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Regulatory Toxicology and Pharmacology 37 (2003) 293–304

Regulatory Toxicology and Pharmacology

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Safety evaluation of lipase produced from *Rhizopus oryzae*: summary of toxicological data

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Received 5 November 2001

Abstract

The toxicity of Lipase D, an enzyme preparation, was evaluated in a series of studies. Lipase D selectively hydrolyzes triglycerides of fatty acids. It also catalyzes the interesterification of edible fats and oils. In a 13-week gavage study, Sprague–Dawley rats received Lipase D at levels of 0, 500, 1000, or 2000 mg/kg body wt./day. A dose dependent decrease in urinary pH was observed, but there were no effects on electrolyte balance, kidney weight, or histology of the kidney. The no-observed-adverse-effect level in rats was 1000 mg/kg body wt./day. In common with other enzyme preparations, Lipase D was not genotoxic. Lipase D was tested in the Ames assay, the mouse lymphoma forward mutation assay, and the chromosome aberration assay. Finally, the particular strain of *Rhizopus oryzae* used to prepare Lipase D was shown to have low to moderate pathogenicity when injected into the tail vein of mice at doses up to 1.3×10^6 colony-forming units (CFU) per animal. No effects were observed when mice received up to 2.2×10^5 CFU by gavage or in their diets daily for 28 days. The results indicate that this particular strain can be handled using ordinary safety practices current in the fermentation industry. These studies support a conclusion that Lipase D is safe when used as described in the processing of dietary fatty acids and glycerides of fatty acids.
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1. Introduction

Lipase D (EC 3.1.1.3) is an enzyme preparation intended for use in the food processing industry, specifically in the production of dietary ingredients derived from fats and oils. Residual denatured enzyme preparation may be present in the finished dietary ingredients at levels no higher than 100 ppm, resulting in dietary exposure in humans that is unlikely to exceed 50 mg/day. The production organism for Lipase D is a strain of *Rhizopus oryzae* (strain 33158, derived from strain 4697, which was first reported in 1941 by the Institute for Fermentation, Osaka (IFO)). *R. oryzae* has been used for many years in the production of tempeh, an Indonesian fermented soybean food (Steinkraus et al., 1960). The production organism itself is not present in the enzyme preparation. The enzyme has been used in the food industry to hydrolyze triglycerides of fatty acids and as a catalyst for the interesterification of fats and oils, designed to improve their physical properties. Fatty acids on the 1,3-position of the glycerol backbone are hydrolyzed and then randomly re-esterified. Lipase enzymes generally have a long and safe history of use in food applications and are present naturally in many foods.

The present studies were conducted on Lipase D to investigate the toxic potential of the enzyme preparation as well as the potential of the production organism to induce a pathogenic response. Specifically, the enzyme preparation was subjected to a 13-week feeding study in rats, and a series of genotoxicity studies to evaluate its safety when present at very low levels in dietary ingredients. The production organism was tested for pathogenicity because, although viable *R. oryzae* will not be present in the finished enzyme preparation, it is considered preferable from the standpoint of worker safety to use a non-pathogenic production organism. This article does not attempt to address worker safety in a comprehensive way. The safety of a lipase enzyme preparation from a different strain of *R. oryzae* was recently established using a similar series of tests

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doi:10.1016/S0273-2300(03)00006-0

(Coenen et al., 1997). According to the decision-tree approach recommended by Pariza and Johnson (2001), Lipase D may be judged safe primarily if the *R. oryzae* production strain lacks toxigenic potential and the NOAEL in the 13-week feeding study is sufficiently high to ensure safety. The focus of this article is the safety of Lipase D when present at low levels in processed foods.

2. Materials and methods

2.1. Preparation and characterization of Lipase D

Lipase D for toxicity testing was produced according to typical procedures for producing such enzymes. Amano's Lipase D enzyme preparation is produced by the controlled fermentation of a selected strain of *R. oryzae*, a naturally occurring fungus that has been subjected to chemical mutagenesis. The fermentation medium is prepared from food-grade ingredients. The lipase is an extracellular enzyme and is separated from the production organism following fermentation by a series of filtration steps. The filtrate is concentrated, and the enzyme preparation precipitated with ammonium sulfate. Lipase D is extracted with water, and the solution is concentrated, microfiltered, freeze dried, crushed, and blended with dextrin to the desired enzymatic activity. Enzyme concentrate without dextrin was used in the safety studies.

Specifications for commercial Lipase D are lipase activity $\geqslant 800{,}000$ U/g[1]; arsenic $\leqslant 3$ ppm; heavy metals (as lead) $\leqslant 30$ ppm; lead $\leqslant 5$ ppm; total viable microbial count $\leqslant 5 \times 10^4$/g; *Escherichia coli* and *Salmonella*, negative in 25 g; coliforms $\leqslant 30$/g; and loss on drying $\leqslant 10.0\%$. Three batches of Lipase D tested negative for aflatoxins B1, B2, G1, and G2 (limit of detection: 0.5 ppb), ochratoxin A (LOD: 0.5 ppb), sterigmatocystin (LOD: 20 ppb), zearalenone (LOD: 50 ppb), and T-2 toxin (LOD: 200 ppb). Antibiotic activity was negative in the analyzed batches.

2.2. Subchronic (13-week) oral toxicity study in rats

The study was performed in accordance with guidelines of the Japanese Ministry of Health and Welfare (JMHW, 1996).

On the basis of a preliminary two-week oral study, in which no treatment-related effects were observed at the highest dose level, 2000 mg/kg body wt./day, groups of 12 male and 12 female Sprague–Dawley SPF rats [Crj:CD(SD)IGS], 6 weeks of age at the initiation of dosing, received Lipase D (activity 3,170,000 U/g) by gavage at levels of 0, 500, 1000, and 2000 mg/kg body wt./day. Additional groups of six males and six females received 0 or 2000 mg/kg body wt./day during the treatment period and a 4-week recovery period. Animals were observed three times daily (twice on Saturdays and holidays) for clinical signs. Body weights and feed consumption were recorded twice weekly.

