PD
S9 mix	time (hr)					
-	48-0	0 (NC)		0.110	0.952	3.11
		Test article	9.88		0.930	3.08
			14.8		0.886	3.01
			22.2		0.666	2.60
			33.3		0.405	1.88
			50.0		0.303	1.46
			75.0		0.248	1.17
		PC			0.436	1.99

NC : Negative Control (water for injection)

PC : Positive Control (mitomycin C)

The number of cells on the plate of each dose was measured using the auto cell counter at the time of start [a] and end [b] of treatment.

PD : Population Doubling was determined as;
[log (Post-treatment number of cells / Initial number of cells)] / log 2

All calculations were carried out using Excel 2010

T-G212

Quality Assurance Statement (1/2)

Study Number: T-G212

Study Title: Chromosome aberration test in cultured mammalian cells treated with β-Galactosidase concentrate

I, the undersigned, hereby declare that this study was conducted in compliance with the following GLP regulation.

- "The Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs", (Ordinance No. 21 of the Ministry of Health and Welfare, Japan, March 26, 1997)
- "OECD Principles of Good Laboratory Practice" (OECD Council: November 26, 1997)

Inspections were conducted as follows.

(b) (6)

September 15, 2016

Minoru Izutsu, M. Sc. Date
Manager, Quality Assurance Unit
BoZo Research Center Inc.

Study-Based Inspections

Items	Inspectors	Dates of Inspection	Dates of Report to Study Director and Management
Protocol	E. Ko	May 18, 2016	May 18, 2016
Protocol Amendment (1)	E. Ko	May 27, 2016	May 27, 2016
Seeding of Cells	E. Ko	May 30, 2016	May 31, 2016
Preparation / Storage (Test Article・Positive Control) / Treatment with Test Article	M. Yoshida	June 2, 2016	June 2, 2016
Preparation of Chromosome Specimens (Fixation)	E. Ko	June 3, 2016	June 3, 2016
Preparation of Chromosome Specimens (Staining)	M. Yoshida	June 6, 2016	June 6, 2016

T-G212

Quality Assurance Statement (2/2)

Items	Inspectors	Dates of Inspection	Dates of Report to Study Director and Management
Observation of Chromosome Specimens	M. Yoshida	June 6, 2016	June 6, 2016
Protocol Amendment (2)	E. Ko	June 16, 2016	June 16, 2016
Protocol Amendment (3)	E. Ko	June 24, 2016	June 27, 2016
Protocol Amendment (4)	M. Yoshida	July 8, 2016	July 8, 2016
Raw Data	E. Ko	August 9, 2016	August 10, 2016
Final Report (Draft) / Figures / Tables / Appendices	E. Ko	August 9, 2016	August 10, 2016
Final Report	E. Ko	September 15, 2016	September 15, 2016

Facility-Based Inspections

Items	Inspectors	Dates of Inspection	Dates of Report to Division Director and Management
Characteristics Test of Cultured Cells	E. Ko	February 19, 2016	
	M. Yoshida	February 22, 2016	
		February 23, 2016	
		February 24, 2016	
		February 26, 2016	
		March 3, 2016	March 3, 2016

APPENDIX 6

TT-160003

Final Report

(English Version)

A 13-WEEK ORAL GAVAGE TOXICITY STUDY OF β-GALACTOSIDASE CONCENTRATE IN RATS

Study Number: TT-160003

Study Period: June 14, 2016 to January 12, 2017

Test Facility
Tsukuba Laboratory, BoZo Research Center Inc.
8 Okubo, Tsukuba-shi, Ibaraki 300-2611, Japan

Sponsor
Amano Enzyme Inc.
1-2-7 Nishiki, Naka-ku, Nagoya-shi, Aichi 460-8630, Japan

BoZo Research Center Inc.
36-7 Oyama-cho, Shibuya-ku, Tokyo 151-0065, Japan

1. GLP Compliance Statement

Study Number:	TT-160003
Study Title:	A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats

Characteristic and stability analysis of the test article and analysis of the test solutions were not contracted, therefore they were not conducted under GLP, but obtained and confirmed its data under non-GLP. Expect for the above mention, the study was conducted in compliance with the following GLP regulations.

- "The Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs" (Ordinance No. 21 of the Ministry of Health and Welfare, Japan, March 26, 1997)

(Sealed in the original)
Atsunori Yafune, Ph.D., D.J.S.O.T.
Study Director
Tsukuba Laboratory, BoZo Research Center Inc.

January 12, 2017
Date

2. Table of Contents

Attached Data

Figures

Tables

Appendices

3. Outline of Study

3.1 Study Number

TT-160003

3.2 Study Title

A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats

3.3 Purpose of Study

The test article was administered to rats by oral gavage for 13 weeks in order to identify the dose level that induced apparent toxic changes and the dose level that induced no toxic changes.

3.4 Sponsor

Amano Enzyme Inc.

1-2-7 Nishiki, Naka-ku, Nagoya-shi, Aichi 460-8630, Japan

3.5 Contractor

BoZo Research Center Inc.

36-7 Oyama-cho, Shibuya-ku, Tokyo 151-0065, Japan

3.6 Testing Facility

Tsukuba Laboratory, BoZo Research Center Inc.

8 Okubo, Tsukuba-shi, Ibaraki 300-2611, Japan

3.7 Study Schedule

Start of study:	June 14, 2016
Allocation of Test Article to the Study Director :	June 23, 2016
Animal receipt:	June 21, 2016
Start of administration:	June 29, 2016 (male) June 30, 2016 (female)
End of administration:	September 27, 2016 (male) September 28, 2016 (female)
Date of terminal necropsy:	September 28, 2016 (male) September 29, 2016 (female)

Completion of study: January 12, 2017

3.8 Study Director

Atsunori Yafune, Ph.D., D.J.S.O.T.

Tsukuba Laboratory, Safety Department, BoZo Research Center Inc.

3.9 Contributors

Study leader: Tetsuya Yamamoto, B.S.

Test Article archiving: Norihiko Nakamura, Ph.D.

Clinical examination: Atsushi Wakita, M.S.

Study leader for preparation of specimens:
Maki Noguchi

Pathology: Kenichiro Kasahara, D.J.S.T.P.

3.10 Unexpected Circumstances That Might Have Affected the Reliability of the Study and Deviations from Protocol

In this study, there were no unexpected circumstances that affected the reliability of the study results nor deviations from the Protocol.

3.11 Archives

The original Protocol (including Protocol amendments), written records, raw data, reports (including the original of the Final Report) and specimens (including the archive samples of the test article) are retained in the archive facility and test article storage room of Tsukuba Laboratory, BoZo Research Center Inc. for a period of 5 years after the Final Report is submitted. At the end of this period, Amano Enzyme Inc. and BoZo Research Center Inc. will discuss and determine the disposition of the said materials. However, biological samples (urine and plasma) that cannot endure long-term preservation were discarded at the end of the study.

3.12 Signature of Study Director and Date

(Sealed in the original) January 12, 2017

Atsunori Yafune, Ph.D., D.J.S.O.T. Date

Tsukuba Laboratory

BoZo Research Center Inc.

4. Summary

The toxicity profile of β-galactosidase concentrate was examined when the test article was orally administered to Sprague-Dawley rats [Crl:CD(SD), 12/sex/group, started at 6 weeks of age] for 13 weeks. The dose solutions at dose levels of 500, 1000 and 2000 mg/kg/day were administered by oral gavage once daily at a dose volume of 20 mL/kg for 13 weeks. A control group was provided and the animals received the vehicle, water for injection.
Observation of clinical signs, body weight and food consumption, ophthalmology and urinalysis (including water consumption), hematology, blood chemistry, necropsy, organ weights, and histopathology were performed.

As a result, no deaths occurred in any group and no test article-related toxic changes were noted in clinical signs, body weight and food consumption, ophthalmology, urinalysis (including water consumption), hematology, blood chemistry, necropsy, organ weight and histopathology.

Based on the results described above, repeated oral dosing of β-galactosidase concentrate in rats for 13 weeks showed no effects observed in any examinations. Therefore, the no-observed-adverse-effect level (NOAEL) of β-galactosidase concentrate was considered to be 2000 mg/kg/day in both sexes under the test conditions.

5. Introduction

This study was conducted at BoZo Research Center Inc. on behalf of Amano Enzyme Inc., in compliance with the following regulations and in accordance with the following guidelines related to the toxicity study and animal welfare.

1) GLP
- "The Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs" (Ordinance No. 21 of the Ministry of Health and Welfare, Japan, March 26, 1997)

2) Toxicity Study Guidelines
- "Guidelines for Toxicity Studies Required for Applications for Approval to Manufacture (Import) Drugs" (Ordinance No. 1, Notification No. 24 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, September 11, 1989)
- "Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals" (Notification 0219, Article No. 4 of the Pharmaceutical and Food Affairs Bureau, Ministry of Health, Labour and Welfare, Japan, 19 February 2010)
- "Partial Revision of Guidelines for Repeated-Dose Toxicity Studies" (Notification No. 655 of the Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare, Japan, April 5, 1999)
- "Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives" (Notification No. 29 of the Environmental Health Bureau, Ministry of Health and Welfare, Japan, March 22, 1996)

3) Animal Welfare
- "Act on Welfare and Management of Animals" (Act No. 105, October 1, 1973, Law No. 50)
- "The Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006)
- "Guidelines for Appropriate Implementation of Animal Experiments" (Science Council of Japan, June 1, 2006)

This study was conducted under the approval of the Institutional Animal Care and Use Committee of the test facility (Approval No. APS16002).

6. Test Materials and Methods

6.1 Test Article and Vehicle

6.1.1 Test Article

The test article was supplied by Amano Enzyme Inc. The information on the test article used in the present study were as follows (Attached Data 1, non-GLP conditions).

Name:	β-Galactosidase Conc.
Lot number:	GFE68-001@K
Origin:	*Cryptococcus terrestris*
Description:	Pale, dark yellow powder
Enzyme activity:	2,550 U/g (β-galactosidase activity, lactose substrate method)
Stability:	Stable for a year under refrigeration after manufacturing. The Study Sponsor analyzed the test article which was returned to the Study Sponsor after the end of animal experiment to confirm the stability of the test article during the period of animal experiment. As a result, the stability of the test article was confirmed during the period of animal experiment (Attached Data 2, non-GLP conditions).
Precautions for handling:	Avoiding inhalation and contact with the eyes or skin
Expiry date:	February 12, 2017 (Production date: February 13, 2016)
Storage conditions:	Preserved in a refrigerator (actual values: 3.0 to 9.8°C, acceptable range: 1 to 10°C)
Storage place:	Test article storage room and test article preparation room at Tsukuba Laboratory
Treatment of remainders:	Approximately 1 g of the test article was stored as an archive sample. The test article remaining after animal experiment was returned to the Sponsor.

6.1.2 Vehicle

Name:	Water for injection
Specification:	Japanese Pharmacopoeia
Manufacturer:	Otsuka Pharmaceutical Factory, Inc.
Lot numbers:	5J99, 5L87, 6B85, 6B92 and 6D98

Storage conditions:	At room temperature
Storage place:	Vehicle preparation room at Tsukuba Laboratory

6.2 Preparation of Dosing Formulations

6.2.1 Vehicle for the Control Group

For the control group, a requisite amount of water for injection was divided into one-day aliquots in brown glass bottles and stored in the same manner as the test formulations.

6.2.2 Preparation of Test Formulations

At maximum, 7 days' aliquot was prepared at one time at 25, 50 and 100 mg/mL and used within 7 days after preparation (the day of preparation was designated as day 0).

1) Preparation of 100 mg/mL

   A requisite amount of the test article was added to a beaker and the test article was washed with approximately 20 % of the final volume of water for injection (unit: mL, test article (g) ×112 / 12). The rest of the water for injection was added and suspended using a stirrer bar. After confirming the suspension conditions, the test formulation was placed at room temperature until the foam depleted to a certain extent. The test formulation was divided into aliquots for one day's use in brown glass bottles while using a stirrer bar.

2) Preparation of 25 and 50 mg/mL

   A requisite amount of the test article to make each dose formulation was gradually added to a beaker with water for injection (approximately 80 % of the final preparation volume) while stirring. After confirming the suspension conditions, the test formulation was placed at room temperature until the foam depleted to a certain extent. This mixture was transferred to a measuring cylinder, and water for injection was added to the specified volume. Test formulations were divided into aliquots for one day's use in brown glass bottles while using a stirrer bar.

6.2.3 Stability of Test Formulations

It was confirmed by the Sponsor that 25, 50 and 100 mg/mL formulations (vehicle: water for injection) were stable and homogeneous for 5 hours in refrigeration or at room temperature and were also stable for 7 days under refrigeration (1 to 10°C) (Attached Data 3-1 and 3-2, non-GLP conditions). In addition, there were stable for freezing and thawing up to three times (Attached Data 3-3, non-GLP conditions).

6.2.4 Storage conditions

The test formulations were stored in a cold place (refrigerator, actual values: 3.2 to 9.8°C, acceptable range: 1 to 10°C).

6.2.5 Verification of Concentration and Homogeneity of Test Formulations

Each test formulation used for administration in Week 1 and Week 13 was sent to the Sponsor (Gifu Laboratory) frozen and subjected to verification of concentration and homogeneity. As a result, the proportion to the nominal value ranged from 93.5 to 103.3% and CV ranged from 0.9 to 2.7%, and the concentration and homogeneity in test formulations were confirmed to be within the acceptable range (concentration: 100 ± 10% of the nominal value, homogeneity: CV within 10%) (Attached Data 4-1 and 4-2, non-GLP conditions).

Analytical method: lactose substrate method

Analyzing facility: Amano Enzyme Inc.

Item determined: β-Galactosidase activity

Number and volume of samples at each concentration:
3 samples/concentration (approximately 2 mL each from upper, middle and lower layers)

Shipping address of test formulations for analysis:
1-6 Tekunopuraza, Kagamihara-shi, Gifu 509-0109, Japan
Gifu Laboratory, Amano Enzyme Inc.
Development of industrial enzyme Department
Masamichi Okada

Shipping dates of test formulations for analysis:
Week 1: June 27, 2016
Week 13: September 20, 2016

6.3 Reason for Choice of Test Animal Species and Strain

Toxicity study guidelines require toxicity studies using rodents. Rats were chosen as they are widely used in safety studies of pharmaceuticals, etc. The strain of rats used in this study was chosen as their nature is well known and ample background data are available.

6.4 Test Animals and Group Allocation

Sixty male and 60 female Sprague-Dawley SPF rats [Crl:CD(SD), Atsugi Breeding Center, Charles River Laboratories Japan Inc.] were obtained at 5 weeks of age and they were quarantined/acclimated for 8 days for males and 9 days for females. During this period, animals were observed for clinical signs such as abnormalities in external appearance, nutritional condition and behavior (once every day), weighed (3 times: 7, 6 and 2 days for males and 8, 7 and 3 days for females before the start of administration) and subjected to ophthalmological examination (for details, see "6.11.4 Ophthalmology"). Animals considered healthy with favorable body weight gain (from body weight first measured in the quarantine period to body weight at group allocation) and no abnormalities in clinical observation or ophthalmological examination were selected and used for the study at 6 weeks of age. There were no animals exhibiting abnormal clinical signs or body weight changes, but some animals with abnormalities in the ophthalmological examination (for details, see foot-note in "6.11.4 Ophthalmology") which were judged to have a potential impact on the study evaluation were excluded from group allocation.

