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Research Section

SAFETY EVALUATION OF LIPASE G FROM *PENICILLIUM CAMEMBERTII*

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Abstract—Lipase G, a partial glycerides eliminating enzyme produced by *Penicillium camembertii*, was subjected to safety evaluation studies to establish its safety when used as a processing aid in the food industry. The toxicological studies on the enzyme included a 90-day gavage study with rats, a mutagenicity study using bacteria, and a pathogenicity study using mice. The no-adverse-effect level from the 90-day gavage toxicity study was 2000 mg/kg body weight/day for rats. There was no evidence of mutagenic potential. The micro-organism was evaluated for pathogenicity using mice and classified as a non-pathogen. Results indicate that the production and use of lipase G may be regarded as safe for the enzyme production worker and the consumer.

INTRODUCTION

Many microbial lipases have been reported (Iwai and Tsujisaka, 1984; Sugiura, 1984) and have received much attention because of their potential use in industry. Using the three basic catalytic activities of lipases (i.e. hydrolysis, esterification and transesterification) various lipases have been used for applications including fat-splitting (Hoq *et al.*, 1985; Linfield *et al.*, 1984), the synthesis of esters, such as acyl sorbitol or acyl homoserin (Chopineau *et al.*, 1988; Nagao and Kito, 1989), the production of a substitute for cocoa butter (Yokozeki *et al.*, 1982), and the resolution of racemic mixtures (Kirchner *et al.*, 1985). The lipase used in each application is selected according to its specificity for substrates such as fatty acid or alcohol, its regiospecificity or stereospecificity, as well as its temperature and pH stability and dependence. A novel lipase (lipase G), which hydrolyses mono- and diacylglycerol was found in the culture filtrate of *Penicillium camembertii.* The taxonomic classification of the source organism together with purification and characterization of the lipase have been described (Yamaguchi and Mase, 1991). Lipase G can be used in industry for the specific hydrolysis or synthesis of partial acylglycerols.

This paper describes the three safety studies of lipase G that have been carried out according to the Japanese guidelines. A 90-day gavage study in rats was performed because of the possibility of process contaminants or fermentation products resulting in residual levels of toxic material in the enzyme preparation. A mutagenicity study with *Salmonella typhimurium* and *Escherichia coli* was carried out because of the possibility of residual levels of mutagens in the enzyme preparation. A pathogenicity study with mice was carried out using spores of *Penicillium camembertii* because of the possibility of human exposure to the organism in the factory during fermentation.

Abbreviation: TOS = total organic solids.

MATERIALS AND METHODS

90-Day gavage study with rats

Test material. A crude preparation of lipase G (LGK, Lot no. 000602) was produced by the commercial process without the final purification and dilution steps. The composition of such a 'tox-batch' ensures that any toxic component will be present at its maximum concentration before any subsequent removal or dilution by further processing.

LGK is a brown powder containing 1.5% lipase G with a fatty smell and has a lipase G activity of 100,222 U/g (emulsified vinyllaurate method). The stability of LGK during the test period was confirmed by measurement of enzyme activity.

The results of analysis of the test material is shown in Table 1. The total organic solids (TOS) content of LGK was 75.76% (Table 1). The commercial preparation of lipase G (with a minimum lipase G activity of 5000 U/g) has a TOS value of 35.36%.

Animals. Sprague–Dawley rats, Crj:CD strain, were obtained from Charles River (Japan). 114 rats

Table 1. Results of the analysis of the crude preparation of lipase G (LGK)

Component	Level in LGK	Analytical method
Total nitrogen	25.0 mg/g	Semi-micro Kjeldahl's method
Protein nitrogen	124 mg/g	Lowry's method
Carbohydrate	75.5 mg/g	Phenol–sulfuric acid method
Water	2.04%	Loss on drying
Ash	22.2%	Loss on ignition at 800°C
Na	1860 ppm	Atomic absorption
K	12,200	Atomic absorption
Mg	255	Atomic absorption
Ca	1070 ppm	Atomic absorption
Cl	1.73%	Mohr's method
P_2O_5	1.79%	Molybdovanadophosphoric acid spectrophotometric method
SO_4	0.49%	Ion chromatography
CO_2	0.1%	Gas chromatography
Lipase G activity	100,222 U/g	Emulsified vinyllaurate method (Yamaguchi, 1991)
Triglyceride lipase activity	32,000 U/g	Lipase kit method (Furukawa *et al.*, 1982)
Esterase activity	291 U/g	pNPL method (Isobe *et al.*, 1988)
Protease activity	7.4 U/g	Folin method
Cyclopiazonic acid	Negative	Lansden, 1986
Total organic solids*	75.76%	

*Total organic solids (TOS) defined as 100 − (A + W + D)%, where A = ash content, W = water content, and D = content of diluents: 100 − (2.04 + 22.2 + 0) = 75.76%. The commercial preparation of lipase G (with a minimum lipase G activity of 5000 U/g) has a TOS value of 100 − (4.52 + 9.73 + 50.39)% = 35.36%.

of each sex were acclimatized for 6 days and then 100 rats of each sex were stratified by body weight, randomly assigned to treatment groups and housed two rats of the same sex to each cage. Commercial pelleted diet (CE-2, Japan CLEA Co., Ltd) and tap water were available *ad lib.* At the start of the administration the average age of both sexes of rats was 5 wk and the weight range was 138.5 to 181.4 g for males and 112.4 to 148.0 g for females.

Test material administration. The test material was administered by gavage once a day for 90–92 days at levels of 0, 500, 1000 and 2000 mg/kg body weight/day as a suspension in distilled water. A polyethylene catheter was inserted into the stomach and LGK was administered in a suspension with a disposable syringe. Control animals were dosed with distilled water alone. At wk 13, 20 animals/sex/group were killed and 10 animals/sex in the highest dose and control groups were maintained untreated for another 31 days (after the dosing period) as a recovery group.

Observations. Throughout the study any signs of ill health, together with any behavioural changes or reactions, caused by the treatment with the test material were recorded for individual animals. A macroscopic examination was made on all rats every day during the dosing period and every day (except holidays) during the recovery period.

Body weight, and food and water consumption. Body weight and food consumption were measured twice a week for all rats, and food conversion efficiency (g body weight gain/g food consumed) was calculated.

Water consumption was measured using 10 rats per sex per group before dosing, 20 rats per group at wk 9 and 11 (dosing period), and 10 rats of each sex per group at wk 17 (recovery period). The rats were housed individually in metabolic cages for 23 hr, after which water consumption was recorded and urine samples were collected.

Urinalysis. The urine samples were examined for colour, and the volume, osmolarity, and creatinine, sodium, potassium and chloride contents were measured. In addition, a Clinitek (Miles Inc.) was used to grade urinary protein, glucose, ketones, bilirubin, occult blood and urobilinogen. Microscopy of the urinary sediments was also carried out.

Auditory test and ophthalmological examination. An auditory test (pinna reflex to a 5800 Hz Galtone whistle) was carried out in the control and highest dose groups at wk 13 and in the recovery group and controls at wk 17.

