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Safety Evaluation of Lipase Produced from *Candida rugosa:* Summary of Toxicological Data

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The toxicity of lipase AY, an enzyme preparation used in lipid hydrolysis to produce flavors, was evaluated in a series of studies. A 13-week dietary toxicity study in Sprague–Dawley (Crj:CD) rats was conducted in which animals received lipase AY in the feed at concentrations of 0, 625, 1250, or 2500 mg/kg body wt. No adverse treatment-related effects were observed. Lack of genotoxic potential was demonstrated by the results of an *in vitro* reverse mutation assay in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and in *Escherichia coli* strain WP2 *uvrA*, by an *in vitro* forward mutation assay in L5178Y mouse lymphoma cells, and by an *in vitro* chromosome aberration test in CHL/IU cells derived from fibroblasts of the lungs of Chinese hamsters. Finally, the particular strain of *Candida rugosa*, the yeast strain used to prepare lipase AY, has been shown to be nonpathogenic upon a single injection into the tail vein of rats of viable spores at doses up to 1.5×10^7 colony-forming units per animal. The results of these studies demonstrate that the enzyme preparation may be considered safe to workers and consumers when employed in the production of flavors from fats.

INTRODUCTION

Lipase AY, or triacylglycerol lipase (EC 3.1.1.3), is an enzyme preparation intended for use as a processing aid in the food industry. The enzyme is produced from a selected strain of the naturally occurring yeast *Candida rugosa*. Lipase AY nonselectively hydrolyzes triglycerides to produce free fatty acids and glycerol. In particular, when acting on milk fat, the lipase enzyme releases free fatty acids that have desirable flavor characteristics. The enzyme has been used to prepare food flavoring ingredients from milk for about 20 years, both in Japan and the United States. Production of the desired flavor includes a heat treatment phase intended to inactivate the enzyme after it has achieved the desired modification.

Lipase derived from *C. rugosa* is listed in the 4th edition of the *Food Chemicals Codex* (Committee on Food Chemicals Codex, Institute of Medicine, 1996) as an enzyme used in dairy products, confectionery, and for flavor development in processed foods.

The present studies were conducted on lipase AY to investigate the toxic potential of the enzyme preparation as well as the potential of the production organism to induce a pathogenic response. The studies were done in accordance with guidelines issued by the Ministry of Health and Welfare of Japan and guidelines of the OECD.

MATERIALS AND METHODS

Preparation and Characterization of the Test Material

Lipase AY enzyme preparation for toxicity testing was produced according to typical procedures for producing such enzymes. When fermentation was complete, the fermentation broth was filtered to remove biomass solids. The filtrate was concentrated by ultrafiltration and filtered through a microfine ceramic filter. Ethanol was added to precipitate the enzyme, which was collected by centrifugation and dried under vacuum. The resulting solid, lipase AY concentrate (LAYO), was used as the test substance. A commercial product, lipase AY30, is prepared by blending the concentrate with dextrin to a desired activity of at least 30,000 units per gram. (One lipase AY unit is defined as the amount of enzyme preparation that can release one micromole equivalent of fatty acids per minute under assay conditions.)

Specifications for the test material are lipase activity, $\geq$30,000 units/g; arsenic, $\leq$3 ppm; lead, $\leq$10 ppm; heavy metals as lead, $\leq$40 ppm; coliforms, $\leq$30/g; anaerobic sulfite reductants, $\leq$50,000 germs/g; *Escherichia coli*, negative in 25 g; *Salmonella*, negative in 25 g; total microbial count, $\leq 5 \times 10^4$/g; antibacterial activity, negative; and mycotoxins, negative. Three batches of lipase AY30 tested negative for aflatoxins B_1, B_2, G_1, and G_2; ochratoxin A; sterigmatocystin; zearalenone; and T-2 toxin.