After selection of animals on the basis of the above observations and examinations during the quarantine/acclimation period, the animals were ranked according to their body weight on the day of group allocation (2 days for males and 3 days for females before the start of administration) and assigned in such a way that group mean body weight was comparable. Forty-eight animals of each sex were assigned by a combination of the block placement method and random sampling method using a computer (requisite number of groups was composed by the block placement method and test groups and individual animal numbers were assigned at random). Individual body weight at the start of administration ranged from 209 to 246 g in males and 144 to 179 g in females, these were within the permissible range (Mean±20%).

Animals remaining after group allocation were excluded from the study and were effectively utilized for education and research.

6.5 Animal Husbandry

The animal room (Room No. D309) was maintained at a temperature of 22 ± 3°C (measured values: 21.5 to 25.5°C) [Note 1)], relative humidity of 50 ± 20% (measured values: 44 to 99%) [Note 1, Note 2)], air ventilation at 10 to 20 changes per hour and 12-hour illumination per day (07:00 to 19:00). Two animals of the same sex were housed in plastic solid floor cages (W 280 × D 440 × H 210 mm) with bedding [ALPHA-dri (Shepherd Specialty Papers, Inc.), Lot Nos. 01116、02116、04116 and 05116]. Animals were allowed free access to pelleted diet CR-LPF (radiation-sterilized, Oriental Yeast Co., Ltd., Lot Nos.: 160229 and 160516 in a stainless-steel

feeder) and tap water (Tsukuba City Water: via water bottle). As environmental enrichment, 7979C.CS certified/irradiated Diamond Twists (Envigo RMS, Inc., Lot Nos.: 7979C-040715P and 7979C-090915P) were supplied to rats once a week during the period of animal experiment, and analytical reports of Diamond Twists were obtained and copies were filed after verifying that there were no abnormalities which could have affected the study results.

Note 1: In 22 August 2016, the temperature and humidity of the animal room was up to 25.5°C and 99% or more due to breakdown of the boiler and air-cooled chiller affected by power outage from a typhoon. However, there were no effect on the evaluation of the study since the time of deviation was a short time (approximately a hour) and no abnormalities was observed in any animals in clinical sighs.

Note 2: In 23 August 2016, the humidity of the animal room was up to 72% due to the malfunction of the temperature regulator. However, there was no effect on the evaluation of the study since the time of deviation was a short time (approximately 5 minutes) and the excursion was minor.

6.6 Contaminants in Feed, Bedding and Drinking Water

The following analysis reports on contaminants in the feed, bedding and water were obtained and copies were filed after verifying that there were no abnormalities which could have affected the study results.

Analytical reports of feed (all the lots used: Eurofins Food and Product Testing)

Analytical reports of waster in accordance with the Waterworks Law (Kotobiken Medical Laboratories, Inc.)

Analytical report of bedding (all the lots used, N·P Analytical Laboratories)

6.7 Animal Identification and Cage Labeling

Animals were individually identified by ear tags for small animals with sequential numbers and attached on arrival. After group allocation, all cages had labels, color-coded according to test groups, displaying the study number, administration route, dose level, sex, animal number, ear tag number and scheduled date of necropsy. Additionally, marking the tail of an animal was performed to easily identify within the same cage.

6.8 Reason for Administration Route, Method, Period and Frequency of Administration and Rationale for the Selection

The oral route was selected as the test article was intended to be used for processing food and the administration period was set at 13 weeks. The frequency of administration was once daily (7 times/week), which is normal for repeated-dose toxicity studies.

Dose volume was set at 20 mL/kg body weight and the test formulation was administered orally by gavage using a flexible stomach tube (between 08:01 and 13:08). Animals in the control group received the vehicle (water for injection) in the same manner. Individual dose

volume (display unit: 0.1mL) was calculated based on the animal's most recently measured body weight.

6.9 Dose Levels and Group Composition

Three dose levels were set at 500, 1000 and 2000 mg/kg/day, and a total of 4 groups including a control group were provided. Each group consisted of 12 males and 12 females. Group composition is shown in the following Text Table 1.

Text Table 1. Group Composition

Group	Dose Level (mg/kg/day)	Dose Concentration (mg/mL)	Dose Volume (mL/kg/day)	Sex	No. of Animals	Animal Number
Control	0	0	20	Male Female	12 12	1001-1012 1101-1112
Low	500	25	20	Male Female	12 12	2001-2012 2101-2112
Middle	1000	50	20	Male Female	12 12	3001-3012 3101-3112
High	2000	100	20	Male Female	12 12	4001-4012 4101-4112

6.10 Rationale for Selection of Dose Levels

The dose levels were selected on the basis of the results in a 2-week oral toxicity preliminary study of β-galactosidase concentrate in rats (BoZo Research Center Inc., Study No. C-TT160001, dose levels: 0, 500, 1000 and 2000 mg/kg/day) [1)]. In that study, there were no apparent toxic changes even at 2000 mg/kg/day. Therefore, the high dose level was set at 2000 mg/kg/day, which was regarded as the maximum feasible dose in oral gavage studies, and the middle and low dose levels were set at 1000 and 500 mg/kg/day, with a common ratio of 2.

6.11 Methods of Observation and Examination

Designation of experimental days was as follows:

Day 1 of administration: Starting day of administration

Week 1 of administration: From Day 1 to Day 7 of administration

6.11.1 Clinical Observations

All animals were observed for clinical signs such as abnormalities in external appearance, nutritional condition, posture, behavior and excretions, 3 times a day during the administration period: before dosing, immediately after and 1 to 3 hours after dosing (however, twice a day on Saturdays and holidays: before and immediately after dosing).

6.11.2 Body Weight

All animals were weighed twice in Week 1 of administration, on Days 1 and 7 of administration, and thereafter once a week, every 7 days, prior to dosing (between 07:36 and 10:15). On the day of necropsy, body weight was also recorded in order to calculate relative organ weight.

6.11.3 Food Consumption

For all cages, food consumption was recorded twice in Week 1 of administration, on Days 1 and 7 of administration, and thereafter once a week, every 7 days, prior to dosing (between 07:47 and 10:34) on each day. One day's food consumption on Day 1 was recorded on the starting day of administration from the previous day, 6 days' cumulative food consumption was recorded on Day 7, from Day 1 to Day 7, and thereafter 7 days' cumulative food consumption was recorded every 7 days to calculate one day's consumption per animal (each cage).

6.11.4 Ophthalmology

The examination was done before the start of the administration period (during the acclimation period) and after dosing in Week 13 (Day 90 for males and Day 89 for females) of administration. Before the start of the administration, all animals received were examined, and the animals with abnormalities which could affect the toxicological evaluation were excluded from group allocation [Note]. In Week 13 of administration, 6 males and 6 females in each group (intra-group numbers: 1 to 6) were examined. The procedure is shown below.

First, Mydrin P (Santen Pharmaceutical Co., Ltd., Lot No.: M499201) was applied to the eyes to dilate the pupils, and then the anterior portion, optic media and fundus oculi were observed using an indirect ophthalmoscope (Omega 500, HEINE Optotechnik GmbH & Co. KG).

Note: 8 males and 7 females with abnormalities which were judged to have a potential impact on the toxicological evaluation (hemorrhage of iris, synechia, persistent pupillary membrane, focal opacity in lens, persistent hyaloid artery, not observation in the transparent body, retinal fold, hyperreflectivity in fundus and not observation in the fundus.)

6.11.5 Urinalysis (including Water Intake)

Urinalysis was done in Week 13 (Day 87 and 88 for males and Day 86 and 87 for females) of administration.

All animals were individually accommodated in a steel cage with a urine collector after dosing on the day of urinalysis, and 4-hour urine samples were collected under deprivation of food but free access to water and then 20-hour urine samples were collected with free access to food and water. Items listed in the following Text Table 2 were examined.

In addition, one-day water intake from the previous day was measured using a water bottle for each animal (1 g = 1 mL).

Text Table 2. Items, Methods, Equipment, etc. in Urinalysis

1) Examination on 4-hour Urine	
Item	Method
pH	test strips Multistix[a)]
protein	test strips Multistix[a)]
ketones	test strips Multistix[a)]
glucose	test strips Multistix)[a)]
occult blood	test strips Multistix[a)]
bilirubin	test strips Multistix[a)]
urobilinogen	test strips Multistix[a)]
color	macroscopic examination
sediments	microscopic examination
urine volume (4-hour)[Note 1]	volumetry using a graduated centrifuge tube (unit: mL)
2) Examination on 20-hour Urine	
Item	Method
urine volume (20-hour)[Note 1]	volumetry using a graduated centrifuge tube (unit: mL)
osmotic pressure	freezing point method [b)] (unit: mOsm/kg)
sodium[Note 2]	ion selective electrode method [c)] (unit: mmol/24h)
potassium[Note 2]	ion selective electrode method [c)] (unit: mmol/24h)
chloride[Note 2]	ion selective electrode method [c)] (unit: mmol/24h)
Equipment used a): Urinary Chemical Analyzer Clinitek Advantus™ (Siemens Healthcare Diagnosis, Inc.) b): Automatic Osmometer OSMO STATION OM-6060 (Arkray, Inc.) c): Clinical Laboratory System TBA-120FR (Toshiba Medical Systems)	
Note 1: Urine volume (mL/24h) was calculated by totaling the amount of 4-hour and 20-hour urine. Note 2: One day's amount of excretion was calculated from the concentration determined on the 20-hr urine and the volume of 24-hr urine.	

6.11.6 Hematology

At the time of terminal necropsy on the day following the end of the administration period, all animals deprived of food overnight prior to blood collection were subjected to abdominal incision under isoflurane anesthesia and blood samples (approximately 1 mL) were collected from the abdominal aorta into blood collection tubes (SB-41: Sysmex Corp.) containing an anticoagulant (EDTA-2K). The following parameters in Text Table 3 1) were determined. For determining parameters described in Text Table 3 2), plasma obtained by centrifuging (set at room temperature, 2,380 ×g, for 10 minutes) the blood samples (0.9 mL) treated with 3.2 % sodium citrate tubes (3.2 % sodium citrate blood collection tubes: Insepack II-W Sekisui Medical co., Ltd., 1 vol sodium citrate solution/9 vol blood) that were separately collected in the same way was used. May-Grünwald-Giemsa stained-smears from all animals were prepared as reserve in case of microscopic examination, but they were not examined because there were no circumstances which required it.

Text Table 3. Items, Methods, Equipment, etc. of Hematology

1) Examination on EDTA-2K-treated blood samples		
Item	Method	Unit
red blood cell count (RBC)	dual angle laser flow-cytometric measurement[a)]	10^4(E4)/μL
hemoglobin (HGB)	modified cyanmethemoglobin method[a)]	g/dL
hematocrit (HCT)	calculated from RBC and MCV[a)]	%
mean corpuscular volume (MCV)	dual angle laser flow-cytometric measurement[a)]	fL
mean corpuscular hemoglobin (MCH)	calculated from RBC and HGB[a)]	pg
mean corpuscular hemoglobin concentration (MCHC)	calculated from HGB and HCT[a)]	g/dL
reticulocyte (Retic)	laser flow-cytometric measurement with RNA stain[a)]	10^9(E9)/L
platelet count (PLT)	dual angle laser flow-cytometric measurement[a)]	10^4(E4)/μL
white blood cell count (WBC)	dual angle laser flow-cytometric measurement[a)]	10^2(E2)/μL
differential leukocyte count[Note)]	peroxidase flow-cytometric measurement and dual angle laser flow-cytometric measurement[a)]	10^2(E2)/μL
2) Examination on plasma samples obtained from sodium citrate-treated blood samples		
Item	Method	Unit
prothrombin time (PT)	clot method[b)]	s
activated partial thromboplastin time (APTT)	clot method[b)]	s
Equipment used a): ADVIA2120i Hematology System (Siemens Healthcare Diagnostics Inc.) b): Coagulometer ACL Elite Pro (Instrumentation Laboratory)		
Note: Lymphocyte (LYMP), neutrophil (NEUT), eosinophil (EOS), basophil (BASO), monocyte (MONO) and large unstained cell (LUC).		

6.11.7 Blood Chemistry

Blood samples (approximately 2 mL) collected at the same time as for hematological examination were collected into test tubes containing heparin (Venoject II-Heparin Sodium, for 3 mL, Terumo Corporation). The blood sample was centrifuged (set at room temperature, 2,380 ×g, for 10 minutes) and the supernatant was re-centrifuged under the same conditions to obtain the plasma sample for analysis. The following items in Text Table 4 were determined.

Text Table 4. Items, Methods, Equipment, etc. of Blood Chemistry

Item	Method	Unit
AST	UV-rate method[a)]	IU/L
ALT	UV-rate method[a)]	IU/L
LDH	UV-rate method[a)]	IU/L
γ-GTP	L-γ-glutamyl-3-carboxy-4-nitroanilide method[a)]	IU/L
ALP	Bessey-Lowry method[a)]	IU/L
total cholesterol (T-CHO)	CEH-COD-POD method[a)]	mg/dL
triglyceride (TG)	LPL-GK-GPO-POD method[a)]	mg/dL
phospholipids (PL)	PLD-ChOD-POD method[a)]	mg/dL
total bilirubin (T-BIL)	bilirubin oxidase method[a)]	mg/dL
glucose (GLU)	glucose dehydrogenase method[a)]	mg/dL
blood urea nitrogen (BUN)	urease-LEDH method[a)]	mg/dL
creatinine (CRNN)	creatininase-creatinase-sarcosine oxidase-POD method[a)]	mg/dL
sodium (Na)	ion selective electrode method[a)]	mmol/L
potassium (K)	ion selective electrode method[a)]	mmol/L
chloride (Cl)	ion selective electrode method[a)]	mmol/L
calcium (Ca)	OCPC method[a)]	mg/dL
inorganic phosphorus (P)	Molybdic acid method[a)]	mg/dL
total protein (TP)	biuret method[a)]	g/dL
albumin (ALB)	BCG method[a)]	g/dL
A/G ratio (A/G)	calculated from total protein and albumin	
Equipment used a): Clinical Laboratory System TBA-120FR (Toshiba Medical Systems Corporation)		

6.11.8 Pathological Examination

6.11.8.1 Necropsy

At the terminal necropsy, all animals were sacrificed by exsanguination via the abdominal aorta after blood sampling, and the external appearance and all the organs and tissues in the cranial, thoracic and abdominal cavities were carefully examined macroscopically.

6.11.8.2 Organ Weights

For all animals, the following organs were weighed (absolute organ weight), and the relative organ weight per 100 g body weight was calculated based on the absolute organ weight and the animals' terminal body weight. Paired organs indicated by * were weighed separately, but evaluation was done on the total value of the right and left organs.

brain, pituitary, thyroid (including parathyroid)*, adrenal*, thymus, spleen, heart, lung

(including bronchus), salivary gland (submandibular + sublingual)*, liver, kidney*, testis*, prostate, seminal vesicle*, ovary* and uterus

6.11.8.3 Histopathology

All organs/tissues shown below were removed from all animals and fixed in phosphate buffered 10% formalin. However, eyeballs and optic nerves were fixed in 3% glutaraldehyde/2.5% formalin and the testes and epididymides were fixed in Bouin's solution and then preserved in phosphate buffered 10% formalin. All the organs/tissues were embedded in paraffin and sectioned, and hematoxylin and eosin (H & E)-stained specimens were prepared. Of these, all organs/tissues of animals in the control and high dose groups were examined microscopically. The results showed no test article-related changes were observed in any organs/tissue, thus histopathology examination in the low and middle dose groups was not conducted.