A chromoretinographical examination was made with a funduscopic camera on six rats per group (excluding the recovery group) just before the dosing

Table 2. Water consumption by male and female rats administered LGK orally for 90–92 days

	Water consumption (ml)†					
	Administration period				Recovery period	
	LGK dose (mg/kg body weight)				LGK dose (mg/kg body weight)	
Gender	0 (controls)	500	1000	2000	0 (controls)	2000
Male	18.2 ± 8.1	18.6 ± 9.0	18.8 ± 11.2	23.9 ± 9.1*	12.2 ± 6.8	11.6 ± 6.3
Female	19.3 ± 8.4	20.0 ± 5.7†	16.6 ± 8.6‡	23.6 ± 9.9	11.0 ± 4.8	15.5 ± 9.8

†Values shown are those determined at wk 11 (administration period) and wk 17 (recovery period) and are means ± SD for groups of 20 rats (administration period) or 10 rats (recovery period). The value marked with an asterisk differs significantly (two-tailed Student's *t*-test or Aspin-Welch *t*-test) from the corresponding control value (*$P < 0.05$).
‡n = 19 (one was excluded because of incorrect treatment of the sample).

Table 3. Analysis of urine of male rats administered LGK orally for 90–92 days

Parameter or component	Administration period				Recovery period	
	LGK dose (mg/kg body weight)				LGK dose (mg/kg body weight)	
	0 (controls)	500	1000	2000	0 (controls)	2000
Volume (23 hr) (ml)	18.4 ± 4.6	18.2 ± 6.7	18.6 ± 8.0	21.4 ± 6.6	11.8 ± 2.9	14.6 ± 3.7
Osmolarity (mOsm/kg)	1272 ± 349	1403 ± 386	1475 ± 451	1272 ± 299	1575 ± 375	1302 ± 306
Creatinine (mg/100 ml)	94.1 ± 16.8	104.7 ± 31.6	105.6 ± 30.7	88.2 ± 25.2	150.7 ± 31.3	123.4 ± 31.7
Na^+ (mEq/litre)	65.9 ± 18.5	74.6 ± 22.4	98.6 ± 32.4***	106.5 ± 27.8***	57.9 ± 30.4	46.8 ± 19.1
K^+ (mEq/litre)	195.9 ± 44.5	205.7 ± 52.4	219.8 ± 67.2	188.3 ± 44.8	196.2 ± 61.8	163.9 ± 52.0
Cl^- (mEq/litre)	120.5 ± 38.6	128.3 ± 45.1	147.5 ± 64.5	129.3 ± 45.1	93.9 ± 56.2	70.7 ± 36.3
pH	7.6 ± 0.6	7.5 ± 0.6	7.6 ± 0.8	7.8 ± 0.5	8.0 ± 1.0	8.1 ± 0.8
Protein:						
−	4	0	0	0	0	0
±	8	2	2	2	1	0
+	8	13 **	10 **	13 **	4	7
+ +	0	3	5	3	4	2
+ + +	0	2	3	2	1	1
Glucose:						
−	20	20	20	20	10	10
±	0	0	0	0	0	0
Ketone body:						
−	16	6	0	5	1	2
±	3	9 **	10 ***	7 **	6	6
+	1	5	10	8	3	2
Bilirubin:						
−	20	20	20	20	10	10
+	0	0	0	0	0	0
Occult blood:						
−	18	15	17	15	8	9
±	1	5	2	3	2	0
+	1	0	0	0	0	1
+ +	0	0	1	2	0	0
Urobilinogen:						
0.1 EU/100 ml	20	20	20	20	10	10
1.0 EU/100 ml	0	0	0	0	0	0
Sediment						
Erythrocytes						
−	16	13	14	15	6	10
±	4	7	5	3 *	4	0
+	0	0	0	0	0	0 *
+ +	0	0	0	0	0	0
+ + +	0	0	1		0	0
Leucocytes						
−	12	12	8	9	8	7
±	8	8	12	11	2	3
+	0	0	0	0	0	0
Squamous epithelial cells						
−	2	8	7	9	4	5
±	18	12	13	11	6	5
+	0	0	0	0	0	0
Round epithelial cells						
−	20	20	20	20	10	9
±	0	0	0	0	0	1
+	0	0	0	0	0	0
Small round epithelial cells						
−	17	19	18	18	10	10
±	3	1	2	2	0	0
+	0	0	0	0	0	0
Transitional epithelial cells						
−	20	20	20	20	9	10
±	0	0	0	0	1	0
Waxy cast						
−	20	20	19	20	10	10
±	0	0	1	0	1	0

EU = Ehrlich unit

Grades were as follows:

Protein: − = negative, ± = trace, + = 30 mg/100 ml, + + = 100 mg/100 ml, + + + = ⩾300 mg/ml.

Glucose: − = negative, ± = 0.1 g/100 ml, + = 0.25 g/100 ml, + + = 0.5 g/100 ml, + + + = ⩾1 g/100 ml.

Ketone body: − = negative, ± = 5 mg/100 ml, + = 15 mg/100 ml, + + = 40 mg/100 ml, + + + = 80 mg/100 ml, + + + + = ⩾160 mg/100 ml.

Bilirubin: − = negative, + = small amount, + + = moderate amount, + + + = large amount.

Occult blood: − = negative, ± = trace, + = small amount, + + = moderate amount, + + + = large amount.

Sediment (*count/litre*)

Leucocytes: − = 0, ± = 0 > 3, + = 3 > 9, + + = 9 > 15, + + + = ⩾15.

Erythrocytes: − = 0, ± = 0 > 4, + = 4 > 14, + + = 14 > 24, + + + = ⩾24.

Others: − = 0, ± = 0 > 2, + = 2 > 7, + + = 7 > 12, + + + = ⩾12.

Values shown are those determined at wk 11 (administration period) and wk 17 (recovery period) and are means ± SD for groups of 20 rats (administration period) or 10 rats (recovery period). Those marked with asterisks differ significantly (two-tailed Student's t-test or Aspin–Welch t-test) from the corresponding control values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$). For details of urine collection periods, see Materials and Methods.