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Acute Oral Toxicity Studies in Rats and Mice

Groups of 10 male and 10 female Slc:ddy mice and 10 male and 10 female Slc:SD rats received a water suspension by gavage of 0, 1250, 2500, or 5000 mg/kg body wt Lipase AY Concentrate (activity 79,700 units/g). Animals were observed daily over 14 days for mortality and clinical signs. Body weights were recorded on days 0, 1, 2, 3, 7, 10, and 14. After the 14 days, the animals were killed under anesthesia, and the thoracic and abdominal viscera were visually examined.

Subchronic (13-Week) Toxicity Study in Rats

Primary test. Six groups of 10 male and 10 female Sprague–Dawley rats (Crj:CD), 6 weeks of age at the initiation of the dosing, received a water suspension of the test substance once daily by gavage (lipase AY concentrate, activity 79,700 units/g). Based on the results from the acute toxicity study, dosages were set at 0, 625, 1250, and 2500 mg/kg body wt. An additional two groups received 0 ("recovery control") or 2000 mg/kg body wt ("recovery") throughout the 13 weeks and were examined during a 30-day recovery period.

Repeat test. Due to a dose-dependent increase in potassium (K^+) excretion observed in both sexes, the test was repeated with males only. Two groups of 6 males received 0 or 2500 mg/kg body wt of the test substance for 90 days and were examined during a 60-day recovery period.

Primary test. Animals were observed for clinical signs at least once daily during the administration period and once daily, except holidays, during the recovery period. Body weights and feed consumption were recorded twice weekly. In the 13th week of the study and in the final week of the recovery period, an auricular reflex examination by Galton's whistle was performed on all animals. Eyes were examined daily. On the day of necropsy, all animals except those in the recovery groups had eye examinations under anesthesia, and photographs of the retina were taken after mydriasis was induced. Urine was collected from each animal on 1 day during Weeks 11–13 and, from animals in the recovery groups, during the 4th week of the recovery period; complete urinalysis was performed. Parameters included color, sodium, potassium, chloride, specific gravity, pH, total protein, glucose, ketone bodies, occult blood, bilirubin, urobilinogen, and sediment.

On the day after the end of the administration period and the recovery period, blood samples were taken from the posterior vena cava and from the abdominal aorta. After samples were taken, the animals were exsanguinated under anesthesia. All animals were subjected to complete necropsy after death. Hematology and serum clinical chemistry were conducted on all animals from each dose level using standard methods. Hematology parameters included erythrocyte count, leukocyte count, differential leukocyte, platelet count, prothrombin time, partial thromboplastin time, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and reticulocyte. Clinical chemistry parameters included total protein, albumin, creatinine, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase, alkaline phosphatase, glucose, total cholesterol, free cholesterol, cholesterol ester ratio, triglycerides, phospholipids, bilirubin, urea nitrogen, uric acid, calcium, sodium, potassium, chloride, and inorganic phosphorous.

The following organs were dissected and weighed: brain, pituitary gland, thyroid glands, submaxillary glands, thymus, heart, lungs, liver, spleen, kidneys, adrenal glands, testes, and seminal vesicles and prostate or ovaries and uterus. The weighed organs and the following additional organs were fixed in formalin and embedded with paraffin. Additional organs included in the histopathology examination were eyeballs, harderian glands, tongue, lacrimal glands, bronchia, trachea, aorta of the chest, esophagus, pancreas, stomach, duodenum, jejunum, ileum, colon, cecum, rectum, mesenteric lymph nodes, urinary bladder, sternum, spinal cord, femur (including bone marrow), skin (including mammary glands), vagina, and muscle of the thigh (including peripheral nerves). Organs from animals in the 2500 mg/kg body wt group, the control group, and the two recovery groups were examined microscopically.

Repeat test. Animals were observed for clinical signs daily, except holidays. Body weights and feed consumption were recorded twice weekly. Urine was collected 0–4 and 4–22 h after dosing on Days 30, 60, and 90 and on Days 30 and 60 during the recovery period. Urine was analyzed for Na^+, K^+, and Cl^-.

Statistical analysis. Significance of differences between various parameters for treated groups and controls was assessed by Student's *t*-test or modified (Aspin–Welch) *t* test.