Paired organs indicated by * were removed bilaterally, but H&E-stained specimens and microscopy was performed unilaterally. For the parathyroid, preparation of histopathological specimen was done bilaterally together with the thyroid since the parathyroid was occasionally not found on the slide due to its small size, and microscopic examination was performed unilaterally.

cerebrum, cerebellum, spinal cord (thoracic), sciatic nerve*, eyeball*, optic nerve*, harderian gland*, pituitary, thyroid*, parathyroid*, adrenal*, thymus, spleen, submandibular lymph node, mesenteric lymph node, heart, thoracic aorta, trachea, lung (including bronchus), tongue, esophagus, stomach, duodenum, jejunum, ileum (including Peyer's patch), cecum, colon, rectum, submandibular gland*, sublingual gland*, liver, pancreas, kidney*, urinary bladder, testis*, epididymis*, prostate, seminal vesicle*, ovary*, uterus, oviduct*, vagina, mammary gland (inguinal)*, sternum (including bone marrow), femur (including bone marrow)*, femoral skeletal muscle*, skin (inguinal)*, nasal cavity and zymbal gland*

Other than the above, the site for animal identification (ear auricle attached ear tag) and larynx were removed and preserved.

The parathyroid of either side was not found on the section of 1 male in the low dose group and 1 female in the middle and high dose group each; however, it was judged to have no effect for study evaluation since the number of cases for evaluation were assured and there were no test article-related effects in the parathyroid and no target organ.

6.11.9 Statistical Analysis

Numerical data including body weight, food consumption, water consumption in the urinalysis, quantitative data of urinalysis, hematology, blood chemistry and organ weight were calculated mean ± standard deviation and assessed statistically according to the procedures described below. First, an analysis of variance was conducted by the Bartlett test [2] (significance level: 1%). When the data were homogeneous, Dunnett's test[3, 4] was applied to compare between the control and dose groups (significance levels: 5% and 1%, two-tailed). When the data were heterogeneous, Steel's test [5] was applied (significance levels: 5% and 1%, two-tailed). All analyses were performed by SAS Release 9.1.3 (SAS Institute Inc.).

7. Results

7.1 Mortality

No deaths occurred in any group in either sex.

7.2 Clinical Signs

The results are shown in Tables 1-1 to 1-14 and Appendices 1-1 to 1-56.

No abnormal signs were observed in any animal in either sex.

7.3 Body Weight

The results are shown in Figures 1 and 2, Tables 2-1 to 2-4 and Appendices 2-1 to 2-8.

No treatment-related changes were observed in any group in either sex.

7.4 Food Consumption

The results are shown in Figures 3 and 4, Tables 3-1 to 3-4 and Appendices 3-1 to 3-8.

No treatment-related changes were observed in any group in either sex.

7.5 Ophthalmology

The results are shown in Tables 4-1 and 4-2 and Appendices 4-1 to 4-8.

No treatment-related changes were observed in any group in either sex.

Hyper-reflectivity in the fundus was observed in 1 female at 500 mg/kg/day; however, this was an incidental change because it was not dose-related.

7.6 Urinalysis (including Water Consumption)

The results are shown in Tables 5-1 to 5-18 and Appendices 5-1 to 5-24.

No treatment-related changes were observed in any group in either sex.

A statistically significant increase in chloride were recorded in males at 2000 mg/kg/day and a statistically significant increase in potassium were recorded in males at 500 and 2000 mg/kg/day; however, it was judged to be of no toxicological significance because there were minimal changes and there were no toxic changes in chloride and potassium in the plasma.

7.7 Hematology

The results are shown in Tables 6-1 to 6-8 and Appendices 6-1 to 6-24.

No treatment-related changes were observed in any group in either sex.

A statistically significant decrease in reticulocytes was recorded in females at 500 and 1000 mg/kg/day and a statistically significant decrease in hematocrit was recorded in females at 1000 mg/kg/day; however, they were incidental changes because they were not dose-related.

7.8 Blood Chemistry

The results are shown in Tables 7-1 to 7-8 and Appendices 7-1 to 7-16.

No treatment-related changes were observed in any group in either sex.

A statistically significant decrease in chloride and increase in phosphate were recorded in males at 2000 mg/kg/day; however, they were judged to be no toxicological significance since the individual values were mostly within the historical control data (chloride; N=49: Mean 106 mmol/L, Mean±2SD 104~108 mmol/L, P; N=49: Mean 5.5 mg/dL, Mean±2SD 4.7~6.3 mg/dL).

7.9 Organ Weights

The results are shown in Tables 8-1 to 8-4 and Appendices 8-1 to 8-24 (absolute weight), Tables 9-1 to 9-4 and Appendices 9-1 to 9-24 (relative weight).

No treatment-related changes were observed in any group in either sex.

A statistically significant increase in relative weight of liver was recorded in females at 2000 mg/kg/day; however, it was a minimal change only in the relative weight.

7.10 Necropsy

The results are shown in Tables 10-1 and 10-2 and Appendices 10-1 to 10-96.

No treatment-related changes were observed in either sex.

The gross findings observed in Tables and Appendices were judged to be incidental based on their incidence.

7.11 Histopathological Examination

The results are shown in Tables 11-1 to 11-9 and Appendices 10-1 to 10-96.

No treatment-related changes were observed in either sex.

The histopathological findings observed in Tables and Appendices were judged to be incidental because there was no bias in their incidence or histopathological aspects.

8. Discussion

The toxicity profile of β-galactosidase concentrate was examined when the test article was orally administered to Sprague-Dawley rats [Crl:CD(SD), 12/sex/group, started at 6 weeks of age] for 13 weeks. The dose solutions at dose levels of 500, 1000 and 2000 mg/kg/day were administered by oral gavage once daily at a dose volume of 20 mL/kg for 13 weeks. A control group was provided and the animals received the vehicle, water for injection.
Observation of clinical signs, body weight and food consumption, ophthalmology and urinalysis (including water consumption), hematology, blood chemistry, necropsy, organ weights, and histopathology were performed.

As a result, no deaths occurred in any group and no test article-related toxic changes were noted in clinical signs, body weight and food consumption, ophthalmology, urinalysis (including water consumption), hematology, blood chemistry, necropsy, organ weight and histopathology.

Based on the results described above, repeated oral dosing of β-galactosidase concentrate in rats for 13 weeks showed no effects observed in any examinations. Therefore, the no-observed-adverse-effect level (NOAEL) of β-galactosidase concentrate was considered to be 2000 mg/kg/day in both sexes under the test conditions.

9. References

1) Yafune A.: A 2-week oral gavage toxicity preliminary study of β-galactosidase concentrates in rats. (BoZo Research Center Inc., Study number: C-TT160001), 2016.
2) Snedecor GW, Cochran WG. Statistical methods, 8th ed. Ames: Iowa State University Press; 1989.
3) Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc 1955; 50: 1096-121.
4) Dunnett CW. New tables for multiple comparisons with a control. Biometrics 1964; 20: 482-91.
5) Steel RGD. A multiple comparison rank sum test: Treatments versus control. Biometrics 1959; 15: 560-72.

TT-160003
Attached Data 1

Test Material Data Sheet

Identity	:	β –Galactosidase concentrate
Specification	:	β –Galactosidase
Origin	:	*Cryptococcus terrestris*
Lot No.	:	GFE68-001@K
Appearance	:	Pale yellowish brown powder
Activity	:	2,550 u/g （β –Galactosidase activity, Lactose substrate method)
Loss on drying	:	4.9 % (Drying method, 100g, 105°C)
Ash	:	5.1 % (JSFA* method)
Lead	:	Not more than 1 μg/g (FCC method)
Total viable aerobic count	:	1.8 x 10^2 cfu/g (SCD Agar plate method)
Coliforms	:	< 3.0 CFU/g (FDA BAM)
Escherichia coli	:	Negative/25g (FDA BAM)
Salmonella	:	Negative/25g (FDA BAM)
Antibiotic activity	:	Negative (JECFA method)
Storage conditions	:	Refrigeration
Date of production	:	February 13, 2016
Expiration date	:	February 12, 2017

*: Japanese Standards of Food Additives

(b) (6)

Atsushi Mizutani April 8, 2016

Quality Assurance Dept., Amano Enzyme Inc.

TT-160003
Attached Data 2

Stability of the test material during examination period

Test No. : TT160003
Test material : β-Galactosidase concentrate
Lot No. : GFE68-001@K
Assay parameter : β-Galactosidase activity, Lactose substrate method
Assayers : Masamichi Okada / R & D Dept., Amano Enzyme Inc.
Date of assays : 2016/3/4 (Before), 2016/ 12/12 (After)
Storage condition : Refrigaration

Results :

		Activity (u/g)	Residual activity[a] (%)
Before		2,550	–
After	TU160164-1	2,330	91
	TU160164-2	2,320	91
	TU160164-3	2,360	93
	TU160164-4	2,410	95
	TU160164-5	2,450	96
	TU160164-6	2,420	95
	TU160164-7	2,520	99

a) : acceptable range is 100±15%

Conclusion :

The test material was stable during the examination period.

(b) (6)

Atsushi Mizutani December 13, 2016
Quality Assurance Dept., Amano Enzyme Inc.

Stability and Homogeneity of Test Material

Test material : β-Galactosidase concentrate
Lot No. : GFE68-001@K
Assay Parameter : β-Galactosidase activity, Lactose substrate method
Assayer : Masamichi Okada / R & D Dept.
Date of assay : March 7, 2016

Results :

	Room temperature						Ice cold					
Concentration	0hrs		3hrs		5hrs		0hrs		3hrs		5hrs	
25mg/mL	1	59	1	62	1	63	1	59	1	61	1	62
	2	61	2	63	2	63	2	61	2	61	2	62
	3	60	3	61	3	62	3	60	3	61	3	61
	Average	60	Average	62	Average	63	Average	60	Average	61	Average	62
	S.D.	1.0	S.D.	1.0	S.D.	0.6	S.D.	1.0	S.D.	0.0	S.D.	0.6
	R. A.*	100	R. A.	103	R. A.	104	R. A.	100	R. A.	102	R. A.	103
	C.V.**	1.7	C.V.	1.6	C.V.	0.9	C.V.	1.7	C.V.	0.0	C.V.	0.9
50mg/mL	1	125	1	121	1	125	1	125	1	121	1	121
	2	124	2	125	2	124	2	124	2	122	2	123
	3	120	3	122	3	125	3	120	3	122	3	122
	Average	123	Average	123	Average	125	Average	123	Average	122	Average	122
	S.D.	2.6	S.D.	2.1	S.D.	0.6	S.D.	2.6	S.D.	0.6	S.D.	1.0
	R. A.	100	R. A.	100	R. A.	101	R. A.	100	R. A.	99	R. A.	99
	C.V.	2.2	C.V.	1.7	C.V.	0.5	C.V.	2.2	C.V.	0.5	C.V.	0.8
100mg/mL	1	249	1	250	1	246	1	249	1	244	1	245
	2	249	2	250	2	245	2	249	2	245	2	248
	3	249	3	246	3	243	3	249	3	246	3	243
	Average	249	Average	249	Average	245	Average	249	Average	245	Average	245
	S.D.	0.0	S.D.	2.3	S.D.	1.5	S.D.	0.0	S.D.	1.0	S.D.	2.5
	R. A.	100	R. A.	100	R. A.	98	R. A.	100	R. A.	98	R. A.	99
	C.V.	0.0	C.V.	0.9	C.V.	0.6	C.V.	0.0	C.V.	0.4	C.V.	1.0

* : Percentage of residual activity (vs. 0 hours, Acceptable range: 100±10%)
** : Coefficient of variation (Acceptable range: ≦10%)

Conclusion : Test material was stable and homogenous in each dilutions or storage periods under both room temperature and ice cold.

(b) (6)

Atsushi Mizutani December 21, 2016
Quality Assurance Dept. Amano Enzyme Inc.

TT-160003
Attached Data 3-2

Stability and Homogeneity of Test Material (long term)

Test material : β-Galactosidase concentrate
Lot No. : GFE68-001@K
Assay Parameter : β-Galactosidase activity, Lactose substrate method
Assayer : Masamichi Okada / R & D Dept.
Date of assay : March 4, 2016

Results :

	Refregaration (4℃)					
Concentration	0 day		3 day		7 day	
25mg/mL	1	59	1	60	1	63
	2	61	2	61	2	62
	3	61	3	62	3	63
	Average	60	Average	61	Average	63
	S.D.	1.2	S.D.	1.0	S.D.	0.6
	R. A.*	100	R. A.	101	R. A.	104
	C.V.**	1.9	C.V.	1.6	C.V.	0.9
50mg/mL	1	118	1	124	1	123
	2	120	2	125	2	124
	3	121	3	128	3	122
	Average	120	Average	126	Average	123
	S.D.	1.5	S.D.	2.1	S.D.	1.0
	R. A.	100	R. A.	105	R. A.	103
	C.V.	1.3	C.V.	1.7	C.V.	0.8
100mg/mL	1	226	1	249	1	245
	2	231	2	250	2	246
	3	223	3	245	3	248
	Average	227	Average	248	Average	246
	S.D.	4.0	S.D.	2.6	S.D.	1.5
	R. A.	100	R. A.	109	R. A.	109
	C.V.	1.8	C.V.	1.1	C.V.	0.6

* : Percentage of residual activity (vs. 0 hours, Acceptable range: 100±10%)
** : Coefficient of variation (Acceptable range: ≦10%)

Conclusion : Test material was stable and homogenous in each dilutions or storage p
under refregaration.

(b) (6)

Atsushi Mizutani / July 28, 2016
Quality Assurance Dept. Amano Enzyme Inc.

TT-160003
Attached Data 3-3

Stability and Homogeneity of Test Material by Freezing (−20℃) and Thawing

Test material : β−Galactosidase concentrate
Lot No. : GFE68−001@K
Assay Parameter : β−Galactosidase activity, Lactose substrate method
Assayer : Masamichi Okada / R & D Dept.
Date of assay : March 4, 2016

Results :

	Number of cycles							
Concentration	0		1		2		3	
25mg/mL	1	59	1	59	1	60	1	60
	2	61	2	62	2	62	2	61
	3	61	3	61	3	61	3	64
	Average	60	Average	61	Average	61	Average	62
	S.D.	1.2	S.D.	1.5	S.D.	1.0	S.D.	2.1
	R. A.*	100	R. A.	101	R. A.	101	R. A.	102
	C.V.**	1.9	C.V.	2.5	C.V.	1.6	C.V.	3.4
50mg/mL	1	118	1	118	1	124	1	121
	2	120	2	121	2	123	2	118
	3	121	3	119	3	123	3	122
	Average	120	Average	119	Average	123	Average	120
	S.D.	1.5	S.D.	1.5	S.D.	0.6	S.D.	2.1
	R. A.	100	R. A.	100	R. A.	103	R. A.	101
	C.V.	1.3	C.V.	1.3	C.V.	0.5	C.V.	1.7
100mg/mL	1	226	1	245	1	248	1	250
	2	231	2	249	2	249	2	249
	3	223	3	249	3	241	3	244
	Average	227	Average	248	Average	246	Average	248
	S.D.	4.0	S.D.	2.3	S.D.	4.4	S.D.	3.2
	R. A.	100	R. A.	109	R. A.	109	R. A.	109
	C.V.	1.8	C.V.	0.9	C.V.	1.8	C.V.	1.3

* : Percentage of residual activity (vs. 0 hours, Acceptable range: 100±10%)
** : Coefficient of variation (Acceptable range: ≦10%)

eriods

Conclusion : Test material was stable and homogenous in each dilutions at least 3 times freezing and thawing cycle.