Table 4. Analysis of urine of female rats administered LGK orally for 90–92 days

Parameter or component	Administration period				Recovery period	
	LGK dose (mg/kg body weight)				LGK dose (mg/kg body weight)	
	0 (controls)	500	1000	2000	0 (controls)	2000
Volume (23 hr) (ml)	15.6 ± 6.6	14.0 ± 4.7†	12.5 ± 5.0†	16.6 ± 6.2	7.0 ± 2.4	10.3 ± 6.9
Osmolarity (mOsm/kg)	1329 ± 386	1528 ± 382†	1523 ± 348†	1341 ± 474	1442 ± 296	1278 ± 560
Creatinine (mg/100 ml)	65.5 ± 23.2	72.6 ± 16.9†	80.1 ± 25.8†	66.2 ± 24.0	144.7 ± 45.7	116.2 ± 61.5
Na^+ (mEq/litre)	77.0 ± 21.7	88.2 ± 17.2†	103.3 ± 23.4***†	110.5 ± 38.8**	57.1 ± 30.6	53.2 ± 25.6
K^+ (mEq/litre)	204.2 ± 58.0	224.3 ± 51.3†	225.2 ± 55.2†	201.0 ± 64.3	162.7 ± 50.8	149.7 ± 77.3
Cl^- (mEq/litre)	153.9 ± 58.3	178.1 ± 56.4†	199.8 ± 74.7*†	184.2 ± 89.3	87.1 ± 53.8	92.1 ± 65.4
pH	7.7 ± 0.9	7.9 ± 0.8	7.1 ± 0.6*	7.6 ± 0.8	7.3 ± 0.8	8.2 ± 1.1
Protein:						
−	17	9	2	3	1	2
±	1	6	7	1	2	2
+	2	4	9 ***	12 ***	5	0
+ +	0	1	2	3	2	6
+ + +	0	0	0	1	0	0
Glucose:						
−	20	20	20	20	10	10
±	0	0	0	0	0	0
Ketone body:						
−	20	20	20	19	8	10
±	0	0	0	1	2	0
+	0	0	0	0	0	0
Bilirubin:						
−	20	20	20	20	9	10
±	0	0	0	0	0	0
+	0	0	0	0	1	0
Occult blood:						
−	20	19	19	20	10	10
±	0	1	1	0	0	0
+	0	0	0	0	0	0
Urobilinogen:						
0.1 EU/100 ml	20	20	20	20	10	10
1.0 EU/100 ml	0	0	0	0	0	0
Sediment						
Erythrocytes						
−	19	19	18	19	9	9
±	1	1	2	1	1	1
+	0	0	0	0	0	0
Leucocytes						
−	16	17	14	11	9	7
±	4	3	6	9	1	3
+	0	0	0	0	0	0
Squamous epithelial cells						
−	3	2	3	3	5	2
±	17	18	17	16	5	8
+	0	0	0	1	0	0
+ +	0	0	0	0	0	0
Round epithelial cells						
−	19	20	20	19	10	10
±	1	0	0	1	0	0
+	0	0	0	0	0	0
Small round epithelial cells						
−	19	17	16	20	10	10
±	1	3	4	0	0	0
+	0	0	0	0	0	0

EU = Ehrlich unit

Grades were as follows:

Protein: − = negative, ± = trace, + = 30 mg/100 ml, + + = 100 mg/100 ml, + + + = ⩾300 mg/ml.

Glucose: − = negative, ± = 0.1 g/100 ml, + = 0.25 g/100 ml, + + = 0.5 g/100 ml, + + + = ⩾1 g/100 ml.

Ketone body: − = negative, ± = 5 mg/100 ml, + = 15 mg/100 ml, + + = 40 mg/100 ml, + + + = 80 mg/100 ml, + + + + = ⩾160 mg/100 ml.

Bilirubin: − = negative, + = small amount, + + = moderate amount, + + + = large amount.

Occult blood: − = negative, ± = trace, + = small amount, + + = moderate amount, + + + = large amount.

Sediment (count/litre)

Leucocytes: − = 0, ± = 0 > 3, + = 3 > 9, + + = 9 > 15, + + + = ⩾15.

Erythrocytes: − = 0, ± = 0 > 4, + = 4 > 14, + + = 14 > 24, + + + = ⩾24.

Others: − = 0, ± = 0 > 2, + = 2 > 7, + + = 7 > 12, + + + = ⩾12.

†n = 19 (one value was excluded because of incorrect treatment of the sample).

Values are means ± SD for groups of 20 rats (administration period) or 10 rats (recovery period), and those marked with asterisks differ significantly (two-tailed Student's *t*-test or Aspin-Welch *t*-test) from the corresponding control values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$). For details of urine collection periods, see Materials and Methods.

Table 5. Haematological examination of male and female rats treated orally with LGK for 90 days

	Administration period				Recovery period	
	LGK dose (mg/kg body weight)				LGK dose (mg/kg body weight)	
Parameter†	0 (controls)	500	1000	2000	0 (controls)	2000
			Males			
WBC ($\times 10^2/\mu l$)	44 ± 16	37 ± 11	40 ± 12	47 ± 17	28 ± 11	28 ± 8
RBC ($\times 10^4/\mu l$)	927 ± 49	918 ± 60	927 ± 39	931 ± 45	889 ± 30	923 ± 35*
PL ($\times 10^4/\mu l$)	107.7 ± 16.7	112.5 ± 13.2	108.7 ± 13.6	108.5 ± 10.5	106.6 ± 11.6	109.8 ± 15.1
Haemoglobin (g/100 ml)	16.2 ± 0.7	16.0 ± 0.9	16.2 ± 0.6	16.3 ± 0.9	15.6 ± 0.3	16.0 ± 0.7
Haematocrit (%)	49.9 ± 2.6	48.6 ± 2.9	49.4 ± 2.3	49.7 ± 3.2	48.2 ± 1.2	49.3 ± 2.2
Reticulocyte (‰)	14.8 ± 6.4	16.0 ± 7.3	15.2 ± 7.6	15.7 ± 6.8	21.5 ± 10.5	19.0 ± 6.2
MCV (fl)	53.8 ± 2.0	53.0 ± 2.0	53.2 ± 1.1	53.3 ± 1.6	54.3 ± 2.3	53.4 ± 1.7
MCH (pg)	17.5 ± 0.6	17.5 ± 0.6	17.4 ± 0.3	17.5 ± 0.4	17.5 ± 0.6	17.3 ± 0.5
MCHC (g/100 ml)	32.6 ± 0.7	32.9 ± 0.5	32.7 ± 0.7	32.8 ± 0.6	32.3 ± 0.4	32.4 ± 0.5
PT (sec)	16.1 ± 1.9	15.5 ± 1.6	16.0 ± 2.4	15.1 ± 1.2	14.0 ± 0.2	14.1 ± 0.6
APTT (sec)	23.4 ± 1.5	24.4 ± 1.7*	24.0 ± 1.3	22.8 ± 1.2	24.2 ± 1.7	24.3 ± 1.8
			Females			
WBC ($\times 10^2/\mu l$)	23 ± 7	25 ± 7	23 ± 7	23 ± 7	21 ± 5	17 ± 4
RBC ($\times 10^4/\mu l$)	802 ± 34	821 ± 26	824 ± 40	807 ± 40	751 ± 54	752 ± 72
PL ($\times 10^4/\mu l$)	113.5 ± 9.8	113.8 ± 8.6	116.6 ± 14.1	114.7 ± 10.9	103.5 ± 17.0	111.0 ± 25.0
Haemoglobin (g/100 ml)	15.1 ± 0.6	15.3 ± 0.4	15.5 ± 0.6*	15.2 ± 0.6	14.7 ± 0.5	14.7 ± 0.6
Haematocrit (%)	45.5 ± 2.0	46.5 ± 1.4	47.1 ± 2.2*	46.0 ± 2.0	42.5 ± 3.1	42.5 ± 3.8
Reticulocyte (‰)	15.0 ± 8.1	17.9 ± 8.4	14.6 ± 8.9	16.9 ± 7.8	10.2 ± 4.7	13.4 ± 5.0
MCV (fl)	56.7 ± 1.7	56.7 ± 1.0	57.1 ± 1.6	57.0 ± 1.7	56.6 ± 1.2	56.7 ± 2.1
MCH (pg)	18.8 ± 0.5	18.7 ± 0.3	18.8 ± 0.6	18.8 ± 0.7	19.7 ± 1.9	19.8 ± 1.6
MCHC (g/100 ml)	33.2 ± 0.5	33.0 ± 0.4	32.9 ± 0.9	33.0 ± 1.0	34.8 ± 3.3	34.8 ± 2.5
PT (sec)	13.5 ± 0.4	13.7 ± 0.4	13.6 ± 0.3	13.5 ± 0.3	13.5 ± 0.3	13.5 ± 0.2
APTT (sec)	19.7 ± 1.0	19.3 ± 0.8	19.5 ± 1.1	19.6 ± 1.1	19.7 ± 1.0	19.8 ± 1.2