Reverse Mutation Test on Bacteria

Mutagenicity was evaluated in four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and a strain of *E. coli* (WP2 *uvrA*) in accordance with the OECD guidelines (OECD, 1995a). In a preliminary test, lipase AY concentrate, having an activity of 153,000 units/g, did not induce mutations, with or without metabolic activation, in any of the tested strains and did not inhibit the growth of any of the strains at 5000 μg/plate. Therefore, the highest dose was set at 5000 μg/plate, and four lower doses were set by serial dilution. Plating was in triplicate at each dose level. The study was conducted with and without activation and was repeated to provide confirmatory data.

Positive controls consisted of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide for the TA100 and TA98 strains, 9-aminoacridine hydrochloride for the TA1537 strain, sodium azide for the TA1535 strain, N-ethyl-N′-nitro-N-nitrosoguanidine for the *E. coli* WP2 *uvrA* strain, and 2-aminoanthracene for all tester strains in the presence of metabolic activation. The substance was judged to be mutagenic if the mean number of revertants on the plates was two or more times the mean number of the negative control plates, and the number of revertants increased with increasing dose.

Chromosome Aberration Test

The potential of lipase AY to induce chromosome aberrations was tested using CHL/IU cells, derived from fibroblasts of the lungs of Chinese hamsters. The study was carried out in accordance with the OECD guidelines (OECD, 1995b). Based on a growth inhibition range-finding test, lipase AY solution concentrations were set at 1250, 2500, and 5000 μg/mL (lipase AY concentrate, activity 142,000 units/g). Mitomycin C was the positive control in the test conducted in the absence of metabolic activation; benzo[*a*]pyrene was the positive control in the test conducted with metabolic activation. Physiological saline was used as the negative control. Suspensions of CHL cells in a culture medium were seeded to petri dishes and incubated for 72 h. The test solution, the positive control, or the negative control were then added, and the media were incubated for 6, 24, or 48 h. In the 6-h test, after exposure to the test substance the cells were washed and incubated in fresh culture medium for an additional 18 h. The 6-h test was also carried out with metabolic activation using S-9 mix. Two hours before termination, colcemid was added and the incubation continued.

After incubation, trypsin was added to the culture media, and cells were separated by centrifugation. The cells were swollen with 0.075 M potassium chloride hypotonic solution, fixed in a 3:1 mixture of methanol and acetic acid, and air-dried on a glass slide. The dried cells were stained with 3.0% Giemsa staining solution, and 100 well-spread metaphases were analyzed microscopically at 600× or 1000× magnification. Cells from two petri dishes were examined. Chromosomal aberrations were recorded as structural aberrations or polyploid cells. Structural aberrations were classified as chromatid or chromosome gaps, chromatid or chromosome breaks, chromatid or chromosome exchange, and "other" (Environmental Mutagen Society of Japan, 1988). Significance of the differences between frequencies of cells with structural aberrations in the various treated groups and the positive and negative control groups was assessed using the χ^2 test. 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, 1987). The test substance was considered clastogenic if (1) the frequency of the structural or numerical aberrations was statistically higher in treated cells than in controls, (2) the assay was considered positive by Ishidate's criterion, and (3) the aberrations were dose dependent.

Forward Mutation at the Thymidine Kinase (TK) Locus in L5178Y Mouse Lymphoma Cells

The ability of lipase AY to induce a mutation at the TK locus of mouse lymphoma cells was evaluated in duplicate assays. L5178Y $tk^{+/-}$-3.7.2C cells were treated with 1.3, 1.8, 2.5, 3.5, or 5.0 mg/mL lipase AY concentrate (activity 153,000 units/g), based on a range-finding cytotoxicity test. The test was conducted with and without metabolic activation. Methylmethanesulfonate and cyclophosphamide served as positive controls for the nonactivation and activation assays, respectively. Cells were incubated in solutions of the test substance for 3 h and then washed and resuspended in nutrient media and seeded into flasks. After 48 h, the subcultured cells were suspended in nutrient media with 3 μg/mL trifluorothymidine (TFT), and 200 μL of the suspension was distributed into 96 multiwell plates (2000 cells/well). TFT-resistant colonies were counted for the mutation assay between days 11 and 13 after plating. Relative total growth (RTG), expressed as a percentage of control, was calculated from the cell growth rates during the 2 days of expression of the mutant phenotype and the relative cloning efficiency. Results of the assay were evaluated according to procedures developed by the United Kingdom Environmental Mutagen Society (UKEMS) (Robinson *et al.*, 1989).