(b) (6)

Atsushi Mizutani July 28, 2016
Quality Assurance Dept. Amano Enzyme Inc.

TT-160003
Attached Data 4-1

Content of the test article in the test solutions

Test No.	: TT-160003
Test material	: Solutions of β-Galactosidase concentrate (25, 50 and 100 mg/mL)
Solvent	: Water for injection
Assay parameter	: β-Galactosidase activity (Lactose substrate method)
Assayers	: Masamichi Okada / R&D Dept., Amano Enzyme Inc.
Date of acceptance	: 2016/ 6/ 28
Date of assays	: 2016/ 6/ 29
Storage condition	: Refrigeration

Results :

Concentration (mg/mL)	Activity (u/mL)			Residual activity[e] (%)
	Original value[a]	Measured value		
25	60	57 58 55	57[b] 1.5[c] 2.7[d]	95.0
50	123	113 118 114	115 2.6 2.3	93.5
100	249	235 238 239	237 2.1 0.9	95.2

a) : Measured on March 7, 2016 b) : Mean c) : Standard deviation
d) : Coefficient of variation (Acceptable range: ≦10%)
e) : Acceptable range is 100±10%

Each test solution was homogenous and included the correct amount of the test material specified in the protocol.

(b) (6)

Atsushi Mizutani December 21, 2016
Quality Assurance Dept.
Amano Enzyme Inc.

Content of the test article in the test solutions

Test No. : TT-160003
Test material : Solutions of β-Galactosidase concentrate (25, 50 and 100 mg/mL)
Solvent : Water for injection
Assay parameter : β-Galactosidase activity (Lactose substrate method)
Assayers : Masamichi Okada / R&D Dept., Amano Enzyme Inc.
Date of acceptance : 2016/ 9/ 21
Date of assays : 2016/ 11/ 7
Storage condition : Refrigeration

Results :

Concentration (mg/mL)	Activity (u/mL)			Residual activity[e] (%)
	Original value[a]	Measured value		
25	60	61	62[b]	103.3
		62	0.6[c]	
		62	0.9[d]	
50	123	120	123	100.0
		124	2.6	
		125	2.2	
100	249	241	240	96.4
		242	2.6	
		237	1.1	

a) : Measured on March 7, 2016b) : Mean c) : Standard deviation
d) : Coefficient of variation (Acceptable range: ≦10%)
e) : Acceptable range is 100±10%

Each test solution was homogenous and included the correct amount of the test material specified in the protocol.

(b) (6)

Atsushi Mizutani November 16, 2016
Quality Assurance Dept.
Amano Enzyme Inc.

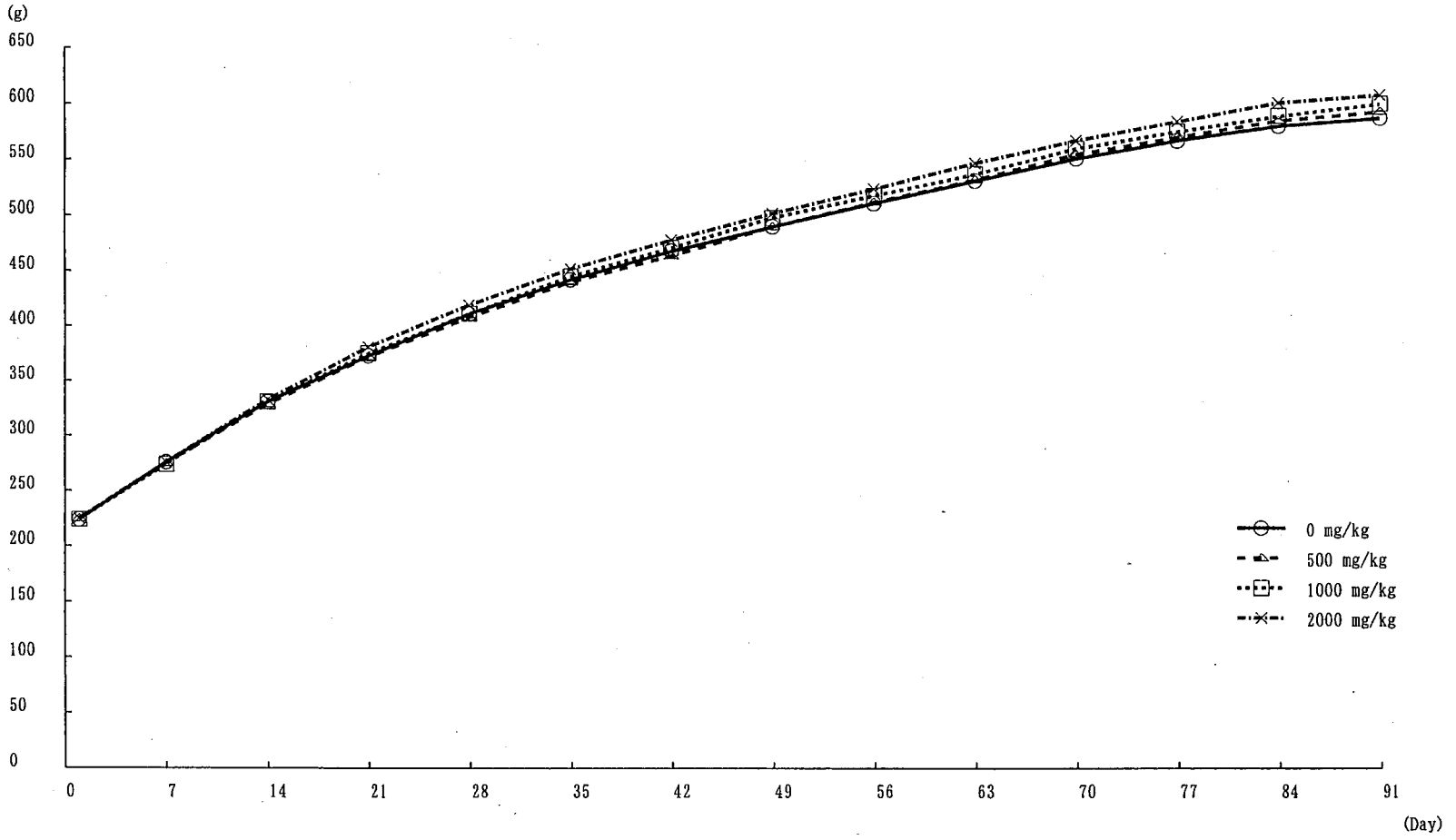


Fig. 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats
Body Weight - Male

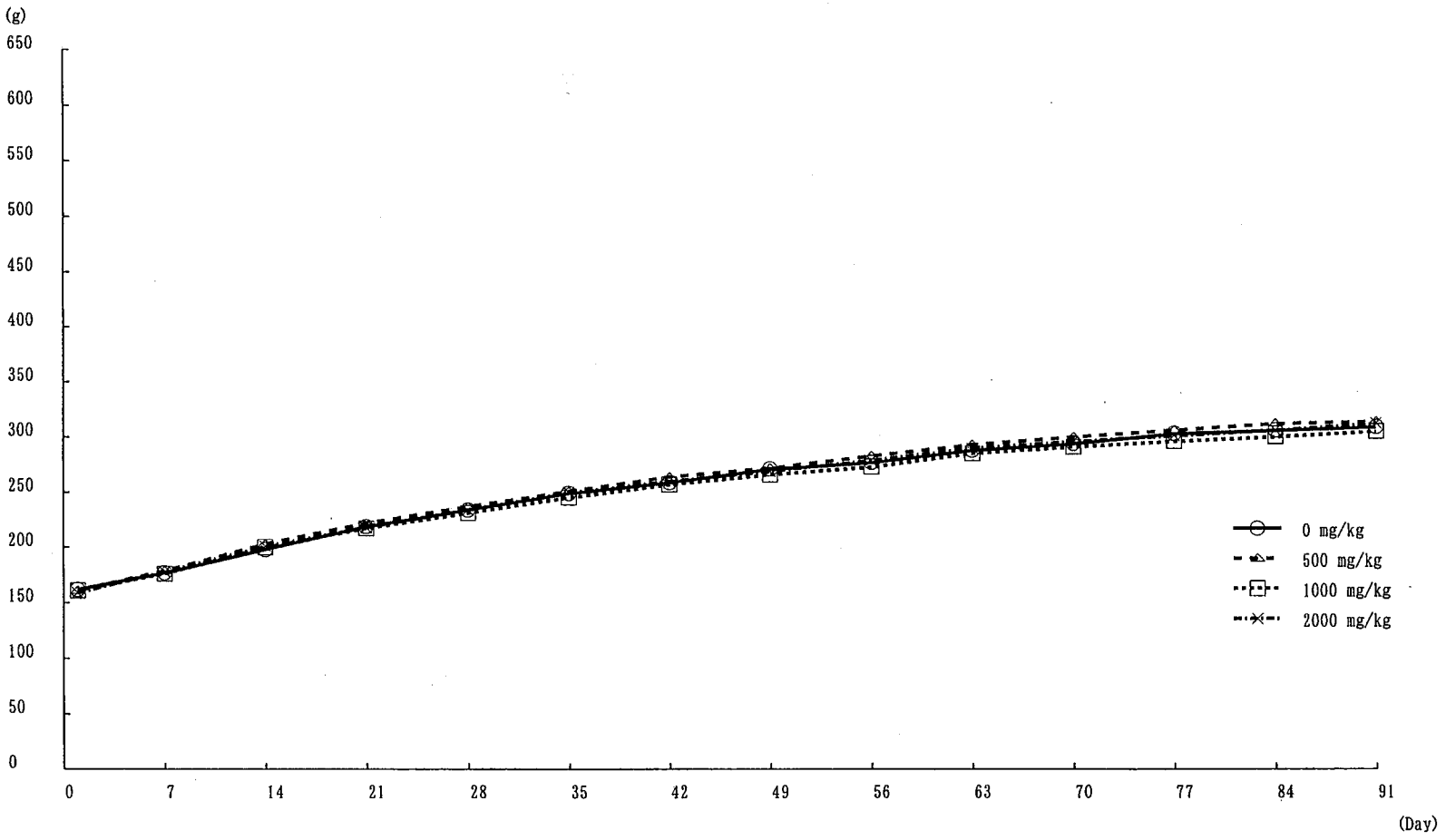


Fig. 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats
Body Weight - Female

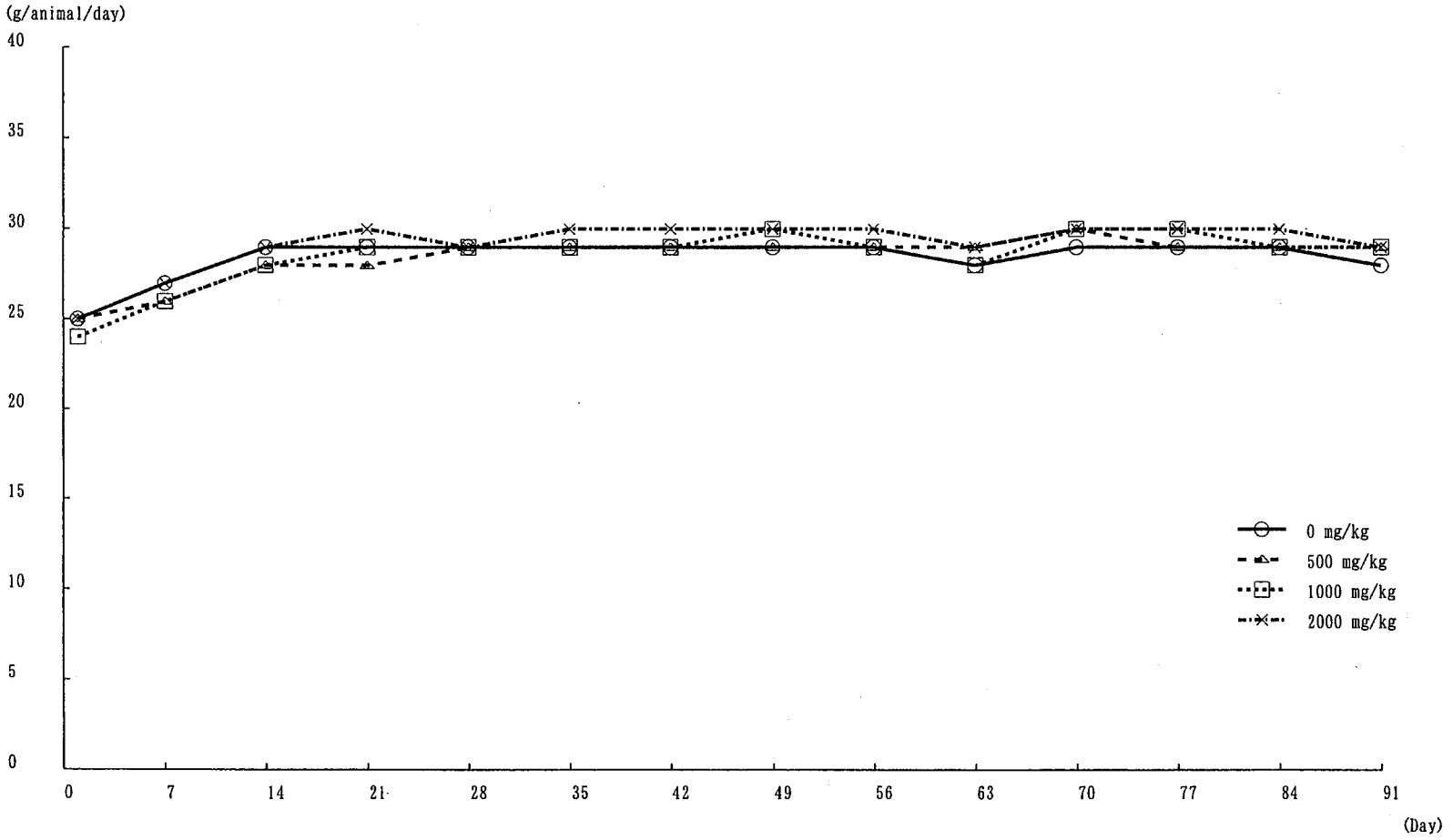


Fig. 3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats
Food Consumption - Male