†Abbreviations: WBC = white blood cell count; RBC = red blood cell count; PL = platelet count; MCV = mean cell volume; MCH = mean cell haemoglobin; MCHC = mean cell haemoglobin concentration; PT = prothrombin time; APTT = activated partial thromboplastin time.

Values are means ± SD for groups of 20 rats (administration period) or 10 rats (recovery period). Values marked with an asterisk differ significantly (two-tailed Student's t-test or Aspin-Welch t-test) from the corresponding control value (* $P < 0.05$).

period, on 20 rats per group at the end of the dosing period (excluding four rats in each of the control, low- and mid-dose groups, and five rats in the high dose group, because of the breakdown of the funduscopic camera), and on 10 rats per group at the end of the recovery period.

Haematology and clinical chemistry. When the rats were killed at the end of the dosing or recovery period, blood samples were collected from the abdominal aorta and the vena cava of all of the rats (which had been starved for 18 hr before blood sampling). The following haematological measurements were made: total and differential white blood cell counts, red blood cell count, platelet count, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, prothrombin time, and activated partial thromboplastin time.

Serum samples were obtained by centrifugation of blood, and analysed for total protein, creatinine, glutamatic–pyruvic transaminase, alkaline phosphatase, glucose, cholesterol (total and free), triglycerides, phospholipid γ-glutamyl transpeptidase, total bilirubin, blood urea nitrogen, uric acid, calcium, inorganic phosphorus, sodium, potassium and chloride ion. Electrophoretic separation of the serum protein was also carried out.

Absolute and relative organ weights. The absolute and relative (to body weight) weights of the following organs were recorded: brain, pituitary gland, thyroid/parathyroid glands, submandibular/sublingual glands, thymus, lungs, spleen, adrenal glands, heart, liver, kidneys, testes, seminal vesicles, prostate, ovaries/fallopian tubes and uterus.

Pathological examination. At the end of the dosing or recovery period the rats were starved for 18 hr and then killed by exsanguination under barbiturate anaesthesia. They were examined macroscopically and then specimens of the organs that were weighed, plus the tissues and organs listed below, were prepared for histopathological examination: Harderian glands, lacrimal glands, trachea, oesophagus, mesenteric lymph nodes, stomach, jejunum, caecum, rectum, spinal cord, bladder, skin from mammary gland region, vagina, sciatic nerve with skeletal muscle, eyes, tongue, aorta, pancreas, duodenum, ileum, colon, sternum, femur with bone marrow. Specimens were preserved in buffered 10% formalin solution and embedded in paraffin wax. Sections, 3-μm thick, were cut and stained with haematoxylin and eosin. Sternum and femur with bone marrow were decalcified and treated in the same way.

Statistical analysis. Data on body weight, water consumption, food consumption, food conversion efficiency, haematology, biochemistry and organ weights were analysed by Student's t-test, if the variance was non-significant ($P < 0.05$), or the Aspin Welch t-test (two-tailed, if the variance was significant). Non-parametric data from urinalysis was assessed by the chi-square test.

Bacterial mutagenicity test

Test material and positive control solutions. LGK, of the same specification as for the 90-day study, was used.

LGK was diluted with distilled water to give solutions containing 100,000, 50,000, 25,000, 12,500, 6250 or 3130 μg/ml and tested at a maximum concentration of 5000 μg/plate. The positive controls of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine, 9-aminoacridine hydrochloride and 2-aminoanthracene were dissolved in dimethyl sulfoxide.

Test system. Salmonella typhimurium strains TA1535 and TA100 (for base-pair substitutions) and TA1537 and TA98 (for frameshift mutations) and *Escherichia coli* strain WP2uvrA (for base-pair substitutions) were used. These cultures were tested both with and without metabolic activation using S-9 fraction of the rat liver.

Test procedure. Each experiment was carried out using triplicate plates by the preincubation method (Ames *et al.*, 1975).

Pathogenicity study

The assessment of pathogenicity of a fungal source organism is important from the point of view of safety during factory production, but it is not a strict requirement for safety data in support of an enzyme when no fungal organisms or spores will contact the food.

However, it is generally preferable not to use pathogenic organisms for enzyme production and the study described here was designed to examine the pathogenic potential of the source organism.

Test material. Penicillium camembertii spores were used.

The procedures were carried out under sterile conditions. After 5 days of culture, 20 ml Tween 80

Table 6. Clinical chemical analysis of male and female rats treated orally with LGK for 90 days