Rat Pathogenicity Study on Production Organism

Because lipase AY is an enzyme derived from *C. rugosa*, the safety of the strain itself was evaluated by injection into mice. Spores of *C. rugosa* AYL-2 were shaken at 25°C for 25 h in a glucose-based medium. The yeast was separated by centrifugation, washed, and suspended in 25 mL saline. Viable cells were determined to be 1.5×10^8 colony-forming units (CFU)/mL. Three suspensions of 1.5×10^8, 1.5×10^6, and 1.5×10^4 CFU were prepared by dilution. Four-week-old SPF Slc:ICR mice received 0.1 mL of one of the three *C. rugosa* suspensions by injection into the tail vein, with 10 animals for each dose. An additional five mice served as controls. The animals were observed daily for a period of 14 days. At the end of this time, the mice were sacrificed, and independent sections of the brain, liver, and kidney were examined for viable yeast and histopathology.

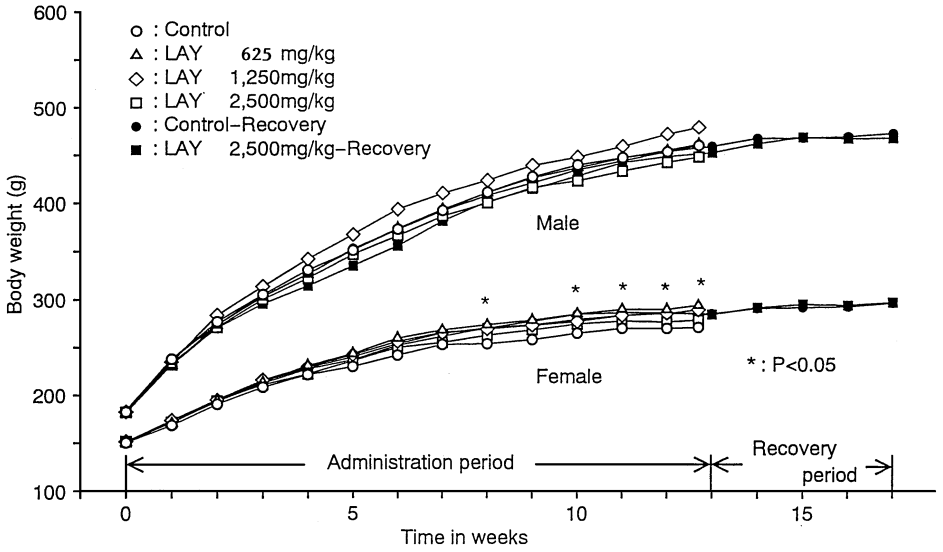

FIG. 1. Body weight changes in rats administered lipase AY orally for 13 weeks.

RESULTS

Short-Term Studies in Rats and Mice

No animals died during the test period. No effects on mean body weights could be ascribed to the test substance, and no adverse macroscopic findings were observed at autopsy.

Subchronic Toxicity Study in Rats

No deaths were observed in any of the animals in the primary test. Occasional injury of the upper jaw was found in almost all groups and ascribed to the stainless-steel inner lid of the feeder. In the repeat test, one animal from the 2500 mg/kg body wt group died from starvation (Day 54) resulting from the same type of injury to the upper jaw.

Mean weekly body weights for the primary test are shown in Fig. 1. In the primary test, mean body weights of females in the 625 mg/kg body wt group during the final 4 weeks were significantly higher than corresponding weights of the controls; but no such trend was observed in females from the two higher dose groups. Lower feed consumption relative to controls was observed in males from the 2500 mg/kg body wt group (Fig. 2), but reduced consumption was not observed with the recovery 2500 mg/kg body wt group nor in the animals of the repeat test.