Prior to administration of the first dose, the eyes of all animals were examined macroscopically, and the anterior portion, transparent body, and fundus oculi were examined using an opthalmoscope. In week 13 of administration and the final week of recovery, similar examinations were performed on six animals of each sex in each group. In addition, photographs of the fundus oculi of the animals in the control and high dose groups were taken with a fundus camera after applying a mydriatic agent.

Urine samples were collected over a 24-h period on days 29–30, 86–88, and day 22–23 of recovery. Urinalysis parameters included external appearance, urine volume, pH, total protein, ketone bodies, glucose, occult blood, bilirubin, urobilinogen, sediment, osmolality, Na^+, K^+, Cl^-, and *N*-acetyl-β-glucosaminidase (NAG). On the day following the end of the administration or recovery period, after the rats were fasted for 16–19 h, blood samples were taken from the abdominal aorta. The animals were then exsanguinated under anesthesia and necropsied. All organs and tissues in the cephalic, thoracic, and abdominal cavities were macroscopically examined. Hematology and serum clinical chemistry were conducted on all animals. Hematology parameters included erythrocytes (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), reticulocyte ratio, platelet count, leukocytes (WBC), differential leukocyte count, prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen. Clinical chemistry parameters included aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), total cholesterol (TC), triglycerides (TG), phospholipids (PL), total bilirubin (BIL), glucose (GLU), blood urea nitrogen (BUN), creatinine (CRE), Na^+, K^+, Cl^-, Ca^{2+}, inorganic phosphate, total protein, albumin (Alb), albumin/globulin ratio (A/G), and protein fractions (globulin). Standard methods were used in the analyses.

The following organs were weighed: brain, pituitary, thyroids (including parathyroids), salivary glands (submandibular and sublingual), adrenals, thymus, spleen, heart, lungs (including bronchus), liver, kidneys, testes, epididymis, seminal vesicle, prostate, ovaries, and uterus. In addition to these organs, the following organs or tissues were also preserved, usually in 10% formalin, embedded in paraffin, sectioned and stained: cerebrum, cerebellum, spinal cord (cervical, thoracic, and lumbar),

[1] One lipase unit is defined as the amount of enzyme that can release one-tenth μmol equivalent of fatty acid per minute upon incubation of olive oil with the enzyme solution at 37 °C.

sciatic nerve, eyeball, optic nerve, Harderian gland, submandibular lymph node, mesenteric lymph node, thoracic aorta, trachea, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, urinary bladder, vagina, mammary glands, bone and bone marrow (sternum and femur), femoral muscle, skin (inguinal), oviduct, zymbal's gland, and any organ or tissue having gross lesions. In addition, the ear (auricle), larynx, and nasal cavity were fixed and preserved but not sectioned. Organ and tissue sections from all animals in the control and highest dose groups in the main and recovery groups and organs showing macroscopic lesions in any dose group were examined microscopically. Because of the role of the pancreas in lipase synthesis and the kidney in processing products of lipase metabolism and excess lipase protein, the kidney and pancreas of all males were examined microscopically.

2.2.1. Statistical analysis

Numerical data obtained for body weights, food consumption, water intake, urinalysis, hematology, clinical chemistry, and organ weights were analyzed for homogeneity of variance using Bartlett's test (level of significance, 1% two-tailed); and significant differences between the groups were analyzed using Dunnett's test. Heterogeneous data were assessed using a Dunnett-type method to compare the difference of the group mean rank between the control group and each dose group (level of significance: 5 and 1%, two-tailed). If the number of groups was 2 (control group and 1 recovery group), data were analyzed for homogeneity of variance using the *F*-test (levels of significance: 5%, one-tailed). For homogeneous data, significant differences between the groups were analyzed by Student's *t* test. Heterogeneous data were analyzed using Aspin–Welch's *t* test (levels of significance: 5 and 1%, two-tailed, for both tests). For qualitative data of urinalysis, comparison was made between the control group and each dose group by a cumulative χ^2 method (level of significance, 5 and 1% two-tailed).

2.3. Reverse mutation test in bacteria (Ames assay)

Mutagenicity of Lipase D was evaluated in four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and in *E. coli WP2uvrA*. The tests were conducted according to OECD Guidelines (OECD, 1995); the Japanese Ministry of Labor (JML, 1985); and the Japanese Ministry of Health and Welfare (JMHW, 1989). In a preliminary test, no growth inhibition was observed by Lipase D (activity 2,784,000 U/g) on any of the tester strains, with or without metabolic activation, at the highest level tested, 5000 μg/plate, the OECD recommended maximum test concentration for non-cytotoxic substances. Therefore, in the final tests, 5000 μg/plate was selected as the maximum test dose, with lower doses decreasing in half multiples (0, 313, 625, 1250, and 2500 μg/plate). Plating was in triplicate at each dose, and two independent assays were conducted. Positive controls consisted of 2-(2-furyl)-3-(5-nitro-2-furyl)-3-(5-nitro-2-furyl)acrylamide for the TA98 and TA100 strains, 9-aminoacridine hydrochloride for the TA1537 strain, sodium azide for the TA1535 strain, *N*-ethyl-*N′*-nitro-*N*-nitrosoguanidine for the *E. coli* strain, and 2-aminoanthracene for all tester strains in the presence of metabolic activation (S-9 mix). The substance was judged to be mutagenic when the mean number of revertant colonies on the plates containing the test substance was two or more times the mean number of revertant colonies on plates containing the solvent control (DMSO) and the number of revertant colonies was dose-related.