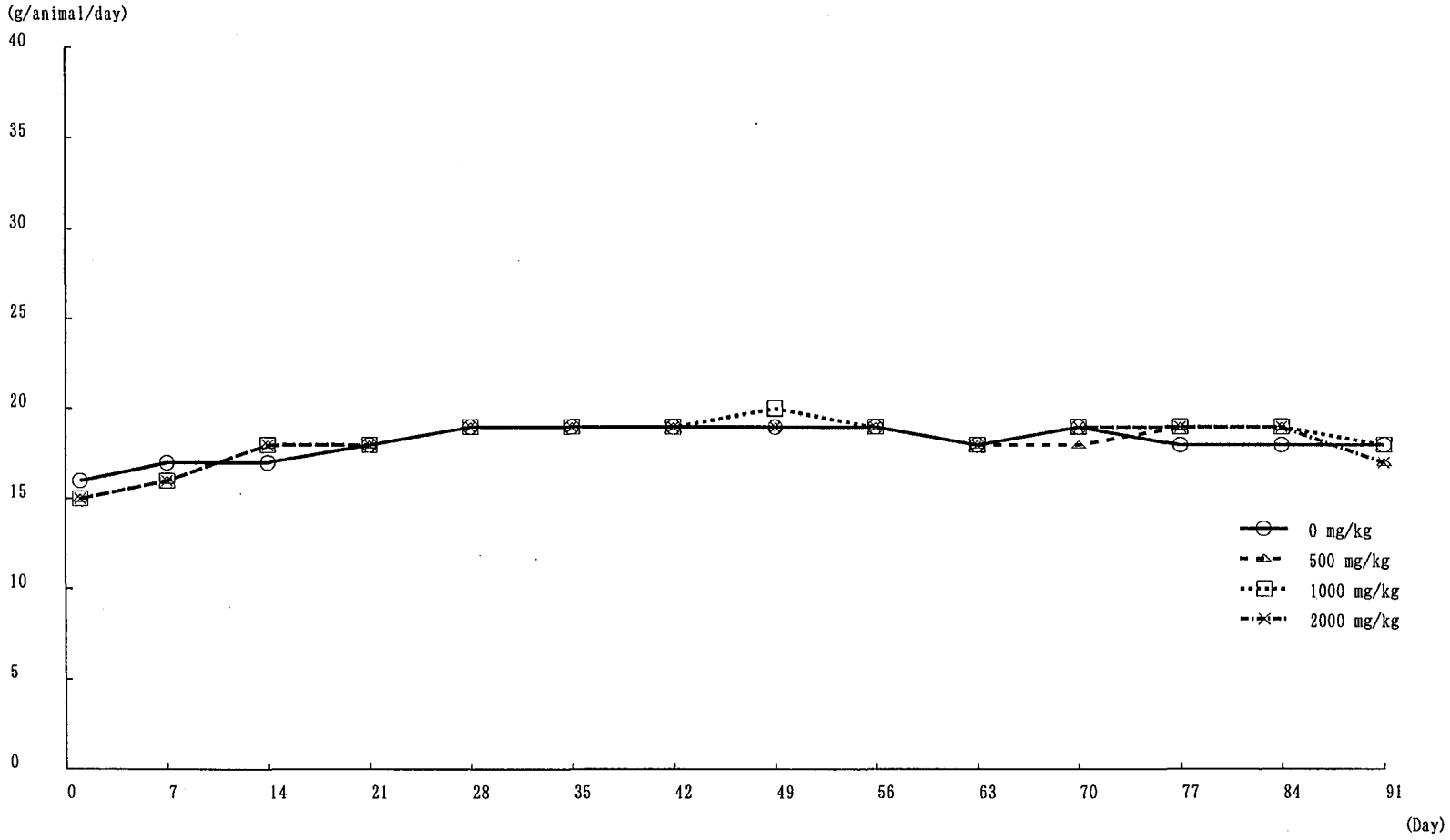


Fig. 4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats
Food Consumption - Female

Table 1-1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Male

Dose mg/kg	Findings	Day of administration 1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Male

Dose mg/kg	Findings	Day of administration													
		15	16	17	18	19	20	21	22	23	24	25	26	27	28
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Male

Dose mg/kg	Findings	Day of administration													
		29	30	31	32	33	34	35	36	37	38	39	40	41	42
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Male

Dose mg/kg	Findings	Day of administration													
		43	44	45	46	47	48	49	50	51	52	53	54	55	56
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-5 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Male

Dose mg/kg	Findings	Day of administration													
		57	58	59	60	61	62	63	64	65	66	67	68	69	70
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-6 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Male

Dose mg/kg	Findings	Day of administration													
		71	72	73	74	75	76	77	78	79	80	81	82	83	84
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-7 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Male

Dose mg/kg	Findings	Day of administration 85	86	87	88	89	90	91	92#
0	n	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12

#:Day of necropsy

Table 1-8 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Female

Dose mg/kg	Findings	Day of administration 1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-9 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Female

Dose mg/kg	Findings	Day of administration													
		15	16	17	18	19	20	21	22	23	24	25	26	27	28
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-10 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Female

Dose mg/kg	Findings	Day of administration													
		29	30	31	32	33	34	35	36	37	38	39	40	41	42
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-11 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Female

Dose mg/kg	Findings	Day of administration													
		43	44	45	46	47	48	49	50	51	52	53	54	55	56
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-12 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Female

Dose		Day of administration													
mg/kg	Findings	57	58	59	60	61	62	63	64	65	66	67	68	69	70
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-13 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Female

Dose mg/kg	Findings	Day of administration													
		71	72	73	74	75	76	77	78	79	80	81	82	83	84
0	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Table 1-14 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Clinical signs
Female

Dose mg/kg	Findings	Day of administration 85	86	87	88	89	90	91	92#
0	n	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12
500	n	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12
1000	n	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12
2000	n	12	12	12	12	12	12	12	12
	No abnormal findings	12	12	12	12	12	12	12	12

#:Day of necropsy

Table 2 - 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Body weight
Sex : Male Unit : g

Test Article Dose	Day	1	7	14	21	28	35	42	49	56
β-Galactosidase 0 mg/kg	Mean	224	276	331	373	411	442	468	490	511
	S.D.	8	11	16	21	27	32	36	38	40
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase 500 mg/kg	Mean	225	275	329	372	408	440	464	490	512
	S.D.	9	15	22	30	35	40	43	47	49
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase 1000 mg/kg	Mean	224	274	331	375	411	445	471	498	518
	S.D.	10	15	24	33	40	45	49	53	54
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase 2000 mg/kg	Mean	225	276	333	381	419	452	478	502	524
	S.D.	7	10	14	18	23	27	32	35	37
	n	12	12	12	12	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 2 - 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Body weight
Sex : Male Unit : g

Test Article Dose	Day	63	70	77	84	91
β-Galactosidase 0 mg/kg	Mean	531	551	567	580	587
	S.D.	42	45	46	49	48
	n	12	12	12	12	12
β-Galactosidase 500 mg/kg	Mean	532	555	570	585	593
	S.D.	51	55	54	58	57
	n	12	12	12	12	12
β-Galactosidase 1000 mg/kg	Mean	537	560	575	589	600
	S.D.	60	60	62	64	68
	n	12	12	12	12	12
β-Galactosidase 2000 mg/kg	Mean	547	567	584	601	608
	S.D.	39	40	43	44	44
	n	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 2 - 3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Body weight
Sex : Female
Unit : g

Test Article Dose	Day	1	7	14	21	28	35	42	49	56
β-Galactosidase 0 mg/kg	Mean	162	177	198	219	234	249	259	271	277
	S.D.	9	8	11	12	17	17	21	22	23
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase 500 mg/kg	Mean	161	179	203	222	237	251	264	272	283
	S.D.	9	13	16	21	26	32	31	36	38
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase 1000 mg/kg	Mean	161	176	200	217	231	245	257	266	273
	S.D.	9	13	18	22	24	26	25	31	32
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase 2000 mg/kg	Mean	159	178	201	218	234	250	260	269	279
	S.D.	11	13	15	17	18	17	21	21	22
	n	12	12	12	12	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 2 - 4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Body weight
Sex : Female Unit : g

Test Article Dose	Day	63	70	77	84	91
β-Galactosidase 0 mg/kg	Mean	288	294	303	306	309
	S.D.	27	30	31	32	34
	n	12	12	12	12	12
β-Galactosidase 500 mg/kg	Mean	293	300	306	312	314
	S.D.	41	41	42	43	45
	n	12	12	12	12	12
β-Galactosidase 1000 mg/kg	Mean	285	291	296	300	305
	S.D.	33	31	35	34	36
	n	12	12	12	12	12
β-Galactosidase 2000 mg/kg	Mean	290	296	301	306	312
	S.D.	19	19	20	19	21
	n	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 3 - 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Food consumption
Sex : Male Unit : g/animal/day

Test Article Dose	Day	1	7	14	21	28	35	42	49	56
β-Galactosidase 0 mg/kg	Mean	25	27	29	29	29	29	29	29	29
	S.D.	1	1	1	2	2	2	2	2	2
	n	6	6	6	6	6	6	6	6	6
β-Galactosidase 500 mg/kg	Mean	25	26	28	28	29	29	29	29	29
	S.D.	1	1	1	2	2	2	1	1	1
	n	6	6	6	6	6	6	6	6	6
β-Galactosidase 1000 mg/kg	Mean	24	26	28	29	29	29	29	30	29
	S.D.	1	1	2	3	3	3	3	3	2
	n	6	6	6	6	6	6	6	6	6
β-Galactosidase 2000 mg/kg	Mean	25	27	29	30	29	30	30	30	30
	S.D.	2	1	1	2	2	2	3	3	3
	n	6	6	6	6	6	6	6	6	6

No significant difference in any treated groups from control group.

Table 3 - 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Food consumption
Sex : Male Unit : g/animal/day

Test Article Dose	Day	63	70	77	84	91
β-Galactosidase 0 mg/kg	Mean	28	29	29	29	28
	S.D.	2	2	2	3	2
	n	6	6	6	6	6
β-Galactosidase 500 mg/kg	Mean	29	30	29	29	29
	S.D.	1	1	0	1	1
	n	6	6	6	6	6
β-Galactosidase 1000 mg/kg	Mean	28	30	30	29	29
	S.D.	3	2	2	3	2
	n	6	6	6	6	6
β-Galactosidase 2000 mg/kg	Mean	29	30	30	30	29
	S.D.	2	2	3	2	2
	n	6	6	6	6	6

No significant difference in any treated groups from control group.

Table 3 - 3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Food consumption
Sex : Female Unit : g/animal/day

Test Article Dose	Day	1	7	14	21	28	35	42	49	56
β-Galactosidase 0 mg/kg	Mean	16	17	17	18	19	19	19	19	19
	S.D.	1	1	1	0	1	1	1	1	1
	n	6	6	6	6	6	6	6	6	6
β-Galactosidase 500 mg/kg	Mean	15	16	18	18	19	19	19	19	19
	S.D.	2	1	1	2	2	2	2	2	2
	n	6	6	6	6	6	6	6	6	6
β-Galactosidase 1000 mg/kg	Mean	15	16	18	18	19	19	19	20	19
	S.D.	2	2	2	2	1	1	2	1	1
	n	6	6	6	6	6	6	6	6	6
β-Galactosidase 2000 mg/kg	Mean	15	16	18	18	19	19	19	19	19
	S.D.	1	1	1	1	1	1	1	1	1
	n	6	6	6	6	6	6	6	6	6

No significant difference in any treated groups from control group.

Table 3 - 4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Food consumption
Sex : Female Unit : g/animal/day

Test Article Dose	Day	63	70	77	84	91
β-Galactosidase 0 mg/kg	Mean	18	19	18	18	18
	S.D.	1	1	1	1	2
	n	6	6	6	6	6
β-Galactosidase 500 mg/kg	Mean	18	18	19	19	17
	S.D.	2	2	2	2	2
	n	6	6	6	6	6
β-Galactosidase 1000 mg/kg	Mean	18	19	19	19	18
	S.D.	1	1	1	1	1
	n	6	6	6	6	6
β-Galactosidase 2000 mg/kg	Mean	18	19	19	19	17
	S.D.	1	1	1	1	1
	n	6	6	6	6	6

No significant difference in any treated groups from control group.

Table 4 - 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Ophthalmology (Week 13)
Sex : Male

Findings	Dose (mg/kg)	0	500	1000	2000
	No. of animals	6	6	6	6
Ophthalmoscopy					
No abnormality		6	6	6	6

Table 4 - 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Ophthalmology (Week 13)
Sex : Female

Findings	Dose (mg/kg)	0	500	1000	2000
	No. of animals	6	6	6	6
Ophthalmoscopy					
No abnormality		6	5	6	6
Fundus oculi					
Hyperreflectivity in fundus, focal		0	1	0	0

Table 5 - 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		pH										Protein					
			5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0≤		-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean																
	S.D.																
	n	12	0	0	0	0	0	0	0	5	7	12	1	7	4	0	0
β-Galactosidase 500 mg/kg	Mean																
	S.D.																
	n	12	0	0	0	0	0	1	2	9	0	12	5	6	1	0	0
β-Galactosidase 1000 mg/kg	Mean																
	S.D.																
	n	12	0	0	0	0	0	0	0	8	4	12	3	5	3	1	0
β-Galactosidase 2000 mg/kg	Mean																
	S.D.																
	n	12	0	0	0	0	0	0	5	7	0	12	3	7	2	0	0

Protein) -:Negative, +/-:15, 1+:30, 2+:100, 3+:≥300 mg/dL

Table 5 - 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		Ketones	-	+/-	1+	2+	3+	Glucose	-	1+	2+	3+	4+
β-Galactosidase 0 mg/kg	Mean												
	S.D.												
	n	12	4	7	1	0	0	12	12	0	0	0	0
β-Galactosidase 500 mg/kg	Mean												
	S.D.												
	n	12	10	2	0	0	0	12	12	0	0	0	0
β-Galactosidase 1000 mg/kg	Mean												
	S.D.												
	n	12	6	5	1	0	0	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean												
	S.D.												
	n	12	7	5	0	0	0	12	12	0	0	0	0

Ketones) -:Negative, +/-:5, 1+:15, 2+:40, 3+:80 mg/dL
Glucose) -:Negative, 1+:100, 2+:250, 3+:500, 4+:≥1000 mg/dL

Table 5 - 3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		Oc.Blood						Urobili.					Bilirubin				
			-	+/-	1+	2+	3+		+/-	1+	2+	3+		-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean																
	S.D.																
	n	12	7	5	0	0	0	12	12	0	0	0	12	12	0	0	0
β-Galactosidase 500 mg/kg	Mean																
	S.D.																
	n	12	4	7	1	0	0	12	12	0	0	0	12	12	0	0	0
β-Galactosidase 1000 mg/kg	Mean																
	S.D.																
	n	12	4	7	1	0	0	12	12	0	0	0	12	12	0	0	0
β-Galactosidase 2000 mg/kg	Mean																
	S.D.																
	n	12	3	9	0	0	0	12	12	0	0	0	12	12	0	0	0

Oc.Blood) -:Negative, +/-:0.015, 1+:0.062, 2+:0.135, 3+:0.405 mg/dL
Urobili.) +/-:0.1~1.0, 1+:2.0, 2+:4.0, 3+:≥8.0 Ehrlich U/dL
Bilirubin) -:Negative, 1+:0.8, 2+:1.6, 3+:3.2 mg/dL
Oc.Blood : Occult blood Urobili. : Urobilinogen