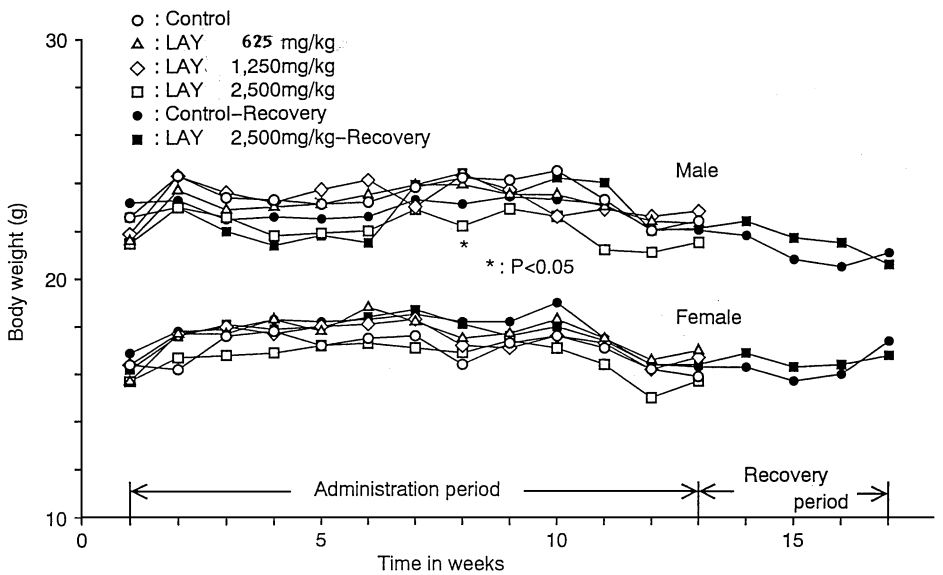

FIG. 2. Food consumption of rats administered lipase AY orally for 13 weeks.

TABLE 1
Selected Urinalysis Parameters in Sprague–Dawley Rats Treated with Lipase AY for 13 weeks

	Males (10 per group)				Females (10 per group)			
Parameter	0 mg/kg	625 mg/kg	1250 mg/kg	2500 mg/kg	0 mg/kg	625 mg/kg	1250 mg/kg	2500 mg/kg
Na^+(mEq/L)	29.5 ± 27.2[a]	14.0 ± 7.9	21.3 ± 16.2	39.7 ± 33.9	39.9 ± 18.6	23.7 ± 13.2*	30.0 ± 14.6	26.6 ± 13.7
K^+(mEq/L)	182 ± 109	227 ± 109	279 ± 83*	569 ± 183***	221 ± 118	357 ± 182	451 ± 218**	527 ± 264**
Cl^-(mEq/L)	44.5 ± 29.1	44.4 ± 16.8	59.7 ± 28.5	136.4 ± 88.9*	49.4 ± 13.5	58.6 ± 36.0	101.2 ± 61.6*	95.4 ± 60.9*
Occult blood[b]	1(1)	—	1(3)	1(1), 1(3)	—	—	1(4)	—
				Recovery period				
Na^+(mEq/L)	18.6 ± 19.3			35.7 ± 38.7	31.1 ± 14.0			24.7 ± 11.0
K^+(mEq/L)	195 ± 146			390 ± 153**	211 ± 124			585 ± 172***
Cl^-(mEq/L)	48.3 ± 30.6			83.3 ± 49.4	48.9 ± 35.1			82.7 ± 41.4
Occult blood	1(1)			2(1), 2(4)	—			—

[a] Mean ± SD.
[b] Occult blood, number of animals (severity): 0 = no effect; 1 = equivocal; 2 = low; 3 = moderate; 4 = heavy.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

No treatment-related auditory or ophthalmologic abnormalities were observed.