	Administration period				Recovery period	
	LGK dose (mg/kg body weight)				LGK dose (mg/kg body weight)	
Parameter	0 (controls)	500	1000	2000	0 (controls)	2000
			Males			
Total protein (g/100 ml)	5.8 ± 0.3	6.0 ± 0.3	6.0 ± 0.2	5.9 ± 0.2	5.9 ± 0.2	5.9 ± 0.3
Creatinine (mg/100 ml)	0.65 ± 0.06	0.62 ± 0.04*	0.64 ± 0.05	0.62 ± 0.05	0.70 ± 0.07	0.68 ± 0.04
GOT (IU/litre)	124 ± 23	132 ± 37	119 ± 16	134 ± 33	114 ± 27	135 ± 16
GPT (IU/litre)	47 ± 13	48 ± 31	46 ± 12	46 ± 14	42 ± 10	48 ± 13
Alkaline phosphatase (IU/litre)	208 ± 34	198 ± 36	184 ± 25*	199 ± 29	168 ± 24	165 ± 13
Glucose (mg/100 ml)	158 ± 44	147 ± 11	154 ± 12	150 ± 14	172 ± 18	167 ± 15
Total cholesterol (mg/100 ml)	41 ± 9	44 ± 8	43 ± 7	43 ± 8	46 ± 9	49 ± 17
Free cholesterol (mg/100 ml)	8 ± 2	9 ± 2	9 ± 2	9 ± 2	9 ± 3	10 ± 4
E:T ratio	0.80 ± 0.02	0.80 ± 0.03	0.80 ± 0.02	0.80 ± 0.02	0.80 ± 0.02	0.80 ± 0.01
Triglyceride (mg/100 ml)	50 ± 12	46 ± 21	42 ± 16	35 ± 12***	50 ± 14	54 ± 24
Phospholiid (mg/100 ml)	83 ± 12	85 ± 12	83 ± 10	83 ± 9	85 ± 12	90 ± 21
γ-GTP (IU/litre)	0.21 ± 0.20	0.53 ± 0.68	0.25 ± 0.37	0.33 ± 0.44	0.36 ± 0.16	0.36 ± 0.14
Total bilirubin (mg/100 ml)	0.13 ± 0.01	0.13 ± 0.02	0.13 ± 0.01	0.12 ± 0.02	0.12 ± 0.02	0.11 ± 0.01
Blood urea nitrogen (mg/100 ml)	17.2 ± 1.7	17.4 ± 1.7	17.9 ± 2.4	17.7 ± 2.5	15.8 ± 2.5	15.3 ± 1.5
Uric acid (mg/100 ml)	1.3 ± 0.8	1.2 ± 0.3	1.2 ± 0.2	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.1
Ca^{2+} (mg/100 ml)	9.2 ± 0.5	9.2 ± 0.2	9.1 ± 0.2	9.2 ± 0.2	9.0 ± 0.2	8.9 ± 0.3
Inorganic P (mg/100 ml)	7.4 ± 0.8	7.4 ± 0.6	7.3 ± 0.7	7.7 ± 0.8	6.4 ± 0.5	6.5 ± 0.6
Na^{+} (mEq/litre)	141.2 ± 0.9	140.6 ± 1.3	140.8 ± 1.2	140.6 ± 1.1	131.9 ± 1.1	132.0 ± 1.0
K^{+} (mEq/litre)	4.6 ± 1.0	4.6 ± 0.3	4.4 ± 0.2	4.4 ± 0.2	4.2 ± 0.2	4.3 ± 0.2
Cl^{-} (mEq/litre)	111.4 ± 2.2	112.3 ± 1.5	111.4 ± 1.5	111.8 ± 1.3	99.2 ± 0.9	99.3 ± 1.7
			Females			
Total protein (g/100 ml)	6.3 ± 0.4	6.1 ± 0.4	6.3 ± 0.2	6.2 ± 0.2	6.4 ± 0.4	6.2 ± 0.3
Creatinine (mg/100 ml)	0.71 ± 0.08	0.68 ± 0.07	0.72 ± 0.08	0.70 ± 0.07	0.76 ± 0.05	0.71 ± 0.07
GOT (IU/litre)	119 ± 32	108 ± 17	110 ± 26	112 ± 19	135 ± 24	106 ± 19**
GPT (IU/litre)	39 ± 16	36 ± 10	32 ± 11	34 ± 12	47 ± 17	37 ± 16
Alkaline phosphatase (IU/litre)	89 ± 24	89 ± 18	97 ± 25	91 ± 21	69 ± 13	70 ± 14
Glucose (mg/100 ml)	138 ± 15	139 ± 11	147 ± 16	143 ± 10	158 ± 13	155 ± 10
Total cholesterol (mg/100 ml)	59 ± 15	54 ± 10	57 ± 9	64 ± 12	64 ± 11	63 ± 11
Free cholesterol (mg/100 ml)	17 ± 4	15 ± 3	16 ± 3	18 ± 4	17 ± 4	16 ± 3
E:T ratio	0.72 ± 0.02	0.72 ± 0.01	0.72 ± 0.02	0.72 ± 0.02	0.74 ± 0.02	0.74 ± 0.01
Triglyceride (mg/100 ml)	40 ± 10	35 ± 10	35 ± 11	40 ± 13	38 ± 13	42 ± 8
Phospholipid (mg/100 ml)	129 ± 21	120 ± 18	126 ± 13	134 ± 19	122 ± 20	119 ± 14
γ-GTP (IU/litre)	0.61 ± 0.44	0.61 ± 0.28	0.66 ± 0.30	0.66 ± 0.24	0.49 ± 0.27	0.56 ± 0.34
Total bilirubin (mg/100 ml)	0.14 ± 0.03	0.13 ± 0.02	0.14 ± 0.02	0.14 ± 0.03	0.14 ± 0.02	0.12 ± 0.02
Blood urea nitrogen (mg/100 ml)	20.3 ± 3.1	21.1 ± 2.6	21.1 ± 2.2	21.8 ± 4.3	17.2 ± 3.7	17.5 ± 4.9
Uric acid (mg/100 ml)	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2
Ca^{2+} (mg/100 ml)	9.2 ± 0.3	9.1 ± 0.3	9.2 ± 0.2	9.1 ± 0.2	9.1 ± 0.3	9.0 ± 0.2
Inorganic P (mg/100 ml)	6.2 ± 0.9	6.7 ± 1.0	6.4 ± 1.1	6.5 ± 0.7	5.6 ± 0.7	5.8 ± 0.7
Na^{+} (mEq/litre)	138.0 ± 1.4	138.1 ± 1.2	138.5 ± 1.2	137.9 ± 1.4	138.6 ± 0.9	139.0 ± 0.6
K^{+} (mEq/litre)	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.2	4.1 ± 0.2	4.1 ± 0.2	4.1 ± 0.3
Cl^{-} (mEq/litre)	111.6 ± 2.0	111.1 ± 1.7	111.2 ± 2.3	110.8 ± 2.4	112.9 ± 1.4	112.6 ± 1.3

GOT = glutamic–oxaloacetatic transaminase GPT = glutamatic–pyruvic transaminase E:T ratio = ester cholesterol:total cholesterol γ-GTP = γ-glutamyl transpeptidase

Values are means ± SD for groups of 20 rats (administration period) or 10 rats (recovery period), and those marked with asterisks differ significantly (two-tailed Student's *t*-test or Aspin–Welch *t*-test) from the corresponding control values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$).

Table 7. Relative organ weights of male and female rats administered LGK orally for 90–92 days