2.4. Mouse lymphoma cell gene mutation assay

Lipase D (activity 2,046,000 U/g) was tested for its potential to induce forward mutations at the thymidine kinase locus (TK+/−) in mouse lymphoma L5178 cells. The study conformed to guidelines of the OECD (OECD, 1984), US EPA (EPA, 1983), and the European Union (EEC, 1988).

In two independent tests, Lipase D was added to cell suspensions, at concentrations determined on the basis of a preliminary cytotoxicity test, and gently shaken for 3 h. Sterile deionized water served as the negative control. Methyl methanesulfonate (MMS) was the positive control in the absence of metabolic activation. 3-Methylcholanthrene (MC) was the positive control with metabolic activation. After 3 h, the cells were washed and then placed in a growth medium for 48 h to allow expression of induced mutants. Samples were checked for suspension growth at 24 and 48 h. At the end of this period, cells were assessed for viability by plating in triplicate onto agar plates, incubating for 12 days, and counting the colonies. Mutant frequencies were assessed in similar fashion except that the nutrient media contained trifluorothymidine (TFT), which only permitted growth of mutant TK−/− colonies.

2.4.1. Statistical analysis

Data were analyzed by weighted analysis of variance following the methods described by Arlett et al. (1989). Criteria for a positive response were: (1) an increase in mutant frequency in treated cultures of at least 100 mutants per 10^6 survivors, relative to the concurrent control; (2) a statistically significant increase in mutant frequency; (3) evidence of a dose relationship over at least two dose levels; (4) demonstration of reproducibility in any increase in mutant frequency; and (5) the observed increases in mutant frequency must lie outside the historical control range

with a corresponding relative total growth (RTG) of approximately 20%.

2.5. Chromosome aberration test

The potential of Lipase D to induce chromosome aberrations was tested using CHL/IU cells, derived from fibroblasts from the lungs of Chinese hamsters. The study was conducted in accordance with OECD guidelines (OECD, 1983) and guidelines of the Japanese Ministry of Health and Welfare (JMHW, 1989).

Cultured cells were incubated in the presence of Lipase D for 6 h, with and without S-9 mix, and, in a separate test, for 24 and 48 h without S-9 mix. In the 6-h test, cells were washed with phosphate buffered saline, then cultured in fresh medium for a further 18 h.

Based on a growth inhibition range-finding test, Lipase D (activity 2,660,000 U/g) treatment doses were set at a maximum 0.80 mg/mL for the 24- and 48-h treatment groups; a maximum of 0.10 mg/mL for the 6-h treatment with S-9 mix; and a maximum of 5.0 mg/mL for the 6-h treatment without S-9 mix. Positive controls were mitomycin C (0.05 μg/mL), for tests without metabolic activation, and cyclophosphamide (5 μg/mL), for tests with activation.

Four petri dishes were used for each dose. Two were used for measurement of cell confluency; chromosome specimens were prepared from the other two dishes. Two hours prior to harvesting, colcemid was added. Cells were harvested by centrifugation, followed by hypotonic treatment for 30 min with 0.075 M KCl, washing in fixative (3:1 solution of methanol and acetic acid), and air drying on glass slides. The dried cells were stained with 3% Giemsa solution, and 200 well-spread metaphases were observed for structural aberrations in each dose group (800 metaphases were examined for polyploidy).

Aberrations were recorded as structural aberrations and polyploid cells. Structural aberrations were classified as chromatid or chromosome gaps, chromatid or chromosome breaks, or chromatid or chromosome exchanges (Environmental Mutagen Society of Japan, 1988).

2.5.1. Statistical analysis

The number of cells bearing aberrations in the treated groups and the solvent control groups were compared using Fisher's exact probability test ($p < 0.05$) with Bonferroni's correction for multiple comparisons. In addition, the criterion developed by Ishidate was applied: the assay was judged "negative" if aberrations were below 5%, "equivocal" between 5 and 10%, and "positive" above 10% (Ishidate et al., 1984). The test substance was considered to be clastogenic if the frequency of the structural aberrations was statistically higher in treated cells than in controls and if the assay was positive by Ishidate's criterion.

2.6. Pathogenicity study on *Rhizopus oryzae*

Because Lipase D is an enzyme preparation derived from *R. oryzae*, pathogenicity studies were conducted on the particular strain. One common pathogenicity test involves intravenous injection of the organism into mice. Because adverse effects were observed upon intravenous injection into the tail vein, additional pathogenicity studies were performed reflecting more realistic routes of exposure. A series of five separate pathogenicity studies were conducted using SPF Slc:ICR female mice:

1. Spores of *R. oryzae* were injected once into the tail veins of 4 groups of 10 mice in doses ranging from 0 to 1.3×10^6 colony forming units (CFU) per animal.
2. Spores were administered in a single dose by gavage to 2 groups of 10 mice at doses of 0 or 1.3×10^7 CFU/animal.
3. Spores were incubated in culture medium for 3 days. The culture fluid was then administered in a single gavage dose to 2 groups of 10 mice at doses of 0 or 2.2×10^5 CFU/animal.
4. The same culture fluid was administered by gavage to 2 groups of 10 mice at doses of 0 or 2.2×10^5 CFU/animal daily for 28 days.
5. Three groups of mice received untreated feed, feed mixed with 1.7×10^5 CFU per gram of feed and subsequently sterilized, or feed containing the same concentration of viable spores for 28 days. Because the feed contained 25% of dried culture medium, the nutritional quality of the feed was expected to be lower; hence, the inclusion of the group receiving sterilized spore-mixed feed as a second control group. The diet was replaced with fresh feed every day.

In the single dose studies, animals were observed for clinical signs and mortality for 14 days after administration and weighed every 3 or 4 days. The same observations were performed over 28 days in the repeated dose studies.