Table 5 - 4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		Color					RBC					
			LY	Y	DY	Other		-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean											
	S.D.											
	n	12	0	12	0	0	12	12	0	0	0	0
β-Galactosidase 500 mg/kg	Mean											
	S.D.											
	n	12	0	12	0	0	12	12	0	0	0	0
β-Galactosidase 1000 mg/kg	Mean											
	S.D.											
	n	12	0	12	0	0	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean											
	S.D.											
	n	12	0	12	0	0	12	12	0	0	0	0

Color) LY:Light yellow, Y:Yellow, DY:Dark yellow, Other:Other color
RBC) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe

Table 5 - 5 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		WBC	-	+/-	1+	2+	3+	Ep.SEC	-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	0	12	0	0	0
β-Galactosidase 500 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	2	10	0	0	0
β-Galactosidase 1000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	1	11	0	0	0
β-Galactosidase 2000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	1	11	0	0	0

WBC) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Ep.SEC) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Ep.SEC : Squamous epithelial cells

Table 5 - 6 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		Ep.SREC	-	+/-	1+	2+	3+	Ep.Oth.	-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 500 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 1000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0

Ep.SREC) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Ep.Oth.) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Ep.SREC : Small round epithelial cells Ep.Oth. : Epithelial others

Table 5 - 7 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		Cast	-	+/-	1+	2+	3+	Cr.PS	-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	10	2	0	0	0
β-Galactosidase 500 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	11	1	0	0	0
β-Galactosidase 1000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0

Cast) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Cr.PS) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Cr.PS : Crystal phosphate salts

Table 5 - 8 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		Cr.CO	-	+/-	1+	2+	3+	Cr.Oth.	-	+/-	1+	2+	3+	Uri.Vol. mL/24h	W.C. mL/24h
β-Galactosidase 0 mg/kg	Mean													16.5	31
	S.D.													5.3	7
	n	12	12	0	0	0	0	12	12	0	0	0	0	12	12
β-Galactosidase 500 mg/kg	Mean													21.4	37
	S.D.													6.0	9
	n	12	12	0	0	0	0	12	12	0	0	0	0	12	12
β-Galactosidase 1000 mg/kg	Mean													17.8	34
	S.D.													5.4	7
	n	12	12	0	0	0	0	12	12	0	0	0	0	12	12
β-Galactosidase 2000 mg/kg	Mean													20.7	38
	S.D.													3.6	9
	n	12	12	0	0	0	0	12	12	0	0	0	0	12	12

Cr.CO) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Cr.Oth.) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
No significant difference in any treated groups from control group.
Cr.CO : Crystal calcium oxalate Cr.Oth. : Crystal others Uri.Vol. : Urine volume W.C. : Water consumption

Table 5 - 9 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Male

Test Article Dose		Osmotic P. mOsm/kg	U-Na mmol/24h	U-K mmol/24h	U-Cl mmol/24h
β-Galactosidase 0 mg/kg	Mean	1790	2.3	4.9	3.6
	S.D.	418	0.6	1.0	0.7
	n	12	12	12	12
β-Galactosidase 500 mg/kg	Mean	1668	2.7	5.8 D2*	4.3
	S.D.	480	0.4	0.8	0.6
	n	12	12	12	12
β-Galactosidase 1000 mg/kg	Mean	1752	2.5	5.2	3.9
	S.D.	262	0.8	1.1	1.0
	n	12	12	12	12
β-Galactosidase 2000 mg/kg	Mean	1774	2.9	6.0 D2*	4.6 D2**
	S.D.	302	0.5	0.8	0.5
	n	12	12	12	12

Significantly different from control : * $P \leq 0.05$, ** $P \leq 0.01$
D2:Dunnett Test Two-Side
Osmotic P. : Osmotic Pressure

Table 5 - 10 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		pH										Protein					
			5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0≤		-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean																
	S.D.																
	n	12	0	0	0	1	1	1	0	6	3	12	12	0	0	0	0
β-Galactosidase 500 mg/kg	Mean																
	S.D.																
	n	12	0	0	1	0	0	2	0	6	3	12	11	1	0	0	0
β-Galactosidase 1000 mg/kg	Mean																
	S.D.																
	n	12	0	0	1	0	0	1	1	5	4	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean																
	S.D.																
	n	12	0	0	1	0	2	1	4	3	1	12	12	0	0	0	0

Protein) -:Negative, +/-:15, 1+:30, 2+:100, 3+:≥300 mg/dL

Table 5 - 11 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		Ketones	-	+/-	1+	2+	3+	Glucose	-	1+	2+	3+	4+
β-Galactosidase 0 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 500 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 1000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0

Ketones) -:Negative, +/-:5, 1+:15, 2+:40, 3+:80 mg/dL
Glucose) -:Negative, 1+:100, 2+:250, 3+:500, 4+:≥1000 mg/dL

Table 5 - 12 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		Oc.Blood						Urobili.					Bilirubin				
			-	+/-	1+	2+	3+		+/-	1+	2+	3+		-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean																
	S.D.																
	n	12	11	1	0	0	0	12	12	0	0	0	12	12	0	0	0
β-Galactosidase 500 mg/kg	Mean																
	S.D.																
	n	12	12	0	0	0	0	12	12	0	0	0	12	12	0	0	0
β-Galactosidase 1000 mg/kg	Mean																
	S.D.																
	n	12	12	0	0	0	0	12	12	0	0	0	12	12	0	0	0
β-Galactosidase 2000 mg/kg	Mean																
	S.D.																
	n	12	12	0	0	0	0	12	12	0	0	0	12	12	0	0	0

Oc.Blood) -:Negative, +/-:0.015, 1+:0.062, 2+:0.135, 3+:0.405 mg/dL
Urobili.) +/-:0.1-1.0, 1+:2.0, 2+:4.0, 3+:≥8.0 Ehrlich U/dL
Bilirubin) -:Negative, 1+:0.8, 2+:1.6, 3+:3.2 mg/dL
Oc.Blood : Occult blood Urobili. : Urobilinogen

Table 5 - 13 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		Color					RBC					
			LY	Y	DY	Other		-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean											
	S.D.											
	n	12	0	12	0	0	12	12	0	0	0	0
β-Galactosidase 500 mg/kg	Mean											
	S.D.											
	n	12	0	12	0	0	12	12	0	0	0	0
β-Galactosidase 1000 mg/kg	Mean											
	S.D.											
	n	12	0	12	0	0	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean											
	S.D.											
	n	12	0	12	0	0	12	12	0	0	0	0

Color) LY:Light yellow, Y:Yellow, DY:Dark yellow, Other:Other color
RBC) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe

Table 5 - 14 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		WBC	-	+/-	1+	2+	3+	Ep.SEC	-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	1	11	0	0	0
β-Galactosidase 500 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	3	9	0	0	0
β-Galactosidase 1000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	0	12	0	0	0
β-Galactosidase 2000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	1	11	0	0	0

WBC) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Ep.SEC) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Ep.SEC : Squamous epithelial cells

Table 5 - 15 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		Ep.SREC						Ep.Oth.					
			-	+/-	1+	2+	3+		-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 500 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 1000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0

Ep.SREC) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Ep.Oth.) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Ep.SREC : Small round epithelial cells Ep.Oth. : Epithelial others

Table 5 - 16 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		Cast						Cr.PS					
			-	+/-	1+	2+	3+		-	+/-	1+	2+	3+
β-Galactosidase 0 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 500 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 1000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0
β-Galactosidase 2000 mg/kg	Mean												
	S.D.												
	n	12	12	0	0	0	0	12	12	0	0	0	0

Cast) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Cr.PS) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Cr.PS : Crystal phosphate salts

Table 5 - 17 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		Cr.CO	-	+/-	1+	2+	3+	Cr.Oth.	-	+/-	1+	2+	3+	Uri.Vol. mL/24h	W.C. mL/24h
β-Galactosidase 0 mg/kg	Mean													13.5	29
	S.D.													6.1	10
	n	12	12	0	0	0	0	12	12	0	0	0	0	12	12
β-Galactosidase 500 mg/kg	Mean													11.5	24
	S.D.													4.0	5
	n	12	12	0	0	0	0	12	12	0	0	0	0	12	12
β-Galactosidase 1000 mg/kg	Mean													11.6	28
	S.D.													3.3	8
	n	12	12	0	0	0	0	12	12	0	0	0	0	12	12
β-Galactosidase 2000 mg/kg	Mean													14.6	30
	S.D.													6.1	7
	n	12	12	0	0	0	0	12	12	0	0	0	0	12	12

Cr.CO) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
Cr.Oth.) -:Negative, +/-:Slight, 1+:Mild, 2+:Moderate, 3+:Severe
No significant difference in any treated groups from control group.
Cr.CO : Crystal calcium oxalate Cr.Oth. : Crystal others Uri.Vol. : Urine volume W.C. : Water consumption

Table 5 - 18 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Urinalysis Stage : Week 13
Sex : Female

Test Article Dose		Osmotic P. mOsm/kg	U-Na mmol/24h	U-K mmol/24h	U-Cl mmol/24h
β-Galactosidase 0 mg/kg	Mean	1478	1.5	3.2	2.3
	S.D.	363	0.4	0.8	0.6
	n	12	12	12	12
β-Galactosidase 500 mg/kg	Mean	1604	1.5	3.0	2.3
	S.D.	321	0.3	0.7	0.5
	n	12	12	12	12
β-Galactosidase 1000 mg/kg	Mean	1503	1.4	2.9	2.2
	S.D.	278	0.2	0.4	0.3
	n	12	12	12	12
β-Galactosidase 2000 mg/kg	Mean	1444	1.7	3.3	2.5
	S.D.	403	0.3	0.8	0.5
	n	12	12	12	12

No significant difference in any treated groups from control group.
Osmotic P. : Osmotic Pressure

Table 6 - 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Hematology Stage : End of administration
Sex : Male

Test Article Dose		RBC 10E4/μL	HGB g/dL	HCT %	MCV fL	MCH pg	MCHC g/dL
β-Galactosidase	Mean	841	14.9	44.0	52.4	17.7	33.9
0 mg/kg	S.D.	34	0.6	2.0	2.3	0.9	0.7
	n	12	12	12	12	12	12
β-Galactosidase	Mean	840	15.1	44.5	53.0	18.0	34.0
500 mg/kg	S.D.	29	0.6	1.8	1.1	0.4	0.2
	n	12	12	12	12	12	12
β-Galactosidase	Mean	826	14.9	43.7	53.0	18.0	34.1
1000 mg/kg	S.D.	34	0.5	1.6	1.8	0.5	0.5
	n	12	12	12	12	12	12
β-Galactosidase	Mean	839	15.2	44.7	53.3	18.1	33.9
2000 mg/kg	S.D.	26	0.5	1.5	1.6	0.7	0.5
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 6 - 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Hematology Stage : End of administration
Sex : Male

Test Article Dose		Retic 10E9/L	PLT 10E4/μL	WBC 10E2/μL	LYMP 10E2/μL	NEUT 10E2/μL	EOS 10E2/μL
β-Galactosidase	Mean	129.9	108.4	91.7	69.6	16.8	1.4
0 mg/kg	S.D.	31.9	10.0	19.2	15.9	5.9	0.5
	n	12	12	12	12	12	12
β-Galactosidase	Mean	124.6	101.9	93.4	67.0	20.8	1.4
500 mg/kg	S.D.	21.9	7.0	18.7	16.5	5.0	0.5
	n	12	12	12	12	12	12
β-Galactosidase	Mean	116.7	104.0	98.5	75.7	17.5	1.4
1000 mg/kg	S.D.	17.9	9.6	25.1	20.5	7.9	0.4
	n	12	12	12	12	12	12
β-Galactosidase	Mean	117.2	104.1	92.1	69.5	16.6	1.4
2000 mg/kg	S.D.	19.3	11.0	14.3	13.2	5.0	0.6
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.
Retic : Reticulocyte

Table 6 - 3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Hematology Stage : End of administration
Sex : Male

Test Article Dose		BASO 10E2/μL	MONO 10E2/μL	LUC 10E2/μL
β-Galactosidase 0 mg/kg	Mean	0.9	2.6	0.5
	S.D.	0.2	1.3	0.2
	n	12	12	12
β-Galactosidase 500 mg/kg	Mean	0.9	2.8	0.5
	S.D.	0.4	0.9	0.3
	n	12	12	12
β-Galactosidase 1000 mg/kg	Mean	1.0	2.5	0.4
	S.D.	0.3	1.0	0.3
	n	12	12	12
β-Galactosidase 2000 mg/kg	Mean	1.0	3.0	0.5
	S.D.	0.2	1.0	0.3
	n	12	12	12

No significant difference in any treated groups from control group.
LUC : Large unstained cells

Table 6 - 4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Hematology Stage : End of administration
Sex : Male

Test Article Dose		PT s	APTT s
β-Galactosidase 0 mg/kg	Mean	13.8	14.9
	S.D.	0.5	2.0
	n	12	12
β-Galactosidase 500 mg/kg	Mean	13.7	15.0
	S.D.	0.5	1.4
	n	12	12
β-Galactosidase 1000 mg/kg	Mean	13.4	13.7
	S.D.	0.5	2.2
	n	12	12
β-Galactosidase 2000 mg/kg	Mean	13.4	13.7
	S.D.	0.7	1.7
	n	12	12

No significant difference in any treated groups from control group.

