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BIOTECHNOLOGY CONTRIBUTING TO FOOD HEALTH AND THE ENVIRONMENT

January 2, 1998

Vincent E. Zenger, Ph.D.
Office of Premarket Approval
Center for Food Safety and Applied Nutrition,
Food and Drug Administration
1110 Vermont Avenue NW
Washington, D.C. 20005

1997 DEC 29 A 11:12

Ref:: GRASP # 5G0413

Dear Dr. Zenger:

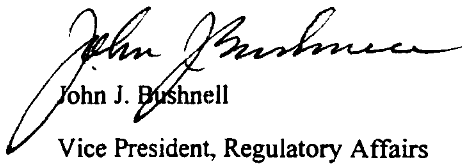
An original reprint of the safety of lipase from *R. oryzae* which I committed to sending to you in my December 19, 1997 letter is attached..

> Safety Evaluation Lipase Derived From *Rhizopus oryzae*: Summary of Toxicological Data; Coenen,T.M.M., Aughton, P., Verhagen, H.; Food and Chemical Toxicology, Vol. 35, No. 3-4, 1997, pp 315-322

If I can be of further assistance, please contact me.

Best wishes for a happy and healthy new year!

Sincerely,



John J. Bushnell
Vice President, Regulatory Affairs

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SUP 1

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Safety Evaluation of Lipase Derived from *Rhizopus oryzae*: Summary of Toxicological Data

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(Accepted 26 September 1996)

Abstract—A lipase enzyme, obtained from *Rhizopus oryzae* produced by a fermentation process was subjected to a series of toxicological tests to document the safety for use as a food additive. The enzyme product was examined for acute, subacute and subchronic oral toxicity, and mutagenic potential. An extensive literature search on the production organism has also been conducted. No evidence of (sub)acute oral toxicity or mutagenic potential was found. Administration of the lipase at dosages of 50, 200 and 1000 mg/kg body weight/day for 90 days did not induce noticeable signs of toxicity. A few minor changes in the chemical composition of the blood in the highest dose group were of no toxicological significance. The no-observed-adverse-effect level of the tox-batch in the subchronic toxicity study was 1000 mg/kg body weight/day. It can be concluded that no safety concerns were identified in the studies conducted with this lipase preparation derived from *R. oryzae* and produced under controlled fermentation conditions. © *1997 Elsevier Science Ltd*

Abbreviations: ALT = alanine aminotransferase; AST = aspartate aminotransferase, DMSO = dimethyl sulfoxide; NOAEL = no-observed-adverse-effect level; OECD = Organisation for Economic Cooperation and Development, PLi = lipase units, SPF = specified pathogen free; TCA = trichloroacetic acid; TOS = total organic solids.

INTRODUCTION

Enzymes from *Rhizopus* species have been used in food production for many years (Broadmeadow *et al.*, 1994; Canadian Food Regulations, 1994; FAO/WHO, 1987). Amylase and glucoamylase enzyme preparations are permitted in or on flour and standardized and unstandardized bakery products (AMFEP, 1992; FDA, 1993). Carbohydrase and protease enzymes derived from *R. oryzae* have been evaluated (JECFA, 1994) and contain the lipase as a small part of the total organic solids (TOS) and thus lipase has indirectly been a non-technical compound in foods.

Lipids are normally present in wheat flour and other bread-making grains. Fat and oil amounts may be as high as 3% of the total weight of the flour, and bakers usually incorporate additional oils and fat-derived emulsifiers (expensive ingredients) to achieve desired technical effects. Added *R. oryzae* lipase can convert the dough oil into mono- and diglycerides. The endogenous lipases in the grain are not adequate to produce the same effect as the added lipase enzyme from *R. oryzae*.

Lipase enzyme from *R. oryzae* has been consumed for many years as a digestive aid without adverse effects. The safety evaluation for that application was based on teratogenicity studies in the rat and rabbit and subchronic oral toxicity studies in dog and rat. The studies resulted in a no-observed-adverse-effect level (NOAEL) for teratogenicity and subchronic oral toxicity of 72,000 lipase U/kg body weight/day (Leuschner, 1974 and 1975a,b,c). Humans have routinely ingested 15–30 mg active lipase TOS and the product has enjoyed a long history on the market. For the proposed application of this lipase, amounts used will be much less than 10 ppm lipase TOS, and this is less than the *R. oryzae* carbohydrase enzyme TOS amount used in starch processing. Even if unrealistically high amounts of bread and cheese are eaten (1 kg each) per day, this would still amount to only 20 mg lipase TOS eaten per day, or an equivalent to about 0.29 mg lipase TOS/kg body weight/day. This unrealistic amount is still about the same as the normal daily dose of the enzyme used in digestive aids, but 10 times lower than reported in a clinical study in which 200 mg lipase TOS per day (75,000 U/day for 2 wk) did not result in adverse acute health effects (Schneider *et al.*, 1985). Realistic consumption of

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bread and cheese will place the ingestion of the lipase well below the amount of digestive aids.

Most safety data of lipase from *R. oryzae* are from the seventies and although the results strongly suggest that this lipase is completely safe for human consumption, an extensive test programme has been performed to confirm the safety of the product.