Animals were sacrificed at the completion of the 14-day observation period for the single dose studies and at the end of the dosing period in the 28-day studies. The brain, lungs, liver, kidneys, and spleen were macroscopically examined and then examined for viable cell counts and histopathology. To determine viable cell counts, slices from these organs were imprinted on agar media and the imprints uniformly spread over the plates. After the media were incubated aerobically at 37 °C for 24 h, colony counts were performed. Histopathological examinations of organ sections were performed in the usual manner. In addition, PAS staining specimens were prepared for investigation of viable spores, germination and vital reaction around spores.

2.6.1. Statistical analysis

Group mean and standard deviation were calculated for animal body weights, food consumption, and viable cell count in each organ. The data were analyzed first by Bartlett's test. If homogeneity of variance was confirmed, the data were further analyzed by one-way F test. Otherwise, Kruskal–Wallis' test was used for further analysis. If a significant difference was found in either test, Dunnet's test was performed if the number of samples was equal in the groups and Scheffe's test was performed if the number of samples was different.

3. Results

3.1. Subchronic (13-week) oral toxicity study in rats

No deaths occurred during the administration or recovery periods, and there were no adverse clinical signs or ophthalmologic abnormalities. Mean food consumption and body weights did not differ significantly from the controls. There were no treatment-related changes in hematology parameters relative to the controls.

Statistically elevated levels of protein and ketone bodies were observed after 5 weeks in the urine of males receiving 2000 mg/kg body wt. Lipase D (Tables 1a, 1b). These effects were not observed in females receiving the same dose, nor were they present after 13 weeks or after recovery. An increase in sedimentary phosphate salts was observed in males in the 2000 mg/kg body wt. group, but the increase was not large, and a similar increase was observed in the control animals of the recovery group. The increase was not observed in females.

A decrease of urine pH was observed at 13 weeks in males dosed at 2000 mg/kg body wt. and in female rats dosed at 500 and 2000 mg/kg body wt. (Tables 1a, 1b). However, there were no changes in electrolytes in urine or serum, BUN, and creatinine levels were unaffected, kidney weights were normal, and no histopathological effects in the kidney were observed that could be related to treatment with the enzyme. Clinical chemistry parameters and mean relative organ weights are shown in Tables 2 and 3, respectively.

An increase in ALT after 13 week was observed in males in the 2000 mg/kg body wt. group ($p < 0.05$), but the increase was not large (14%) and there was no dose dependence. No macroscopic or microscopic changes were observed in the liver. There were no significant changes in BUN, NAG, CRE, GGT, or LDH, which can be markers of kidney damage.

Except for a decrease in the proportion of segmented neutrophils ($p < 0.05$) in males of the 1000 mg/kg body wt. group, mean hematology parameters from treated animals did not differ significantly from the corresponding controls.

Mean absolute organ weights of treated animals did not differ significantly from those of the controls. A decrease in mean relative weight of the heart ($p < 0.05$) was observed in males in the 2000 mg/kg body wt. group, and decreases in mean relative weights of the lung ($p < 0.05$) were observed in all treatment groups. The decreases were slight and there was no dose dependency (Table 3).

Slight or mild tubular basophilia was observed in the kidneys of 3 males in the control and 500 mg/kg body wt. groups, 4 males in the 1000 mg/kg body wt. group and 6 males in the 2000 mg/kg body wt. group. However, there was no increase in severity with dose, and this effect was observed in only 1 female (Table 4). Similarly, slight or mild eosinophilic bodies in the tubular epithelium were observed in 4 control males and in 6 males in the 2000 mg/kg body wt. group, but no cases were observed in females. Slight or mild fibrosis in the islet cells of the pancreas was observed in one male in the 500 mg/kg body wt. group and in 2 males in the 2000 mg/kg body wt. group but in no females. Occasional histological effects observed in other organs were sporadic in nature and not observed in both sexes (Table 4).

3.2. Reverse mutation test in bacteria (Ames assay)

Lipase D did not increase the number of revertant colonies at doses from 313 to 5000 μg/plate with or without metabolic activation.

3.3. Mouse lymphoma cell gene mutation assay

A total of four independent tests were performed, two without S-9 activation and two with S-9 activation. Results are shown in Table 5. In the first test without S-9, a dose-related increase in mutant frequencies was observed, but the increases were not statistically significant. In the second test, there was no dose-related increase in mutant frequency. Similarly, in the experiments with S-9, a dose-related increase in mutant frequencies was observed in the first test, but the increases were not statistically significant. There was no clear dose-related increase in the second test. In all cases, the positive controls, MMS and MC, produced a statistically significant increase in mutant frequencies. It is concluded that the tests did not demonstrate mutagenic potential.

3.4. Chromosome aberration test

Cells with structural aberrations or polyploid cells were not induced in any treatment group with Lipase D. Positive controls (MC or CPA) showed an increased frequency of structural aberrations.

Table 1a
13-Week oral toxicity study of Lipase D in rats selected urinalysis data

Sex	Dose (μg/kg body wt.)	*N*	Mean pH (13 week)	Protein (5 week) [1]				Protein (13 week) [1]					Ketone body (5 week) [2]			Ketone body (13 week) [2]		
				–	+–	+	++	–	+–	+	++	+++	–	+–	+	–	+–	+
Male	0	18	8.2	0	12	6	0	1	10	5	2	0	17	1	0	17	1	0
	500	12	7.8	2	8	2	0	2	6	3	1	0	10	2	0	12	0	0
	1000	12	7.9	0	7	5	0	2	4	5	1	0	9	1	2	12	0	0
	2000	18	7.7[a]	0	5	11	2[a]	1	7	9	0	1	10	5	3[b]	16	2	0
Female	0	18	7.8	7	6	5	0	12	5	1	0	0	14	4	0	18	0	0
	500	12	7.2[a]	7	5	0	0	4	7	0	1	0	12	0	0	11	0	1
	1000	12	7.8	9	3	0	0[a]	3	8	1	0	0	11	1	0	11	1	0
	2000	18	6.8[b]	10	5	3	0	7	10	1	0	0	15	2	1	18	0	0

[1] –, 0–10 mg/dL; +–, 10–20 mg/dL; +, 20–70 mg/dL; ++, 70–200 mg/dL; +++, 200–400 mg/dL [2] –, 0–5 mg/dL; +–, 5–10 mg/dL; +, 10–20 mg/dL; ++, $>$ 20 mg/dL.
[a] $p < 0.05$.
[b] $p < 0.01$.