	Administration period				Recovery period	
	LGK dose (mg/kg body weight)				LGK dose (mg/kg body weight)	
Organ	0 (controls)	500	1000	2000	0 (controls)	2000
Males						
Body weight (g) ...	*459.5 ± 26.6*	*460.9 ± 55.5*	*447.1 ± 47.5*	*437.0 ± 41.4*	*483.8 ± 43.0*	*489.4 ± 43.7*
Brain	487 ± 27	491 ± 51	506 ± 44	503 ± 38	465 ± 40	462 ± 44
Pituitary gland	2.34 ± 0.30	2.47 ± 0.22	2.36 ± 0.25	2.44 ± 0.34	2.30 ± 0.25	2.28 ± 0.45
Thyroid/parathyroid glands	4.74 ± 0.72	4.49 ± 0.81	4.86 ± 0.79	4.90 ± 0.66	4.08 ± 0.59	4.41 ± 0.61
Submandibular/sublingual glands	150 ± 12	145 ± 15	144 ± 13	146 ± 14	149 ± 24	139 ± 20
Thymus	63.8 ± 15.2	61.3 ± 15.5	68.4 ± 13.5	57.6 ± 9.4	44.5 ± 11.2	53.3 ± 10.3
Heart	322 ± 30	330 ± 21	329 ± 43	329 ± 20	301 ± 34	292 ± 19
Lungs	326 ± 20	314 ± 40	333 ± 26	345 ± 26*	318 ± 32	317 ± 21
Liver	2.55 ± 0.18	2.63 ± 0.21	2.52 ± 0.53	2.62 ± 0.15	2.64 ± 0.37	2.61 ± 0.16
Spleen	165 ± 20	163 ± 17	165 ± 19	172 ± 23	147 ± 30	154 ± 16
Kidneys	664 ± 49	678 ± 43	682 ± 41	717 ± 100*	639 ± 58	634 ± 54
Adrenal glands	12.1 ± 1.5	13.7 ± 1.6**	13.5 ± 1.7*	13.3 ± 2.6	12.0 ± 2.1	11.3 ± 1.4
Testes	684 ± 81	728 ± 84	763 ± 75**	752 ± 78**	716 ± 70	685 ± 73
Seminal vesicles	460 ± 89	475 ± 90	440 ± 70	469 ± 100	487 ± 59	481 ± 66
Prostate	223 ± 43	240 ± 54	218 ± 44	249 ± 59	217 ± 29	229 ± 34
Females						
Body weight (g) ...	*270.2 ± 23.5*	*275.8 ± 23.0*	*251.7 ± 24.1*	*273.1 ± 24.7*	*283.8 ± 14.8*	*285.7 ± 21.2*
Brain	772 ± 59	764 ± 62	813 ± 71	764 ± 79	748 ± 64	740 ± 39
Pituitary gland	5.99 ± 1.18	5.55 ± 0.58	5.73 ± 0.72	5.58 ± 0.77	6.04 ± 1.38	5.66 ± 1.13
Thyroid/parathyroid glands	5.97 ± 0.74	5.83 ± 0.82	5.91 ± 1.00	6.13 ± 0.93	5.38 ± 0.48	5.75 ± 0.89
Submandibular/sublingual glands	161 ± 15	159 ± 14	166 ± 14	154 ± 14	151 ± 15	153 ± 13
Thymus	108.1 ± 30.4	95.9 ± 15.5	109.0 ± 19.3	100.2 ± 15.5	79.4 ± 23.6	75.2 ± 15.7
Heart	329 ± 21	337 ± 24	348 ± 33*	338 ± 23	333 ± 30	333 ± 23
Lungs	421 ± 34	427 ± 38	444 ± 40	421 ± 38	428 ± 64	432 ± 24
Liver	2.61 ± 0.19	2.57 ± 0.17	2.66 ± 0.27	2.71 ± 0.14	2.67 ± 0.18	2.66 ± 0.17
Spleen	188 ± 21	195 ± 25	194 ± 23	192 ± 29	180 ± 17	175 ± 19
Kidneys	699 ± 52	721 ± 40	716 ± 65	737 ± 59*	644 ± 38	676 ± 77
Adrenal glands	25.9 ± 3.8	25.8 ± 2.9	27.0 ± 2.3	24.7 ± 2.9	23.2 ± 2.7	22.6 ± 3.7
Ovaries/fallopian tubes	51.2 ± 7.8	55.0 ± 13.5	52.9 ± 8.8	53.7 ± 12.5	51.6 ± 8.1	52.5 ± 8.1
Uterus	213 ± 106	220 ± 87	258 ± 116	272 ± 125	196 ± 60	264 ± 109

Values are mg/100 g body weight, except for liver (g/100 g body weight), and are presented as means ± SD for groups of 20 rats (administration period) or 10 rats (recovery period). Values marked with asterisks differ significantly (two-tailed Student's *t*-test or Aspin-Welch *t*-test) from the corresponding control values (*$P < 0.05$; **$P < 0.01$).

saline solution (0.1%) was poured onto the agar surface and the spores were scraped with a platinum loop to make a suspension. The suspension was filtered through a glass filter. The spores were collected by centrifugation, washed with saline, and then suspended in saline to make three suspensions (10^3, 10^5 and 10^7 CFU/ml).

Animals. Female ICR mice (4 wk old) from Shizuoka Laboratory Animal Center were held in quarantine for 8 days and those showing normal growth at wk 5 were assigned to treatment groups. They were fed a commercial pelleted diet (CE-2, Japan CLEA Co., Ltd) and given tap water *ad lib.* The mice weighed 24.2–28.7 g at the start of the study and were housed five to a cage.

Test procedure. Groups of 10 mice were inoculated with a suspension of 10^2, 10^4 or 10^6 viable spores into the tail vein. After a 14-day observation period the mice were killed and the liver, kidney and brain removed.

A section from each of these organs was then rubbed onto an agar plate and colonies were counted after 48-hr of incubation at 30°C. Organ sections were also prepared for histopathological examination.

RESULTS

90-day gavage study in rats

Clinical signs and body weights. There were no signs of reaction to treatment with the test material during the study. Overall, there were no treatment-related differences in body weight, but males in the 2000-mg/kg dose group on days 84–87 and females in the 1000-mg/kg dose group on days 31–87 showed a significantly reduced weight gain.

Food consumption and food conversion efficiency. There was a small dose-related decrease in food consumption for male rats, which persisted throughout the dosing period in the 1000- and 2000-mg/kg groups. Female rats showed a sporadic reduction in food consumption in the 1000-mg/kg group.

No difference in food consumption was found in either male or female rats at the end of the recovery period.

Food conversion efficiency showed no treatment-related changes.

Table 8. Histopathological findings in male and female rats administered LGK orally for 90–92 days

		Males												Females											
		Administration period						Recovery period						Administration period						Recovery period					
	LGK dose (mg/kg) . . .	0 (controls)			2000			0 (controls)			2000			0 (controls)			2000			0 (controls)			2000		
Organ	Lesion / Lesion grade* . . .	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
Pituitary gland	Cyst	0	0	0	1	0	0	0	0	0	1	0	0	4	0	0	3	1	0	1	1	0	3	0	0
	Adenoma		(0)			(0)			(0)			(0)			(0)			(0)			(0)			(2)	
Harderian glands	Cell infiltration	1	0	0	3	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Lacrimal glands	Cell infiltration	1	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thymus	Haemorrhage	7	0	0	3	0	0	0	0	0	0	0	0	1	0	0	4	0	0	0	1	0	0	0	0
Heart	Myocarditis	1	0	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	1	0	0	1	1	0
	Epicarditis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lungs	Bronchopneumonia	2	0	0	5	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
	Foam cells	3	0	0	4	0	0	0	0	0	1	1	0	2	0	0	0	0	0	2	1	0	0	0	0
	Haemorrhage	4	0	0	2	0	0	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
	Heterotopic ossification	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0
	Arterial wall calcification	8	1	0	13	2	0	6	1	0	4	2	0	7	1	0	3	0	0	7	0	0	4	0	0
Liver	Cell infiltration	4	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Focal necrosis	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Bile duct proliferation	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Fat droplets	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	3	0	0	4	0	0	0	0	0
Spleen	Haemosiderosis	0	0	0	0	0	0	1	0	0	3	1	0	0	0	0	3	0	0	4	2	0	3	2	0
Stomach	Diverticulitis		(0)			(1)			(0)			(0)			(0)			(0)			(0)			(0)	
Pancreas	Acute necrosis	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Haemosiderosis	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0				0			
Kidneys	Chronic pyelonephritis	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Nephritis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pyelitis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Hydronephrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
	Basophilic change of the renal tubule	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Calcification	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Adrenal glands	Vacuolar droplet	1	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Microadenoma		(0)			(1)			(0)			(0)			(0)			(0)			(0)			(0)	
	Haemorrhage	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Lymph nodes	Haemorrhage	0	2	0	2	0	0	0	2	0	2	0	0	1	1	0	0	1	0	2	1	0	1	0	0
	Haemosiderosis	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Prostate	Prostatitis	1	1	0	2	0	0	0	0	0	0	1	0	—	—	—	—	—	—	—	—	—	—	—	—

* + = slight, ++ = moderate, +++ = remarkable, ( ) = non-grade.