A significant and dose-dependent increase in mean K^+ concentrations was observed in the urine of males and females (Table 1). Elevated K^+ levels were also observed in the recovery groups. Occult blood was detected in one male and one female in the 1250 mg/kg body wt group, in one male in the 2500 mg/kg body wt group, and in two males from the 2500 mg/kg body wt recovery group, but the number of these occurrences is within historical controls. In the retest, it was found that the excess K^+ was excreted during the first 4 hours after administration of the dose; no significant difference from control values was observed in urine taken 4–22 h after administration. In contrast to the primary test, no significant differences in K^+ concentrations relative to controls were observed during the recovery period. The elevated K^+ concentrations can be explained by the presence of K^+ at 2.85% in the test substance.

Hematology parameters in male rats did not differ significantly from the corresponding control values. In female rats, the lymphocyte percentage of white blood cells was statistically elevated in the 625 and 1250 mg/kg body wt groups but the corresponding percentage in the control recovery group was higher. Percentage of neutrophils was significantly lower in the 1250 mg/kg body wt group, but the corresponding value in the control recovery group was even lower. Therefore, no toxicological significance is ascribed to these results.

Occasional differences in clinical chemistry parameters were observed (Table 2), but these were not dose dependent and within the normal ranges observed for this strain of rat in the testing laboratory. There were no treatment-related macroscopic changes in any of the dose groups suggestive of toxicity in rats.

Significant differences from controls were occasionally observed in absolute and relative organ weights, but there was no dose–response relationship. The mean absolute weight of the adrenal glands in males in the

TABLE 2
Selected Clinical Chemistry Parameters in Sprague–Dawley Rats Treated with Lipase AY for 13 Weeks

	Males (10 per group)				Females (10 per group)			
Parameter	0 mg/kg	625 mg/kg	1250 mg/kg	2500 mg/kg	0 mg/kg	625 mg/kg	1250 mg/kg	2500 mg/kg
Total cholesterol (mg/dL) [T]	34.8 ± 8.1[a]	35.1 ± 6.3	38.4 ± 6.2	35.3 ± 7.2	66 ± 19.9	55.7 ± 12.1	59.2 ± 10.2	50.0 ± 9.5*
Esterified cholesterol (mg/dL) [E]	29.4 ± 5.9	28.8 ± 4.9	32.1 ± 5.0	29.3 ± 5.5	50.9 ± 14.9	43.1 ± 9.2	45.9 ± 7.1	39.0 ± 7.7*
E/T	0.85 ± 0.03	0.82 ± 0.03*	0.84 ± 0.02	0.83 ± 0.05	0.76 ± 0.02	0.78 ± 0.02	0.78 ± 0.02	0.78 ± 0.03
Akaline phosphatase (ALP) (IU/L)	165.16	190 ± 31*	174 ± 30	196 ± 45	79.6 ± 19.5	83.2 ± 10.9	87.2 ± 10.5	88.0 ± 22.0
				Recovery period				
Total cholesterol (mg/dL)	38.6 ± 10.1			41.8 ± 9.7	60.7 ± 6.7			67.5 ± 12.9
Esterified cholesterol (mg/dL)	31.7 ± 7.3			34.2 ± 7.8	46.4 ± 4.4			51.2 ± 9.5
E/T	0.83 ± 0.04			0.82 ± 0.04	0.77 ± 0.02			0.76 ± 0.01
ALP(IU/L)	136 ± 16			138 ± 23	63.8 ± 7.5			67.0 ± 16.9

[a] Mean ± SD.
* $P < 0.05$.

TABLE 3
Selected Histopathological Findings in Sprague–Dawley Rats after 13-Week Treatment with Lipase AY

		Males (10 animals/group)				Females (10 animals/group)			
				Recovery (30 days)				Recovery (30 days)	
Organ	Finding	0	2500	0	2500	0	2500	0	2500
Eye	Cataract	0	0	0	0	0	3(1)	0	0
Pituitary gland	Adenoma	0	2(1)	0	1(1)	0	0	1(1)	0
Harderian glands	Vacuolar degeneration	1(1)[a]	2(1)	0	0	0	0	0	0
	Cell infiltration	1(1)	4(1)	1(2)	3(1), 1(2)	2(1), 1(2)	0	1(1)	0
Heart	Myocarditis	1(1)	0	1(1)	3(1)	0	0	0	0
Pancreas	Vacuolar degeneration	0	1(1)	0	0	0	0	0	2(1)
	Necrosis	0	0	0	0	0	0	0	2(1)
Spleen	Mononuclear cell leukemia	0	0	0	0	0	1(1)	0	0

[a] Number of animals with finding (mean severity). Mean severity: 1 = minimal; 2 = moderate; 3 = high.