Table 1b
13-Week oral toxicity study of Lipase D in rats selected urinalysis data at 13 weeks

Sex	Dose (μg/kg body wt.)	*N*	Water intake (mL/24 h)	Urine volume (mL/24 h)	Osmolality (mOsm/kg)	Na (mmol/24 h)	K (mmol/24 h)	Cl (mmol/24 h)	NAG (IU/24 h)
Male	0	18	39 ± 7	14.3 ± 5.0	1852 ± 369	1.95 ± 0.66	3.71 ± 1.07	2.38 ± 0.75	0.235 ± 0.116
	500	12	42 ± 10	16.1 ± 3.4	1884 ± 298	2.22 ± 0.51	4.31 ± 0.77	2.77 ± 0.53	0.313 ± 0.105
	1000	12	39 ± 7	14.1 ± 4.5	2085 ± 332	2.07 ± 0.63	4.00 ± 1.03	2.53 ± 0.62	0.255 ± 0.092
	2000	18	41 ± 9	12.8 ± 5.6	2127 ± 363	1.64 ± 0.61	3.35 ± 1.09	2.13 ± 0.74	0.278 ± 0.152
Female	0	18	37 ± 8	10.8 ± 3.7	1826 ± 398	1.36 ± 0.26	2.73 ± 0.53	1.72 ± 0.35	0.171 ± 0.061
	500	12	37 ± 9	8.9 ± 3.6	1802 ± 325	1.03 ± 0.33[a]	2.19 ± 0.59	1.34 ± 0.42	0.136 ± 0.055
	1000	12	39 ± 9	10.9 ± 4.9	1698 ± 447	1.24 ± 0.41	2.33 ± 0.75	1.51 ± 0.57	0.149 ± 0.043
	2000	18	36 ± 9	9.4 ± 5.2	1958 ± 375	1.15 ± 0.46	2.26 ± 0.90	1.47 ± 0.59	0.144 ± 0.055

[a] $p < 0.05$.

Table 2
13-Week oral toxicity study of lipase D in Sprague–Dawley rats selected clinical chemistry values (13-weeks)

Dose (mg/kg)		AST (IU/L)	ALT (IU/L)	LDH (IU/L)	ALP (IU/L)	GGT (IU/L)	TC (mg/dL)	TG (mg/dL)	PL (mg/dL)	BIL (mg/dL)	GLU (mg/dL)	BUN (mg/dL)	CRE (mg/dL)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	Ca (mg/dL)	P (mg/dL)	Alb (g/dL)	A/G
Males ($N = 12$)																				
0	Mean	58	37	46	162	2.2	63	58	97	0.10	136	16	0.63	145	4.7	110	9.5	6.7	2.6	0.73
	SD	8	6	20	31	0.4	14	22	15	0.01	10	3	0.09	1	0.3	3	0.3	1.1	0.2	0.08
500	Mean	45*	39	42	159	2.1	67	99**	107	0.11	145	15	0.62	144	4.5	110	9.6	6.8	2.7	0.71
	SD	13	5	13	28	0.2	15	45	18	0.02	14	2	0.05	1	0.3	3	0.3	1.0	0.1	0.04
1000	Mean	51	38	38	178	1.9	62	82	100	0.11	138	15	0.61	144	4.6	110	9.6	7.1	2.6	0.72
	SD	11	4	10	39	0.2	20	32	21	0.02	16	2	0.06	1	0.2	1	0.2	0.6	0.1	0.07
2000	Mean	55	42*	50	183	2.0	65	69	102	0.10	140	16	0.62	145	4.6	110	9.5	7.1	2.7	0.71
	SD	13	6	18	37	0.2	16	21	19	0.01	16	1	0.06	1	0.3	1	0.4	1.1	0.2	0.06
Females ($N = 12$)																				
0	Mean	70	44	27	94	2.0	77	35	137	0.09	125	18	0.69	143	4.7	112	9.5	5.9	3.1	0.88
	SD	23	21	11	26	0.2	16	7	22	0.01	11	2	0.06	1	0.3	1	0.2	1.0	0.2	0.06
500	Mean	61	36	21	76	1.9	72	36	129	0.09	124	18	0.66	143	4.6	113	9.4	5.7	3.1	0.87
	SD	11	5	4	20	0.6	17	9	24	0.01	11	3	0.05	1	0.3	1	0.2	1.2	0.2	0.09
1000	Mean	70	42	27	92	2.1	82	38	145	0.09	122	18	0.68	142	4.7	113	9.4	5.9	3.0	0.86
	SD	24	15	16	31	0.4	15	8	16	0.01	7	3	0.06	1	0.2	2	0.3	1.3	0.1	0.07
2000	Mean	69	44	24	93	2.2	80	37	137	0.09	122	18	0.68	142	4.5	112	9.4	6.0	3.0	0.83
	SD	14	14	4	28	0.4	18	7	24	0.01	11	3	0.06	1	0.3	2	0.2	1.6	0.2	0.06

* $p < 0.05$.
** $p < 0.01$.