The numbers of affected rats are shown. There were 20 rats per group during the recovery period. Results are shown only for the control and highest dose groups.

Table 9. Bacterial mutagenicity test of LGK

Test substance	With or without S-9 mix	Sample concn (μg/plate)	No. of revertant colonies/plate (mean of three plates)																			
			Base-pair substitutional type												Frameshifting type							
			TA100				TA1535				WP2uvrA				TA98				TA1537			
LGK	−S-9	0	111	100	107	(106)	10	10	11	(10)	18	20	18	(19)	13	14	19	(15)	11	10	14	(12)
		313	103	90	105	(99)	10	10	8	(9)	18	11	14	(14)	13	11	11	(12)	9	9	12	(10)
		625	106	100	109	(105)	10	8	9	(9)	17	11	13	(14)	21	17	12	(17)	8	10	10	(9)
		1250	104	100	109	(104)	12	9	16	(12)	25	20	20	(22)	16	14	10	(13)	12	10	10	(11)
		2500	81	85	90	(85)	11	11	12	(11)	23	18	21	(21)	14	16	14	(15)	16	9	10	(12)
		5000	116	111	102	(110)	13	10	16	(13)	21	22	19	(21)	15	17	16	(16)	10	12	13	(12)
LGK	+S-9	0	88	93	87	(89)	9	10	10	(10)	19	23	21	(21)	18	16	12	(15)	20	14	18	(15)
		313	98	98	107	(101)	9	9	13	(10)	22	21	18	(20)	20	16	18	(18)	14	12	20	(15)
		625	86	85	71	(81)	13	11	11	(12)	19	19	15	(18)	13	25	19	(19)	13	18	10	(14)
		1250	87	88	89	(88)	10	10	11	(10)	20	29	27	(25)	16	15	13	(15)	14	13	17	(15)
		2500	80	86	93	(86)	13	15	10	(13)	19	14	19	(17)	21	14	21	(19)	16	14	14	(15)
		5000	89	81	83	(84)	19	15	17	(17)	18	15	19	(17)	17	22	18	(19)	16	16	15	(16)
Positive controls																						
AF-2	−S-9	*	499	565	489	(518)	—	—	—	—	187	205	212	(201)	370	391	384	(382)	—	—	—	—
ENNG	−S-9	*	—	—	—	—	189	190	186	(188)	—	—	—	—	—	—	—	—	—	—	—	—
9AA	−S-9	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1350	1510	1129	(1330)
Solvent control†	−S-9	—	89	96	85	(90)	10	9	7	(9)	12	10	14	(12)	20	14	15	(16)	10	12	10	(11)
2AA	+S-9	*	2420	2216	1962	(2199)	560	571	586	(572)	1030	1113	1201	(1115)	479	519	435	(478)	505	499	517	(507)
Solvent control†	+S-9	—	75	76	83	(78)	10	13	10	(11)	10	12	12	(11)	19	15	22	(19)	18	12	12	(14)

AF-2 = 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide ENNG = *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine 9-AA = 9-aminoacridine hydrochloride 2-AA = 2-aminoanthracene

*AF-2, 0.01 μg/plate for TA100 and WP2uvrA, 0.1 μg/plate for TA98; ENNG, 5 μg/plate; 9AA, 80 μg/plate; 2AA, 0.5 μg/plate for TA98, 1 μg/plate for TA100, 2 μg/plate for TA1535 and TA1537, 20 μg/plate for WP2uvrA.

†Dimethyl sulfoxide.

There were no colonies on any plates in tests for sterility, with and without S-9 mix, run in parallel with the mutagenicity tests.

Water consumption. Male rats in the 2000-mg/kg dose group showed a small but significant increase in water consumption during the dosing period but not during the recovery period (Table 2). Among the female rats there were no differences between treatment groups throughout the study.

Urinalysis. During the dosing period, there was a dose-related increase in urinary sodium ion in the 1000- and 2000-mg/kg groups of both sexes (Tables 3 and 4). Urinary protein was increased in males of all dose groups and in the females in the 1000- and 2000-mg/kg dose groups. All male dose groups showed an increase in urinary ketones. There was an increase in erythrocytes in male rats in the highest dose group.

Ophthalmoscopic examination and auditory test. There were no abnormal findings in the eyes of test animals, and there was no treatment-related effect on auditory function.

Haematology and clinical chemistry. No treatment-related haematological effects were apparent (Table 5). Serum triglyceride was reduced in the male rats in the 2000-mg/kg dosing group, but there were no other treatment-related effects on clinical chemical parameters (Table 6).

Organ weights. In male rats, the absolute submandibular/sublingual gland weight was reduced in the 1000- and 2000-mg/kg dose groups, and the absolute thymus weight was reduced in the 2000-mg/kg dose group but the effect was reversed in the recovery group. Increases in the weight of the adrenal glands in the 500- and 1000-mg/kg dose groups and an increase in testes weight in the 1000-mg/kg dose group were observed. Female rats showed a small decrease in pituitary gland weight in the 1000-mg/kg dose group; kidney weight was decreased in the 1000-mg/kg dose group, but increased at 2000 mg/kg.

When organ weight is expressed relative to body weight, the relative weight of lungs, kidneys and testes were significantly increased in the males dosed with 2000 mg LGK/kg, and the relative weight of the testes was also increased at 1000 mg/kg (Table 7). The relative weight of the adrenal glands was significantly increased in the male 500- and 1000-mg/kg dose groups but not in the highest dose group. Female rats showed a small but statistically significant increase in heart and kidneys weight in the 1000- and 2000-mg/kg dose groups, respectively.

Pathology. Macroscopic pathological examination showed no treatment-related adverse findings. Histopathological examination revealed several changes that were observed in the treated groups but not in the controls (Table 8): for males, epicarditis (one out of 20 rats), bile duct proliferation (1/20), diverticulitis in the stomach (1/20), chronic pyelonephritis (1/20), nephritis (1/20), pyelitis (1/20), basophilic change of the renal tubule (1/20), and microadenoma in the adrenal glands (1/20) in the 2000-mg/kg group at the end of the treatment period, and acute necrosis in the pancreas (1/10) in the 2000-mg/kg group at the end of the recovery period; for females, adenoma in the pituitary gland (2/10), hydronephrosis (1/10) and haemorrhage in the adrenal glands (1/10) at the end of the recovery period in the highest dose group.