Table 6 - 5 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Hematology Stage : End of administration
Sex : Female

Test Article Dose		RBC 10E4/μL	HGB g/dL	HCT %	MCV fL	MCH pg	MCHC g/dL
β-Galactosidase 0 mg/kg	Mean	815	15.0	43.6	53.6	18.5	34.5
	S.D.	41	0.4	1.7	1.3	0.6	0.6
	n	12	12	12	12	12	12
β-Galactosidase 500 mg/kg	Mean	778 D2**	14.7	42.3	54.5	18.9	34.7
	S.D.	26	0.5	1.4	1.5	0.6	0.4
	n	12	12	12	12	12	12
β-Galactosidase 1000 mg/kg	Mean	780 D2*	14.6	41.9 D2**	53.7	18.7	34.9
	S.D.	25	0.5	1.2	1.5	0.4	0.5
	n	12	12	12	12	12	12
β-Galactosidase 2000 mg/kg	Mean	792	14.7	42.5	53.7	18.6	34.7
	S.D.	21	0.3	0.8	1.7	0.5	0.5
	n	12	12	12	12	12	12

Significantly different from control : * $P \leq 0.05$, ** $P \leq 0.01$
D2:Dunnett Test Two-Side

Table 6 - 6 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Hematology Stage : End of administration
Sex : Female

Test Article Dose		Retic 10E9/L	PLT 10E4/μL	WBC 10E2/μL	LYMP 10E2/μL	NEUT 10E2/μL	EOS 10E2/μL
β-Galactosidase	Mean	93.7	108.4	66.2	50.4	11.2	1.3
0 mg/kg	S.D.	25.5	12.2	15.0	13.3	3.5	0.4
	n	12	12	12	12	12	12
β-Galactosidase	Mean	104.0	110.2	70.5	54.0	12.2	1.1
500 mg/kg	S.D.	30.4	11.7	20.2	17.2	5.0	0.3
	n	12	12	12	12	12	12
β-Galactosidase	Mean	108.3	104.7	64.3	50.4	10.0	1.1
1000 mg/kg	S.D.	23.8	5.9	7.1	7.0	4.6	0.4
	n	12	12	12	12	12	12
β-Galactosidase	Mean	99.8	107.3	77.1	57.9	14.4	1.1
2000 mg/kg	S.D.	26.5	9.5	14.6	14.7	4.1	0.3
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.
Retic : Reticulocyte

Table 6 - 7 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Hematology Stage : End of administration
Sex : Female

Test Article Dose		BASO 10E2/μL	MONO 10E2/μL	LUC 10E2/μL
β-Galactosidase 0 mg/kg	Mean	0.8	2.1	0.4
	S.D.	0.2	0.8	0.2
	n	12	12	12
β-Galactosidase 500 mg/kg	Mean	0.9	1.8	0.3
	S.D.	0.3	0.7	0.2
	n	12	12	12
β-Galactosidase 1000 mg/kg	Mean	0.8	1.8	0.3
	S.D.	0.2	0.5	0.1
	n	12	12	12
β-Galactosidase 2000 mg/kg	Mean	0.8	2.4	0.6
	S.D.	0.2	0.6	0.3
	n	12	12	12

No significant difference in any treated groups from control group.
LUC : Large unstained cells

Table 6 - 8 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Hematology Stage : End of administration
Sex : Female

Test Article Dose		PT s	APTT s
β-Galactosidase 0 mg/kg	Mean	13.2	13.0
	S.D.	1.0	1.4
	n	12	12
β-Galactosidase 500 mg/kg	Mean	13.1	13.0
	S.D.	0.8	1.5
	n	12	12
β-Galactosidase 1000 mg/kg	Mean	13.5	13.2
	S.D.	0.9	1.3
	n	12	12
β-Galactosidase 2000 mg/kg	Mean	12.9	12.7
	S.D.	1.0	1.6
	n	12	12

No significant difference in any treated groups from control group.

Table 7 - 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Blood chemistry Stage : End of administration
Sex : Male

Test Article Dose		AST IU/L	ALT IU/L	LDH IU/L	ALP IU/L	r-GTP IU/L	T-CHO mg/dL
β-Galactosidase	Mean	63	30	69	317	0	77
0 mg/kg	S.D.	14	4	22	61	0	22
	n	12	12	12	12	12	12
β-Galactosidase	Mean	64	31	68	320	0	68
500 mg/kg	S.D.	8	6	12	96	0	12
	n	12	12	12	12	12	12
β-Galactosidase	Mean	59	30	67	300	0	76
1000 mg/kg	S.D.	6	4	11	76	0	19
	n	12	12	12	12	12	12
β-Galactosidase	Mean	62	31	77	318	0	73
2000 mg/kg	S.D.	10	6	19	59	0	11
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 7 - 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Blood chemistry Stage : End of administration
Sex : Male

Test Article Dose		TG mg/dL	PL mg/dL	T-BIL mg/dL	GLU mg/dL	BUN mg/dL	CRNN mg/dL
β-Galactosidase	Mean	94	124	0.1	158	13	0.26
0 mg/kg	S.D.	54	28	0.0	19	1	0.03
	n	12	12	12	12	12	12
β-Galactosidase	Mean	64	111	0.1	145	15	0.29
500 mg/kg	S.D.	20	14	0.0	17	2	0.03
	n	12	12	12	12	12	12
β-Galactosidase	Mean	70	120	0.1	153	14	0.27
1000 mg/kg	S.D.	34	22	0.0	19	2	0.03
	n	12	12	12	12	12	12
β-Galactosidase	Mean	73	118	0.1	146	15	0.26
2000 mg/kg	S.D.	33	12	0.0	13	3	0.03
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 7 - 3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Blood chemistry Stage : End of administration
Sex : Male

Test Article Dose		Na mmol/L	K mmol/L	Cl mmol/L	Ca mg/dL	P mg/dL	TP g/dL
β-Galactosidase	Mean	145	3.7	107	9.9	5.1	6.7
0 mg/kg	S.D.	1	0.2	2	0.3	0.3	0.3
	n	12	12	12	12	12	12
β-Galactosidase	Mean	145	3.5	106	9.9	5.4	6.6
500 mg/kg	S.D.	1	0.2	2	0.2	0.5	0.3
	n	12	12	12	12	12	12
β-Galactosidase	Mean	145	3.7	107	9.8	5.1	6.6
1000 mg/kg	S.D.	1	0.2	1	0.3	0.3	0.3
	n	12	12	12	12	12	12
β-Galactosidase	Mean	145	3.6	105 D2*	10.1	5.5 D2*	6.7
2000 mg/kg	S.D.	1	0.2	1	0.3	0.5	0.3
	n	12	12	12	12	12	12

Significantly different from control : * $P \leq 0.05$
D2:Dunnett Test Two-Side

Table 7 - 4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Blood chemistry Stage : End of administration
Sex : Male

Test Article Dose		ALB g/dL	A/G
β-Galactosidase 0 mg/kg	Mean	2.7	0.7
	S.D.	0.1	0.1
	n	12	12
β-Galactosidase 500 mg/kg	Mean	2.7	0.7
	S.D.	0.1	0.1
	n	12	12
β-Galactosidase 1000 mg/kg	Mean	2.6	0.7
	S.D.	0.1	0.1
	n	12	12
β-Galactosidase 2000 mg/kg	Mean	2.7	0.7
	S.D.	0.1	0.1
	n	12	12

No significant difference in any treated groups from control group.

Table 7 - 5 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Blood chemistry Stage : End of administration
Sex : Female

Test Article Dose		AST IU/L	ALT IU/L	LDH IU/L	ALP IU/L	r-GTP IU/L	T-CHO mg/dL
β-Galactosidase	Mean	64	28	60	170	0	78
0 mg/kg	S.D.	7	6	13	49	0	13
	n	12	12	12	12	12	12
β-Galactosidase	Mean	62	29	57	150	0	86
500 mg/kg	S.D.	9	6	17	26	0	26
	n	12	12	12	12	12	12
β-Galactosidase	Mean	75	35	65	159	0	73
1000 mg/kg	S.D.	46	26	39	33	1	9
	n	12	12	12	12	12	12
β-Galactosidase	Mean	58	30	51	189	0	87
2000 mg/kg	S.D.	4	6	9	65	0	14
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 7 - 6 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Blood chemistry Stage : End of administration
Sex : Female

Test Article Dose		TG mg/dL	PL mg/dL	T-BIL mg/dL	GLU mg/dL	BUN mg/dL	CRNN mg/dL
β-Galactosidase	Mean	32	155	0.1	121	19	0.38
0 mg/kg	S.D.	18	24	0.0	16	5	0.05
	n	12	12	12	12	12	12
β-Galactosidase	Mean	44	165	0.1	120	21	0.39
500 mg/kg	S.D.	42	46	0.0	12	4	0.08
	n	12	12	12	12	12	12
β-Galactosidase	Mean	39	152	0.1	126	20	0.36
1000 mg/kg	S.D.	24	19	0.0	13	3	0.03
	n	12	12	12	12	12	12
β-Galactosidase	Mean	56	176	0.1	133	20	0.33
2000 mg/kg	S.D.	38	27	0.0	9	4	0.04
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 7 - 7 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Blood chemistry Stage : End of administration
Sex : Female

Test Article Dose		Na mmol/L	K mmol/L	Cl mmol/L	Ca mg/dL	P mg/dL	TP g/dL
β-Galactosidase	Mean	144	3.2	107	9.9	3.9	7.0
0 mg/kg	S.D.	1	0.2	1	0.3	0.5	0.3
	n	12	12	12	12	12	12
β-Galactosidase	Mean	143	3.3	106	9.9	4.1	7.0
500 mg/kg	S.D.	1	0.2	1	0.5	0.6	0.6
	n	12	12	12	12	12	12
β-Galactosidase	Mean	144	3.2	107	9.8	4.0	6.9
1000 mg/kg	S.D.	2	0.2	2	0.2	0.6	0.4
	n	12	12	12	12	12	12
β-Galactosidase	Mean	144	3.2	106	10.1	4.1	7.1
2000 mg/kg	S.D.	2	0.2	2	0.3	0.8	0.4
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 7 - 8 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Blood chemistry Stage : End of administration
Sex : Female

Test Article Dose		ALB g/dL	A/G
β-Galactosidase 0 mg/kg	Mean	3.1	0.8
	S.D.	0.2	0.1
	n	12	12
β-Galactosidase 500 mg/kg	Mean	3.1	0.8
	S.D.	0.3	0.1
	n	12	12
β-Galactosidase 1000 mg/kg	Mean	3.1	0.8
	S.D.	0.1	0.1
	n	12	12
β-Galactosidase 2000 mg/kg	Mean	3.2	0.8
	S.D.	0.2	0.1
	n	12	12

No significant difference in any treated groups from control group.

Table 8 - 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Absolute organ weight Stage : End of administration
Sex : Male

Test Article Dose		F.B.W.@@ g	Brain g	Pituitary mg	Thyroid-RL mg	Sa.G.-RL mg	Thymus mg	Heart g	Lung g	Liver g
β-Galactosidase	Mean	566	2.21	11.4	24.5	737	269	1.57	1.48	14.18
0 mg/kg	S.D.	46	0.12	3.1	6.6	67	52	0.17	0.14	1.95
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	571	2.20	12.3	23.2	717	271	1.59	1.51	13.55
500 mg/kg	S.D.	55	0.08	1.8	4.8	98	64	0.13	0.13	1.93
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	579	2.22	13.1	24.2	734	301	1.56	1.50	14.31
1000 mg/kg	S.D.	67	0.09	1.5	5.0	80	72	0.11	0.12	2.58
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	587	2.24	13.0	22.7	738	288	1.55	1.53	14.25
2000 mg/kg	S.D.	45	0.09	2.0	4.5	62	60	0.11	0.08	1.45
	n	12	12	12	12	12	12	12	12	12

Statistical analysis was not done : @@
No significant difference in any treated groups from control group.
Sa.G. : Salivary gland

Table 8 - 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Absolute organ weight Stage : End of administration
Sex : Male

Test Article Dose		Spleen g	Kidney-RL g	Adrenal-RL mg	Testis-RL g	Prostate g	Sem.Ves. g
β-Galactosidase	Mean	0.78	3.34	53	3.52	1.34	1.43
0 mg/kg	S.D.	0.11	0.34	9	0.18	0.20	0.28
	n	12	12	12	12	12	12
β-Galactosidase	Mean	0.82	3.31	58	3.58	1.40	1.56
500 mg/kg	S.D.	0.17	0.40	10	0.29	0.21	0.24
	n	12	12	12	12	12	12
β-Galactosidase	Mean	0.77	3.45	56	3.47	1.37	1.38
1000 mg/kg	S.D.	0.11	0.41	7	0.34	0.29	0.25
	n	12	12	12	12	12	12
β-Galactosidase	Mean	0.81	3.45	54	3.63	1.38	1.45
2000 mg/kg	S.D.	0.06	0.30	7	0.28	0.28	0.24
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.
Sem.Ves. : Seminal vesicle

Table 8 - 3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Absolute organ weight Stage : End of administration
Sex : Female

Test Article Dose		F.B.W.@@ g	Brain g	Pituitary mg	Thyroid-RL mg	Sa.G.-RL mg	Thymus mg	Heart g	Lung g	Liver g
β-Galactosidase	Mean	296	1.98	14.8	17.3	422	249	0.88	1.06	6.56
0 mg/kg	S.D.	32	0.09	2.7	3.1	38	49	0.09	0.08	0.75
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	303	1.93	15.6	15.8	438	248	0.92	1.10	7.00
500 mg/kg	S.D.	42	0.06	2.8	2.4	37	73	0.08	0.09	0.92
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	292	1.97	15.6	15.2	441	231	0.90	1.08	6.92
1000 mg/kg	S.D.	35	0.07	3.4	3.8	46	66	0.08	0.11	0.69
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	298	1.95	15.5	16.3	430	264	0.90	1.09	7.15
2000 mg/kg	S.D.	19	0.06	2.5	3.6	29	44	0.05	0.07	0.47
	n	12	12	12	12	12	12	12	12	12

Statistical analysis was not done : @@
No significant difference in any treated groups from control group.
Sa.G. : Salivary gland

Table 8 - 4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Absolute organ weight Stage : End of administration
Sex : Female

Test Article Dose		Spleen g	Kidney-RL g	Adrenal-RL mg	Ovary-RL mg	Uterus mg
β-Galactosidase	Mean	0.47	1.76	54	72.5	550
0 mg/kg	S.D.	0.08	0.16	6	9.4	114
	n	12	12	12	12	12
β-Galactosidase	Mean	0.49	1.84	57	72.4	601
500 mg/kg	S.D.	0.08	0.21	9	17.5	160
	n	12	12	12	12	12
β-Galactosidase	Mean	0.48	1.85	58	74.4	669
1000 mg/kg	S.D.	0.06	0.24	8	13.2	112
	n	12	12	12	12	12
β-Galactosidase	Mean	0.50	1.83	54	75.6	668
2000 mg/kg	S.D.	0.04	0.14	7	10.3	149
	n	12	12	12	12	12

No significant difference in any treated groups from control group.