3.5. Pathogenicity studies on *Rhizopus oryzae*

No deaths or adverse clinical signs were observed in any of the animals orally receiving *R. oryzae.*

In the 28 day feeding study, weight gain was significantly decreased ($p < 0.05$) in the non-sterilized spore-mixed feed administration group relative to the controls (6.7 versus 9.7 g), but not relative to the animals receiving sterilized spore-mixed feed (8.4 g). Mean feed consumption was higher in the groups receiving the sterilized and non-sterilized spore-mixed feed (5.55 and 5.66 g, respectively, versus 5.19 g for the normal feed group). As reduced weight gain was not observed from repeated dosing of culture fluid, the observed weight reduction in the feeding study is considered to be due to the lower nutritional value of the treated feed. Viable spores were not found in the examined organs. Although occasional histopathological lesions were found in these organs, they could not be related to treatment with the fungi.

In the intravenous study, no death or changes in general conditions were found in the controls and in animals receiving the two lower doses. Mean body weights of the lowest dose group did not differ significantly from controls. In the mid dose group (1.3×10^5 CFU/animal), a significant weight loss was observed on day 7 compared to the controls, but after day 10 there were no significant differences. In the highest dose group (1.3×10^6 CFU/animal), significant weight lowering was observed on days 7 and 10, but there was no significant difference from controls by day 14. Four animals died when 1.3×10^6 CFU were administered. The six surviving animals demonstrated piloerection until day 7, after which no symptoms were noted. Viable spores were found at all doses in the examined organs, except in the brains of animals receiving the lowest dose (Table 6). Histopathological changes were observed in some of the organs in which viable spores were found, and fungal threads were found in the brain of one animal receiving the lowest dose.

4. Discussion

Exposure of rats to Lipase D in feed at dietary levels of 0, 500, 1000, and 2000 mg/kg body wt. for 13 weeks resulted in no deaths, adverse clinical signs, changes in feed consumption, changes in mean body weights, ocular changes, or changes in hematology parameters.

Significant increases in protein ($p < 0.05$) and ketone bodies ($p < 0.01$) in the urine of males receiving 2000 mg/kg body wt. for 5 weeks were not observed at 13 weeks or after the recovery period, and the increases were not observed in females. An increase in sedimentary phosphate, observed in males of the 2000 mg/kg body wt. group at 13 weeks, was not large and not observed in

Table 3
13-Week oral toxicity study of Lipase D in Sprague–Dawley rats mean absolute and relative organ weights (13-weeks)

Dose (mg/kg)		Body weight (g)	Brain (g)	Pituitary (mg)	Thyroid (R + L) (mg)	Salivary gland (R + L) (mg)	Thymus (mg)	Heart (g)	Lung (g)	Liver (g)	Spleen (g)	Kidney (R + L) (g)	Adrenal (R + L) (mg)	Testis (R + L) (g)	Epidid-ymis (R + L) (mg)	Seminal vesical (g)	Prostate (g)
Males ($N = 12$)																	
Absolute organ weights (g or mg)																	
0	Mean	488	2.10	11.9	20.8	665	252	1.40	1.50	11.68	0.76	3.56	57	3.25	1.28	1.38	1.36
	SD	42	0.09	1.1	3.5	67	54	0.12	0.13	1.23	0.16	2.09	7	0.19	0.056	0.22	0.23
500	Mean	512	2.11	11.9	20.4	686	280	1.40	1.47	13.58	0.79	3.16	59	3.14	1.26	1.40	1.35
	SD	51	0.07	1.6	2.6	75	50	0.09	0.13	2.00	0.09	0.24	6	0.21	0.08	0.17	0.18
1000	Mean	526	2.11	12.2	21.3	648	307	1.45	1.51	13.67	0.81	3.37	58	3.27	1.21	1.48	1.31
	SD	62	0.10	1.3	3.9	58	70	0.14	0.17	2.37	0.13	0.33	9	0.25	0.10	0.18	0.20
2000	Mean	517	2.10	12.3	20.9	699	272	1.36	1.47	13.26	0.78	3.34	59	3.26	1.30	1.49	1.37
	SD	65	0.08	0.9	3.3	93	49	0.14	0.14	2.33	0.11	0.43	10	0.30	0.11	0.17	0.23

Relative organ weights (g or mg per 100 g body wt.)

Dose (mg/kg)		Brain (g)	Pituitary (mg)	Thyroid (R + L) (mg)	Salivary gland (R + L) (mg)	Thymus (mg)	Heart (g)	Lung (g)	Liver (g)	Spleen (g)	Kidney (R + L) (g)	Adrenal (R + L) (mg)	Testis (R + L) (g)	Epidid-ymis (R + L) (mg)	Seminal vesical (g)	Prostate (g)
0	Mean	0.43	2.5	4.3	137	52	0.29	0.31	2.39	0.15	0.73	12	0.67	264	0.28	0.28
	SD	0.03	0.2	0.8	15	14	0.01	0.02	0.12	0.02	0.43	1	0.06	28	0.05	0.04
500	Mean	0.42	2.4	4.0	135	55	0.27	0.29*	2.65**	0.16	0.62	12	0.62	247	0.28	0.26
	SD	0.04	0.3	0.5	16	10	0.02	0.02	0.19	0.02	0.04	2	0.07	26	0.04	0.04
1000	Mean	0.40	2.3	4.1	124	59	0.28	0.29*	2.59*	0.16	0.64	11	0.63	246	0.29	0.25
	SD	0.05	0.3	0.8	13	12	0.02	0.02	0.19	0.02	0.06	2	0.08	32	0.05	0.04
2000	Mean	0.41	2.4	4.1	136	53	0.27*	0.29*	2.56	0.15	0.65	11	0.64	253	0.29	0.27
	SD	0.06	0.3	0.6	20	9	0.01	0.02	0.21	0.02	0.04	1	0.06	28	0.06	0.06

Females ($N = 12$)

Absolute organ weights (g or mg)