Bacterial mutagenicity test

Either with or without S-9 mix, LGK caused no increase in the number of revertants (Table 9). All positive controls caused a distinct increase in the number of revertants.

Pathogenicity study

No fungal colonies were observed to grow on the plates after incubation. Histopathological changes were observed in liver and kidney sections but not in the brain sections, indicating that the fungal spores were distributed chiefly to the liver. It was concluded that host defence mechanisms probably rapidly inactivate the spores and that, in view of the absence of colony formation on any plate rubbed with any organ section, *Penicillium camembertii* is non-pathogenic.

DISCUSSION

To certify the safety of lipase G produced by *Penicillium camembertii* a 90-day oral toxicity test in rats and a mutagenicity test of the crude preparation of lipase G (LGK), the intermediate material for lipase G, and a pathogenicity test using its spores were carried out.

No animals died during the study and no clinical signs due to the administration of test material were evident. Sporadic increases or decreases in body weight, food consumption (except male groups) and food conversion efficiency were observed in treated groups of male and female rats, but these were not dose related and were considered not to be due to the test material. A dose-related decrease in food consumption in male rats was not considered to be due to the test material *per se* but food consumption may have been reduced because of the nutrient contribution of the gavaged test material. There was no treatment-related effect in the auditory test, and ophthalmological examination revealed no abnormal findings.

The higher excretion of sodium ion in the 1000- and 2000-mg/kg dose groups seems unlikely to be the result of the direct influence of Na^+ in the test material because far more Na^+ was contained in the chow than in the test material. However, we cannot eliminate the influence of the test material because no significant elevation in urinary Na^+ concentration was observed in the recovery group of either sex. It is also possible that the increase in water consumption caused by the test material contributes to the increase in urinary Na^+ concentration. Since no histopathological abnormalities were observed in the kidney, the increase in urinary excretion of Na^+ must

have been caused by normal metabolization to maintain the equilibrated metabolism in blood.

The increases in protein in the urine of males and females, and of ketone bodies in the urine of males, are considered to be artefacts and of no toxicological significance. Protein is a normal constituent of the urine of rats, with male rats excreting more protein than females (after puberty). The difference in excretion between male and female rats is largely accounted for by $\alpha_{2\mu}$-globulin excretion. The dipstick test, as used in clinical medicine, reacts mainly to the presence of albumin (Stonard, 1990). The excretion of ketone bodies is not thought to be significant because of the lack of a dose relationship in males and because of its non-occurrence in females.

In the haematological examination, there were significant differences in some parameters (i.e. activated partial thromboplastin time in males at 500 mg/kg, haemoglobin and haematocrit in the females at 1000 mg/kg, and red blood cell counts and monocytes in the differential white blood cell counts in the male recovery 2000-mg/kg group). However, these differences were not dose related and all within physiological variations according to our historical control data of the Crj:CD(SD) strain rats.

The dose-dependent decrease in serum triglyceride that was observed in males at the end of the administration period was due to decreased food intake: triglyceride decreased dose dependently, and there was a high correlation ($r = 0.96$) between serum triglyceride (from blood taken just before autopsy after fasting) and final measurements of food intake during the administration period.

At autopsy, no test material-related changes were observed in any dose groups. Swelling of the pituitary gland was observed frequently in the female groups (including the control group), but was at the expected rate of incidence for the Crj:CD(SD) strain of rats. There were no abnormal increases or decreases in organ weights due to the test material at any dosage. Decreases in the absolute weight of the submandibular/sublingual glands in the male groups were dose related but were within normal physiological variations. Significant differences in the relative weight of the adrenal glands and testes in male groups were not dose related.

In the histopathological examination, several of the lesions that were found only in the administration groups have also been found in our historical controls. Diverticulitis in the stomach, chronic pyelonephritis, basophilic change of the renal tubule, microadenoma in the adrenal glands, and acute necrosis in the pancreas were observed for the first time in these rats in our laboratory. However, these lesions were judged not to be due to the test material because of their low incidence (one case for each lesion). A higher incidence of adenoma was observed in the adrenal glands in the male 2000-mg/kg group (one case) and in the pituitary in the female 2000-mg/kg recovery group (two cases) in comparison with our historical control data for animals of the same age. However, we conclude that the above-mentioned lesions occurred spontaneously, and were not caused by the test material, because the incidence of adenoma increases with age.

In order to assure the safety of lipase G (that is, the final preparation) human intake of lipase G from food use should be compared with the amount of lipase G that caused no adverse effect on rats. The human intake of the enzyme can be estimated as follows. In the process of purification of an edible oil, 0.1% (w/w) lipase G is added to the edible oils. After the hydrolysation of partial glycerides with lipase G, water soluble lipase G is separated almost completely from the oily layer. Suppose that 1% of lipase G transfers into the oily layer during water removal, then 10 μg lipase G/oil remains in the oily layer. Since the average consumption of the Western population is 37 g edible oil/person/day (Gurr, 1992), the lipase G intake is estimated to be 0.37 mg/37 g oil/day. The vegetable oil to be treated with lipase G is palm oil, which makes up 11.9% of total vegetable oil consumption (Wilson, 1993). If it is assumed that palm oil makes up 15% of total vegetable oil consumption (which is a little overestimated but allows for some increase in consumption) and that all palm oil is treated with lipase G, the daily total organic solids (TOS) intake for lipase G is 1.96×10^{-2} mg/day according to the following calculation.

Daily intake of lipase G =

$$10\ \mu g/g \times 37\ g/day \times 0.15 = 5.55 \times 10^{-2}\ mg/day$$

TOS of lipase G = 35.36% (see Table 1).
Daily intake of TOS for lipase G =

$$5.55 \times 10^{-2}\ mg/day \times 0.3536 = 1.96 \times 10^{-2}\ mg/day$$

Daily intake of lipase G in TOS level, for a 60-kg person =

$$1.96 \times 10^{-2}/60 = 3.27 \times 10^{-4}\ mg/kg/day$$

Because lipase G is not always used for the treatment of edible oil, this figure is obviously overestimated. In contrast, the non-effective dose in the present toxicity study was 1.516 mg/kg/day in terms of TOS level (TOS of LGK = 75.8%, $2000 \times 0.758 = 1516$ mg/kg/day). Thus the safety margin is approximately 4.6×10^{6}, which is calculated from the non-effective dose (1516 mg/kg/day, TOS level) obtained in our toxicity study of LGK and the estimated daily intake of lipase G (3.27×10^{-4} mg/kg/day, TOS level).

In the mutagenicity test using four types of *Salmonella typhimurium* strains and *Escherichia coli*, LGK had no mutagenic activity.

Lipase G has been analysed for cyclopiazonic acid, aflatoxin B_1, ochratoxin A, penicillic acid, roridin A, zearalenone, roquefortin and penitrem. None of these mycotoxins was found to be present in lipase G.

In the pathogenicity test using spores, *Penicillium camembertii* displayed no apparent pathogenic potential. The fact that *P. camembertii* is one of the fungi that historically have been used for cheese making supports its safety for use in the processing of food.

We conclude that the enzyme preparation lipase G can be considered safe for the processing of edible oils for human consumption.

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