2500 mg/kg body wt group was significantly lower than that of the control group but not significantly different from the mean weight in the control recovery group. The increased mean relative spleen weight observed in males in the 2500 mg/kg body wt group was lower than the mean relative spleen weight in the recovery controls. The reduced absolute and relative spleen weights observed in the female 2500 mg/kg body wt recovery group were within normal ranges observed in the laboratory for this strain of rats.

The occasional histopathological findings (Table 3) frequently develop with aging and are not considered to be related to the test substance. The finding of mononuclear cell leukemia, observed in the spleen from one female of the 2500 mg/kg body wt group, is judged to be incidental.

Reverse Mutation Test

No concentration-related increases in revertant colonies/plate were observed in any *Salmonella* strain or in the *E. coli* strain, either with or without exogenous metabolic activation (Table 4).

Chromosome Aberration Test

No significant increase in the frequencies of polyploid cells or structural aberrations was observed in cells treated with lipase AY concentrate, with or without metabolic activation, at any dosage level at any of

TABLE 4
Reverse Mutation Test with Lipase AY

	Mean Revertant Colonies/Plate with Strain									
	TA 100		TA 1535		WP2 *uvrA*		TA 98		TA 1537	
Concentration (μg/plate)	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9
0	180[a]	178	10	12	18	21	30	38	17	22
313	164	186	10	14	18	22	25	38	14	21
625	162	180	10	12	22	22	26	40	14	18
1250	160	186	11	12	20	23	28	40	17	20
2500	194	178	12	12	20	22	27	35	14	23
5000	184	190	10	14	18	22	28	43	18	21
Positive control[b]	701	517	254	236	1168	610	806	366	583	76

[a] Each result is the average from two experiments. Each experiment was carried out using triplicate plates.
[b] Positive controls: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, 0.01 μg/plate with strain TA 100 −S-9, 0.1 μg/plate with strain TA 98 −S-9; sodium azide, 0.5 μg/plate with strain TA 1535 −S-9; *N*-ethyl-*N′*-nitro-*N*-nitrosoguanidine, 2.0 μg/plate with strain WP2 *uvrA* −S-9; 9-aminoacridine hydrochloride,80 μg/plate with strain TA 1537 −S-9; 2-aminoanthracene, 1.0 μg/plate with strain TA 100 +S-9, 2.0 μg/plate with strain TA 1535 +S-9, 10.0 μg/plate with strain WP2 *uvrA* +S-9, 0.5 μg/plate with strain TA 98 +S-9, 2.0 μg/plate with strain TA 1537 +S-9.

TABLE 5
Chromosome Aberration Test on CHL/IU Cells Treated with Lipase AY (Average from two Petri Dishes)

Compound	Dose (μg/mL)	% Cell survival	% Polyploid cells	% Cells with aberrations	% Cell survival	% Polyploid cells	% Cells with aberrations
		With S-9 mix 6-h treatment			Without S-9 mix 6-h treatment		
Lipase AY	0	100	1.0	0.5	100	0.5	1.5
Lipase AY	1250	96	1.0	1.0	116	1.0	1.0
Lipase AY	2500	94	1.5	1.5	110.5	0.5	1.0
Lipase AY	5000	76	1.5	1.5	96.5	1.5	2.5
BaP	20		0.0	42.5*			
MMC	0.15		—	—		0.0	28.5*
		Without S-9 mix 24-h treatment			Without S-9 mix 48-h treatment		
Lipase AY	0	100	0.5	1.5	100	1.5	1.5
Lipase AY	1250	99	0.5	1.0	91.5	0.0	1.0
Lipase AY	2500	78	0.5	1.5	73	1.0	1.0
Lipase AY	5000	78	1.0	1.5	70.5	1.0	2.0
MMC	0.05		0.0	48*		0.0	55.5*

Note. BaP, benzo[*a*]pyrene. MMC, mitomycin C.
* Significantly different from control ($P < 0.05$) and positive by Ishidate's criteria.

the three incubation times. The maximum percentage of aberrations of a given type was 2.5% gap aberrations in the unactivated 5000 μg/mL group (Table 5). No dose dependence was evident.