Dose (mg/kg)		Body weight (g)	Brain (g)	Pituitary (mg)	Thyroid (R + L) (mg)	Salivary gland (R + L) (mg)	Thymus (mg)	Heart (g)	Lung (g)	Liver (g)	Spleen (g)	Kidney (R + L) (g)	Adrenal (R + L) (mg)	Ovary (R + L) (mg)	Uterus (mg)
0	Mean	286	1.99	15.9	18.0	437	262	0.89	1.12	7.04	0.49	1.96	65	77.7	616
	SD	18	0.10	3.5	3.4	37	63	0.06	0.06	0.66	0.09	0.22	9	9.0	170
500	Mean	302	1.95	17.0	17.1	443	275	0.93	1.15	7.16	0.53	1.91	68	77.7	599
	SD	29	0.07	2.5	3.0	42	51	0.08	0.09	0.80	0.08	0.16	10	7.4	152
1000	Mean	286	1.94	16.8	18.5	423	231	0.89	1.12	7.06	0.50	1.88	72	77.3	662
	SD	24	0.09	2.8	5.1	42	48	0.06	0.06	0.47	0.06	0.13	8	10.7	138
2000	Mean	298	1.99	17.5	18.1	425	237	0.91	1.15	7.30	0.53	1.98	72	78.9	574
	SD	32	0.07	2.8	4.0	47	53	0.10	0.09	0.78	0.06	0.20	9	10.8	102

females. A similar increase was observed in males in the control recovery group. Therefore, these observations are not considered treatment related. A decrease in urine pH was observed at 13 weeks in males of the 2000 mg/kg body wt. group and in females of the 500 and 2000 mg/kg body wt. groups. Since there were no changes in electrolytes in urine or serum or in relevant clinical chemistry parameters, no changes in mean kidney weights, and no treatment-related histological changes in the kidney, the changes in pH are not considered to be toxicologically significant but rather the result of processing increasing concentrations of metabolites.

An increase in ALT, observed after 13 weeks in males of the highest dose group, was not large (14%), and no dose dependent increase was evident. As there was no similar effect in females, and there were no macroscopic or microscopic changes in the liver, the increase is not considered to be related to treatment. There were no significant changes in AST, GGT, or ALP, which may also be indicators of liver toxicity.

Decreases in mean relative weights of the heart and lung, observed in males in the 2000 mg/kg body wt. group, were very slight and not dose dependent (Table 3). The changes in absolute weights were not significant. Hence, the effects are not considered to be treatment-related.

On histological examination, slight or mild tubular basophilia was observed in the kidneys of 3 males in the control and 500 mg/kg body wt. group, 4 males in the 1000 mg/kg body wt. group, and 6 males in the 2000 mg/kg body wt. group. Since the effects were slight and did not increase in severity, and the effect was only observed in one female, the effect was not considered to be treatment-related. The incidence of tubular basophilia in males in the 2000 mg/kg body wt. group is within the range observed in control animals of the same strain and age in the testing laboratory, and is a frequent spontaneous finding in young control Sprague–Dawley rats (Peter et al., 1986). Slight or mild eosinophilic bodies in the tubular epithelium were observed in 4 control males and in 6 males in the 2000 mg/kg body wt. group, but no cases were observed in males in the intermediate dose groups, nor in females. Therefore, the effect is not likely related to treatment. Slight or mild fibrosis in the islet cells of the pancreas was observed in one male in the 500 mg/kg body wt. group and in 2 males in the 2000 mg/kg body wt. group but did not occur in the 1000 mg/kg body wt. group nor in any of the females.

Although the reduced urinary pH and histopathological effects observed in male rats at the highest dose are probably not toxicologically significant, the no-observable-effect level is conservatively set at 1000 mg/kg body wt. The NOEL for female rats is 2000 mg/kg body wt.

In common with most enzyme preparations, Lipase D is not genotoxic.

Relative organ weights (g or mg per 100 g body wt.)

Dose (mg/kg)		Brain (g)	Pituitary (mg)	Thyroid (R + L) (mg)	Salivary gland (R + L) (mg)	Thymus (mg)	Heart (g)	Lung (g)	Liver (g)	Spleen (g)	Kidney (R + L) (g)	Adrenal (R + L) (mg)	Ovary (R + L) (mg)	Uterus (mg)
0	Mean	0.70	5.6	6.3	153	91	0.31	0.39	2.46	0.17	0.68	23	27.2	216
	SD	0.04	1.1	1.1	13	21	0.01	0.02	0.12	0.03	0.05	2	3.2	57
500	Mean	0.65	5.6	5.7	147	92	0.31	0.38	2.37	0.18	0.63	23	26.0	198
	SD	0.05	0.8	1.0	12	19	0.02	0.03	0.12	0.03	0.04	4	4.0	48
1000	Mean	0.68	5.9	6.5	149	81	0.31	0.40	2.47	0.17	0.66	26	27.2	233
	SD	0.06	1.0	2.0	16	13	0.03	0.03	0.13	0.02	0.05	3	4.4	47
2000	Mean	0.67	5.9	6.1	143	80	0.31	0.39	2.45	0.18	0.67	24	26.8	194
	SD	0.07	0.8	1.4	15	19	0.02	0.03	0.16	0.02	0.07	3	4.9	37

*$p < 0.05$; $p < 0.01$.

Table 4
13-Week oral toxicity study of Lipase D in rats selected histopathological findings*

Organ	Finding	Sex	Dose (mg/kg body wt.)			
			0	500	1000	2000
Kidney	Slight or mild tubular basophilia	Male	3/12	3/12	4/12	6/12
		Female	0/12	0/12	0/12	1/12
Kidney	Slight or mild eosinophilic body	Male	4/12	0/12	0/12	6/12
		Female	0/12	—	—	0/12
Kidney	Mild pelvic dilatation	Male	0/12	0/12	0/12	0/12
		Female	0/12	—	—	1/12
Lung (Bronchus)	Mild focal pneumonia	Male	0/12			0/12
		Female	0/12			1/12
Pancreas	Slight or mild islet fibrosis	Male	0/12	1/12	0/12	2/12
		Female	0/12	—	—	0/12
Stomach	Slight or mild erosion, glandular stomach	Male	—	—	2/2**	—
		Female	3/12	1/1**	2/2**	4/12
Prostate	Slight or mild interstitial cell infiltration	Male	3/12	—	—	4/12

* Listed when findings in treated animals in at least one sex exceeded findings in controls.
** Examined after macroscopic finding.