Table 9 - 1 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Relative organ weight Stage : End of administration
Sex : Male

Test Article Dose		F.B.W.@@ g	Brain g/100g	Pituitary mg/100g	Thyroid-RL mg/100g	Sa.G.-RL mg/100g	Thymus mg/100g	Heart g/100g	Lung g/100g	Liver g/100g
β-Galactosidase	Mean	566	0.39	2.0	4.4	131	48	0.28	0.26	2.50
0 mg/kg	S.D.	46	0.03	0.5	1.0	12	10	0.01	0.02	0.20
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	571	0.39	2.2	4.1	126	47	0.28	0.27	2.37
500 mg/kg	S.D.	55	0.04	0.2	0.8	16	9	0.02	0.02	0.20
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	579	0.39	2.3	4.3	128	52	0.27	0.26	2.46
1000 mg/kg	S.D.	67	0.04	0.3	1.1	17	10	0.02	0.03	0.22
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	587	0.38	2.2	3.9	126	49	0.27	0.26	2.42
2000 mg/kg	S.D.	45	0.03	0.3	0.6	15	8	0.01	0.02	0.13
	n	12	12	12	12	12	12	12	12	12

Statistical analysis was not done : @@
No significant difference in any treated groups from control group.
Sa.G. : Salivary gland

Table 9 - 2 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Relative organ weight Stage : End of administration
Sex : Male

Test Article Dose		Spleen g/100g	Kidney-RL g/100g	Adrenal-RL mg/100g	Testis-RL g/100g	Prostate g/100g	Sem.Ves. g/100g
β-Galactosidase	Mean	0.14	0.59	9	0.63	0.24	0.25
0 mg/kg	S.D.	0.02	0.05	2	0.05	0.03	0.06
	n	12	12	12	12	12	12
β-Galactosidase	Mean	0.14	0.58	10	0.64	0.25	0.27
500 mg/kg	S.D.	0.03	0.06	2	0.09	0.04	0.04
	n	12	12	12	12	12	12
β-Galactosidase	Mean	0.13	0.60	10	0.60	0.24	0.24
1000 mg/kg	S.D.	0.01	0.05	1	0.07	0.05	0.06
	n	12	12	12	12	12	12
β-Galactosidase	Mean	0.14	0.59	9	0.62	0.24	0.25
2000 mg/kg	S.D.	0.01	0.05	1	0.05	0.05	0.05
	n	12	12	12	12	12	12

No significant difference in any treated groups from control group.
Sem.Ves. : Seminal vesicle

Table 9 - 3 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Relative organ weight Stage : End of administration
Sex : Female

Test Article Dose		F.B.W.@@ g	Brain g/100g	Pituitary mg/100g	Thyroid-RL mg/100g	Sa.G.-RL mg/100g	Thymus mg/100g	Heart g/100g	Lung g/100g	Liver g/100g
β-Galactosidase	Mean	296	0.68	5.1	5.9	144	84	0.30	0.36	2.22
0 mg/kg	S.D.	32	0.08	1.1	1.0	19	16	0.02	0.03	0.15
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	303	0.65	5.2	5.3	147	82	0.31	0.37	2.32
500 mg/kg	S.D.	42	0.08	1.0	0.9	19	25	0.02	0.03	0.20
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	292	0.68	5.4	5.2	152	79	0.31	0.37	2.38
1000 mg/kg	S.D.	35	0.08	1.2	1.3	12	19	0.02	0.03	0.19
	n	12	12	12	12	12	12	12	12	12
β-Galactosidase	Mean	298	0.66	5.3	5.5	145	89	0.30	0.37	2.40 D2*
2000 mg/kg	S.D.	19	0.05	1.0	1.1	12	15	0.02	0.02	0.15
	n	12	12	12	12	12	12	12	12	12

Statistical analysis was not done : @@
Significantly different from control : * $P \leq 0.05$
D2:Dunnett Test Two-Side
Sa.G. : Salivary gland

Table 9 - 4 A 13-week oral gavage toxicity study of β-galactosidase concentrate in rats TT-160003

Item : Relative organ weight Stage : End of administration
Sex : Female

Test Article Dose		Spleen g/100g	Kidney-RL g/100g	Adrenal-RL mg/100g	Ovary-RL mg/100g	Uterus mg/100g
β-Galactosidase 0 mg/kg	Mean	0.16	0.60	18	24.6	186
	S.D.	0.02	0.07	2	3.3	31
	n	12	12	12	12	12
β-Galactosidase 500 mg/kg	Mean	0.16	0.61	19	23.9	203
	S.D.	0.02	0.05	3	5.3	63
	n	12	12	12	12	12
β-Galactosidase 1000 mg/kg	Mean	0.17	0.64	20	25.6	233
	S.D.	0.03	0.05	4	4.8	48
	n	12	12	12	12	12
β-Galactosidase 2000 mg/kg	Mean	0.17	0.62	18	25.5	225
	S.D.	0.02	0.05	2	3.8	51
	n	12	12	12	12	12

No significant difference in any treated groups from control group.

TT-160003

Table 10-1 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Gross pathological findings

Organs	Sex:	M	M	M	M
	Dose(mg/kg/day):	0	500	1000	2000
Findings	Number:	12	12	12	12
Stomach					
Focus,dark red,glandular stomach		2	1	2	1
Focus,white,serosal		0	0	1	0

M : Male

TT-160003

Table 10-2 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Gross pathological findings

Organs	Sex:	F	F	F	F
	Dose(mg/kg/day):	0	500	1000	2000
Findings	Number:	12	12	12	12
Stomach					
Focus,dark red,glandular stomach		2	0	0	0
Liver					
Focus,dark red		0	0	1	0

F : Female

Table 11-1 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs / Findings	Sex: / Dose(mg/kg/day): / Number:	M 0 12	M 500 12	M 1000 12	M 2000 12
Cerebrum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Cerebellum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Spinal cord,thoracic					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Sciatic nerve					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Eye					
Number examined		12	0	0	12
Not remarkable		11	0	0	12
Dysplasia,retinal		1	0	0	0
minimal		1	0	0	0
Optic nerve					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Harderian gland					
Number examined		12	0	0	12
Not remarkable		11	0	0	11
Cell infiltration		1	0	0	1
minimal		1	0	0	1
Pituitary					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Thyroid					
Number examined		12	0	0	12
Not remarkable		8	0	0	11
Cyst,ultimobranchial		3	0	0	1
minimal		3	0	0	1
Cell infiltration		1	0	0	0
minimal		1	0	0	0
Parathyroid					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Adrenal					
Number examined		12	0	0	12
Not remarkable		11	0	0	12
Cell infiltration,cortical		1	0	0	0
minimal		1	0	0	0
Thymus					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Spleen					
Number examined		12	0	0	12
Not remarkable		6	0	0	5
Hematopoiesis,extramedullary		6	0	0	7
minimal		6	0	0	7

M : Male

Table 11-2 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs / Findings	Sex: / Dose(mg/kg/day): / Number:	M 0 12	M 500 12	M 1000 12	M 2000 12
Lymph node,submandibular					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Lymph node,mesenteric					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Heart					
Number examined		12	0	0	12
Not remarkable		8	0	0	9
Cell infiltration		4	0	0	2
minimal		4	0	0	1
mild		0	0	0	1
Fibrosis,myocardial		1	0	0	2
minimal		1	0	0	2
Aorta,thoracic					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Trachea					
Number examined		12	0	0	12
Not remarkable		11	0	0	10
Cell infiltration,mucosal		1	0	0	2
minimal		1	0	0	2
Lung(bronchus)					
Number examined		12	0	0	12
Not remarkable		8	0	0	6
Mineralization,arterial wall		4	0	0	2
minimal		4	0	0	2
Aggregation,alveolar macrophage		0	0	0	2
minimal		0	0	0	2
Cell infiltration,alveolar		0	0	0	1
minimal		0	0	0	1
Metaplasia,osseous		0	0	0	1
minimal		0	0	0	1
Tongue					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Esophagus					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Stomach					
Number examined		12	1	3	12
Not remarkable		10	0	0	10
Hemorrhage,focal		0	0	0	2
minimal		0	0	0	2
Erosion/Ulcer,glandular stomach		2	1	2	0
minimal		2	1	2	0
Cyst,squamous		0	0	1	0
minimal		0	0	1	0
Hyperplasia,squamous,limiting ridge		2	0	1	0
minimal		2	0	1	0
Intestine,duodenum					
Number examined		12	0	0	12
Not remarkable		12	0	0	11
Erosion/ulcer		0	0	0	1

M : Male

Table 11-3 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs / Findings	Sex: / Dose(mg/kg/day): / Number:				
	Sex:	M	M	M	M
	Dose(mg/kg/day):	0	500	1000	2000
	Number:	12	12	12	12
Intestine,duodenum (continued)					
Erosion/ulcer (continued)					
minimal		0	0	0	1
Intestine,jejunum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Intestine,ileum(Peyer's patch)					
Number examined		12	0	0	12
Not remarkable		11	0	0	11
Mineralization,Peyer's patch		1	0	0	1
minimal		1	0	0	1
Intestine,cecum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Intestine,colon					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Intestine,rectum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Salivary gland,submandibular					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Salivary gland,sublingual					
Number examined		12	0	0	12
Not remarkable		12	0	0	11
Cell infiltration		0	0	0	1
minimal		0	0	0	1
Liver					
Number examined		12	0	0	12
Necrosis,focal		1	0	0	0
minimal		1	0	0	0
Cell infiltration		12	0	0	12
minimal		12	0	0	12
Pancreas					
Number examined		12	0	0	12
Not remarkable		8	0	0	10
Cell infiltration		3	0	0	2
minimal		3	0	0	2
Fibrosis,islet		2	0	0	0
minimal		1	0	0	0
mild		1	0	0	0
Kidney					
Number examined		12	0	0	12
Not remarkable		2	0	0	0
Dilatation,tubular		1	0	0	1
minimal		1	0	0	1
Basophilia,tubular		7	0	0	6
minimal		7	0	0	6
Eosinophilic body,tubular cell		2	0	0	4
minimal		1	0	0	4
mild		1	0	0	0
Urinary cast,hyaline		0	0	0	1

M : Male

Table 11-4 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs / Findings	Sex: / Dose(mg/kg/day): / Number:	M 0 12	M 500 12	M 1000 12	M 2000 12
Kidney (continued)					
Urinary cast,hyaline (continued)					
minimal		0	0	0	1
Mineralization		4	0	0	4
minimal		4	0	0	4
Cell infiltration,interstitial		6	0	0	5
minimal		6	0	0	5
Urinary bladder					
Number examined		12	0	0	12
Not remarkable		10	0	0	10
Cell infiltration,mucosal		0	0	0	1
minimal		0	0	0	1
Cell infiltration,serosal		2	0	0	0
minimal		2	0	0	0
Cell infiltration,muscle layer		0	0	0	1
minimal		0	0	0	1
Testis					
Number examined		12	0	0	12
Not remarkable		11	0	0	9
Degeneration,seminiferous tubular		1	0	0	3
minimal		1	0	0	3
Epididymis					
Number examined		12	0	0	12
Not remarkable		8	0	0	10
Cell infiltration,interstitial		4	0	0	2
minimal		4	0	0	2
Prostate					
Number examined		12	0	0	12
Cell infiltration		12	0	0	12
minimal		11	0	0	11
mild		1	0	0	1
Seminal vesicle					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Mammary gland,inguinal					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Bone+Bone marrow,sternal					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Bone+Bone marrow,femoral					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Skeletal muscle,femoral					
Number examined		12	0	0	12
Not remarkable		5	0	0	4
Degeneration,muscular		7	0	0	8
minimal		7	0	0	8
Skin,inguinal					
Number examined		12	0	0	12
Not remarkable		12	0	0	12

M : Male

Table 11-5 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs	Sex:	M	M	M	M
	Dose(mg/kg/day):	0	500	1000	2000
Findings	Number:	12	12	12	12
Nasal cavity					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Zymbal gland					
Number examined		12	0	0	12
Not remarkable		12	0	0	12

M : Male

Table 11-6 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs	Sex:	F	F	F	F
	Dose(mg/kg/day):	0	500	1000	2000
Findings	Number:	12	12	12	12
Cerebrum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Cerebellum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Spinal cord,thoracic					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Sciatic nerve					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Eye					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Optic nerve					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Harderian gland					
Number examined		12	0	0	12
Not remarkable		11	0	0	11
Cell infiltration		1	0	0	1
minimal		1	0	0	1
Pituitary					
Number examined		12	0	0	12
Not remarkable		11	0	0	11
Cyst,pars distalis		0	0	0	1
minimal		0	0	0	1
Cyst,pars nervosa		1	0	0	0
mild		1	0	0	0
Thyroid					
Number examined		12	0	0	12
Not remarkable		8	0	0	8
Cyst,ultimobranchial		4	0	0	4
minimal		4	0	0	4
Parathyroid					
Number examined		12	0	0	11
Not remarkable		12	0	0	11
No sample		0	0	0	1
Adrenal					
Number examined		12	0	0	12
Not remarkable		9	0	0	10
Cell infiltration,cortical		2	0	0	2
minimal		2	0	0	2
Hypertrophy,cortical cell,focal		1	0	0	0
minimal		1	0	0	0
Thymus					
Number examined		12	0	0	12
Not remarkable		12	0	0	12

F : Female

Table 11-7 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs / Findings	Sex: / Dose(mg/kg/day): / Number:	F 0 12	F 500 12	F 1000 12	F 2000 12
Spleen					
Number examined		12	0	0	12
Not remarkable		11	0	0	10
Hematopoiesis,extramedullary		1	0	0	2
minimal		1	0	0	2
Lymph node,submandibular					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Lymph node,mesenteric					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Heart					
Number examined		12	0	0	12
Not remarkable		11	0	0	10
Cell infiltration		1	0	0	2
minimal		1	0	0	2
Aorta,thoracic					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Trachea					
Number examined		12	0	0	12
Not remarkable		12	0	0	11
Cell infiltration,mucosal		0	0	0	1
minimal		0	0	0	1
Lung(bronchus)					
Number examined		12	0	0	12
Not remarkable		10	0	0	10
Mineralization,arterial wall		1	0	0	2
minimal		1	0	0	2
Aggregation,alveolar macrophage		1	0	0	1
minimal		1	0	0	1
Tongue					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Esophagus					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Stomach					
Number examined		12	0	0	12
Not remarkable		11	0	0	12
Erosion/Ulcer,glandular stomach		1	0	0	0
minimal		1	0	0	0
Hyperplasia,squamous,limiting ridge		1	0	0	0
mild		1	0	0	0
Intestine,duodenum					
Number examined		12	0	0	12
Not remarkable		10	0	0	10
Atrophy,mucosal		1	0	0	0
minimal		1	0	0	0
Erosion/ulcer		1	0	0	2
minimal		1	0	0	2

F : Female

Table 11-8 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs / Findings	Sex: / Dose(mg/kg/day): / Number:	F 0 12	F 500 12	F 1000 12	F 2000 12
Intestine,jejunum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Intestine,ileum(Peyer's patch)					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Intestine,cecum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Intestine,colon					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Intestine,rectum					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Salivary gland,submandibular					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Salivary gland,sublingual					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Liver					
Number examined		12	0	1	12
Necrosis,focal		0	0	1	0
minimal		0	0	1	0
Cell infiltration		12	0	0	12
minimal		12	0	0	12
Pancreas					
Number examined		12	0	0	12
Not remarkable		10	0	0	11
Atrophy,acinar,focal		1	0	0	0
minimal		1	0	0	0
Cell infiltration		1	0	0	1
minimal		1	0	0	1
Kidney					
Number examined		12	0	0	12
Not remarkable		8	0	0	7
Dilatation,tubular		0	0	0	1
minimal		0	0	0	1
Basophilia,tubular		2	0	0	1
minimal		2	0	0	1
Mineralization		2	0	0	3
minimal		2	0	0	3
Cell infiltration,interstitial		2	0	0	2
minimal		2	0	0	2
Urinary bladder					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Ovary					
Number examined		12	0	0	12
Not remarkable		12	0	0	12

F : Female

Table 11-9 A 13-week oral gavage toxicity study of ß-galactosidase concentrate in rats
Histopathological findings

Organs	Sex:	F	F	F	F
	Dose(mg/kg/day):	0	500	1000	2000
Findings	Number:	12	12	12	12
Uterus					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Vagina					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Oviduct					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Mammary gland,inguinal					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Bone+Bone marrow,sternal					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Bone+Bone marrow,femoral					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Skeletal muscle,femoral					
Number examined		12	0	0	12
Not remarkable		8	0	0	9
Degeneration,muscular		4	0	0	3
minimal		4	0	0	3
Skin,inguinal					
Number examined		12	0	0	12
Not remarkable		12	0	0	11
Crust		0	0	0	1
minimal		0	0	0	1
Nasal cavity					
Number examined		12	0	0	12
Not remarkable		12	0	0	12
Zymbal gland					
Number examined		12	0	0	12
Not remarkable		12	0	0	12

F : Female