Forward Mutation in Mouse Lymphoma Cells

A dose-related increase in cytotoxicity (decrease in RTG) was observed in the absence of metabolic activation (Table 6). In the presence of S-9 mix, a decrease in RTG was noted at the highest dose, but there was no clear dose response. None of the cultures treated with lipase AY and cloned in the presence or absence of metabolic activation exhibited a mutant frequency statistically different from that of the controls. The positive controls produced the expected high mutant frequencies.

TABLE 6
Results of Mutation Assay of L5178Y Cells Treated with Lipase AY for 3 h[a]

Concentration (mg/mL)	Without S-9 mix RTG	Without S-9 mix MF ($\times 10^{-6}$)	Without S-9 mix Small colony ratio	Without S-9 mix RTG	Without S-9 mix MF ($\times 10^{-6}$)	Without S-9 mix Small colony ratio (%)
0	100	77.8	34.8	99.8	76.4	46.4
1.3	91.8	77.4	29.2	89.0	71.6	43.4
1.8	95.4	78.4	46.0	97.9	82.8	39.6
2.5	80.8	69.0	29.6	98.6	80.0	48.0
3.5	61.4	86.8	46.1	91.5	88.6	44.6
5	42.2	122.2	47.0	77.6	84.6	56.0
MMS(0.01)	59.3	503.3	60.6	—	—	—
CAA(0.002)	—	—	—	65.2	524.4	64.8

Note. RTG, relative total growth; MF, mutant frequency; MMS, methyl methanesulfonate; CAA, cyclophosphamide.
[a] Values are averages of two experiments, two replicates per experiment.

Pathogenicity Test on Production Organism

No clinical signs were observed in any of the animals during the 14-week observation period. No living yeast was found in any of the organs examined. No histopathological abnormalities were apparent. Therefore, *C. rugosa* AYL-2 has no pathogenic potential.

DISCUSSION AND CONCLUSIONS

Exposure of rats to lipase AY concentrate in the feed at levels of 625, 1250, and 2500 mg/kg body wt for 13 weeks yielded no signs of overt toxicity. Results evaluated and found to be comparable between treated and control groups included survival, body weight gain, feed consumption, opthalmoscopic examination, auditory examination, hematology, clinical chemistry data, organ weights, and macroscopic and microscopic pathology. Elevated K^+ levels in urine were the result of potassium salts present in the enzyme preparation. The repeat test demonstrated that ingested potassium was excreted during the first 4 h. After that time, K^+ levels did not differ significantly from corresponding control levels. Based on all of these results, the highest dose of 2500 mg/kg body wt represents a no-adverse-observed effect level for lipase AY concentrate in rats.

Lipase AY concentrate was shown to have no genotoxic potential when subjected to a battery of tests including bacterial mutation in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and in *E. coli* strain WP2 *uvrA*, *in vitro* chromosome aberration, and forward mutation in L5178Y $tk^{+/-}$ mouse lymphoma cells. Further, the yeast organism producing the enzyme *C. rugosa* has been shown to be nonpathogenic to mice at doses up to 1.5×10^7 CFU/body.

The studies conducted on the lipase AY enzyme preparation satisfy basic data requirements for safety

testing established by the Food and Drug Administration for substances that will be present in the diet at low levels. These studies have confirmed the lack of any oral toxicity, genotoxicity, or other health hazard related to the enzyme preparation or the production organism. Consequently, it may properly be concluded that the lipase AY enzyme preparation is safe for use as a processing aid in food.

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