Table 5
Mouse lymphoma L5178Y cell mutation assay

	Test 1			Test 2		
	Concentration Lipase D concentrate (μg/mL)	Mean RTG (% Control)	Mean mutant frequency per 10^6 survivors	Concentration Lipase D concentrate (μg/mL)	Mean RTG (% Control)	Mean mutant frequency per 10^6 survivors
(−)S9	0	100	139	0	100	150
	80	103	156	120	96	152
	100	72	179	140	81	145
	120	50	213	150	66	139
	140	25	136	160	67	148
	MMS (10 μg/mL)	44	495***	MMS (10 μg/mL)	115	369***
(+)S9	0	100	158	0	100	172
	5	79	159	1	67	168
	10	56	188	2.5	57	182
	15	31	226	5	33	204
	25	18	226	10	19	142
	MC (2.5 μg/mL)	43	923***	MC (2.5 μg/mL)	7	999***

MMS, methyl methanesulfonate; MC, 3-methylcholanthrene.
*** $p < 0.001$.

Table 6
Viable fungi found in organs of mice after intravenous administration of *R. oryzae*

CPU *R. oryzae*/body	Number of mice	Number of remaining viable fungi per plate [number of animals having living fungi]				
		Brain	Lungs	Liver	Spleen	Kidneys
0	10	0	0	0	0	0
1.3×10^4	10	0.0 ± 0.0 [0]	0.3 ± 0.7 [2]	2.4 ± 1.4 [10]	1.4 ± 1.5 [7]	0.4 ± 0.7 [3]
1.3×10^5	10	0.2 ± 0.4 [2]	1.4 ± 2.4 [4]	$12.1 \pm 4/8$ [10]	13.9 ± 4.0 [10]	1.0 ± 1.1 [6]
1.3×10^6	6	0.3 ± 0.8 [1]	12.8 ± 2.3 [6]	17.3 ± 4.1 [6]	19.2 ± 4.8 [6]	4.8 ± 3.0 [6]

Although the manufacturing process of the enzyme preparation ensures that *R. oryzae* will not be present in Lipase D used in food processing, a series of pathogenicity tests were carried out to assess the potential for toxic effects on employees engaged in the manufacture of the preparation. *R. oryzae* has been identified as

causing zygomycosis, also known as mucormycosis, a generally rare infection in humans and animals (Ellis, 1998). The infection has occurred in acidotic diabetics, malnourished children, and severely burned patients, among other immune-compromised individuals. It is not seen in otherwise healthy subjects. The reported potential of *R. oryzae* to cause zygomycosis does not impact our safety evaluation of Lipase D because viable production organisms will not be present in the enzyme preparation or in processed foods. Moreover, even if *R. oryzae* strain 33158 is assumed capable of causing a zygomycosis infection, the infection does not occur in healthy subjects, and the risk of zygomycosis infection in workers exposed to the production organism therefore appears slight. Along with many other fungi, *R. oryzae* is ubiquitous in the environment, and stringent measures are taken in the enzyme industry to ensure that cross-contamination does not occur. Therefore, exposure of production workers to any strain of *R. oryzae*, including the production strain, is much less likely than exposure in the general population.

In the pathogenicity testing described above, treatment-related adverse effects were observed only from intravenous administration. When spores were injected into the tail vein, abnormal symptoms, deaths, decreased weight, and viable fungal cells were observed in all the treated groups. Based on the number of deaths at the highest treatment group (4 out of 10 mice), the LD_{50} is greater than 1.3×10^6 CFU/animal. In contrast, the intravenous LD_{50}'s for known pathogenic fungi, *R. oryzae* (NRRL 3133), *Mucor pusillus* (UI-I, University of Iowa), *R. chinensis* (NRRL 2904), and *R. cohnii* (ATCC 24793) are reported to be 1.7×10^3, 5.8×10^3, 1.3×10^3, and 0.4×10^3 CFU/animal, respectively (Kitz et al., 1983). The LD_{50} of *Aspergillus tamarii*, historically used to ferment foods, such as soy sauce and miso, and known to be non-pathenogenic, is reportedly 1.4×10^5 CFU/animal (Goto et al., 1991), almost 10 times lower than that found for the tester strain of *R. oryzae*.

Although injection of fungi is useful for comparison with other strains, the test should not be considered a measure of true pathogenicity (Pariza and Johnson, 2001). Infections may be produced by many otherwise harmless organisms if they penetrate host barriers, such as intact skin. A true pathogen must be able to cross or evade these barriers. The high intravenous LD_{50} in mice and the lack of any observable oral toxicity in four independent studies indicate that the strain can be handled using ordinary safety practices current in the fermentation industry.

Inhalation exposure to enzymes, particularly proteases, has been associated with allergic reactions in sensitive individuals. The history of this particular organism in the food industry as well as the safe commercial use of Lipase D in Japan for over 5 years without incident suggests that allergy due to this enzyme is not of especial concern. In the enzyme producing industry, due to the potential for cross-contamination, particulate exposure is minimized.

5. Conclusion

It is concluded that the Lipase D enzyme preparation is safe for use as a processing aid in food. Residues of the denatured enzyme in finished food will be in the parts per million range, and dietary exposure will not exceed a few milligram per person per day. The studies conducted on Lipase D satisfy the basic data requirements for the evaluations of enzymes derived from microbial sources. Lipase D is not genotoxic, and the NOAEL in the 90-day feeding study was 1000 mg/kg body wt. day.

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