This review summarizes studies conducted to confirm the safety of lipase from *R. oryzae*. The enzyme product was examined for acute, subacute and subchronic toxicity as well as mutagenic potential.

MATERIALS AND METHODS

The test material (referred to as 'the tox-batch') was produced by the procedure used for the commercial preparation of lipase. The production process is performed according to the requirements of ISO9002 and includes the fermentation process, recovery (downstream processing) and formulation of the product. The purification process was followed by spray-drying to produce the final, non-standardized tox-batch. In Table 1 results of the characterization of the tox-batch by chemical and microbial analysis are presented (van der Lecq and Dalderup, 1995). The stability of the tox-batch during the period of investigations was confirmed by analysis of the enzyme activity. The initial enzyme activity of the tox-batch was approximately 211,000 PLi lipase units/g with a TOS value of 65.8%.

Table 1 Analytical results of the lipase enzyme preparation from *R. oryzae* containing tox-batch[a]

	Result
Identity	
Appearance	
Description	Lump-free powder
Colour	Creamish to tan
Odour	Typical
Identification (SDS–PAGE)	Conforms
Foreign matter	Absent by test
Particle size	D[v > 0.99] = < 0.5 mm
	D[v,0.96] = < 0.25 mm
Enzymatic assay	
Lipase	211,000 PLi/g[b]
Material balance	
Dry matter	97.1%
Total protein (Kjeldahl × 6.25)	32.9%
Total carbohydrates	6.3%
Total fat (NMR)	< 0.1%
TOS[c]	65.8%
Sulfuric ashes	35.2%
Ashes	31.3%
Sodium chloride	27.0%
Residual minerals	
Heavy metals (as Pb)	< 40 ppm
Pb	< 1 ppm
Cd	0.03 ppm
As	0.19 ppm
Hg	< 0.04 ppm
K	405 ppm
Na	10 600 ppm
Ca	10 500 ppm
Mg	5900 ppm
pH (5% solution)	6.5
Toxins	
Aflatoxin B_1	< 10 ppb
Ochratoxin	< 40 ppb
T2 toxin	< 200 ppb
Zearalenone	< 200 ppb
Antimicrobial activity	Negative by test
Microbiological determinations	
Total viable count	110 CFU/g
Moulds	< 10 CFU/g
Enterobacteriaceae	< 10 CFU/g
Salmonella	Absent in 25 g
Escherichia coli	Absent in 25 g
Staphylococcus aureus	Absent in 1 g
Clostridium perfringens	Absent in 1 g
Pseudomonas aeroginosa	Absent in 1 g
Production strain	Absent in 25 g

SDS–PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
NMR = nuclear magnetic resonance CFU = colony-forming units

[a]From van der Lecq and Dalderup (1995).

[b]One lipase unit (PLi) is defined as the amount of enzyme which liberates 1 micromol fatty acids per minute under the conditions of the test.

[c]TOS is defined as 100% − (A + W + D)% where A is the ash content, W is the water content and D is the diluent content. TOS was 65.8% in the tox-batch.

Selection of the tests and methods used in the individual tests were based on the guidelines of the Organisation for Economic Cooperation and Development (OECD, 1984), EEC (EEC, 1992) and FDA guidelines for the Safety Assessment of Direct Food Additives and Color Additives used in Food (FDA Redbook, 1982).

—bacterial gene mutation, Ames test:
OECD 471, EEC B14, USA EPA 40 CFR 1
—mouse micronucleus test:
OECD 474, EEC B12, *FDA Redbook*
—acute oral toxicity in rat:
OECD 401, EEC B1, *FDA Redbook*
—14-day subacute oral toxicity rat:
OECD 407, EEC B7
—90-day subchronic toxicity in rat:
OECD 408, EEC B-L133, *FDA Redbook*.

RESULTS

Oral toxicity studies

Acute oral toxicity in rats

The tox-batch was suspended in purified water (20 mg/kg body weight) and administered once orally at a single oral dose by gavage, to five CD rats of each sex, at 5000 mg/kg body weight. The animals were starved overnight prior to dosing. Mortality and signs of reaction to treatment were recorded during a subsequent 14-day observation period. The animals were killed on the following day and subjected to autopsy.

No mortality and no clinical signs were observed during the study. The body weight gain shown by the animals over the study period was considered to be similar to that expected of normal untreated animals of the same age and strain.

Macroscopic post-mortem examination of the animals at termination did not reveal any abnormalities that were not commonly noted among rats of this age and strain. The oral LD_{50} value of the tox-batch in rats of either sex was established as exceeding 5000 mg/kg body weight (Rees, 1994).

Subacute 14-day oral toxicity (range-finding) in rats

The tox-batch was examined in a 14-day study with four groups of five male and five female young specified pathogen free (SPF)-bred CD rats, which received the tox-batch in purified water, by oral gavage, at doses of 0 (control), 20, 100, 500 and 1000 mg/kg body weight/day.

Tox-batch preparations in vehicle appeared to be stable and homogeneous and sufficiently accurate concentrations were encountered for the purpose of this study. There were no changes in clinical appearance, body weight gain, food consumption, efficiency of food utilization and organ weight considered to be related to treatment. In two males of the 1000 mg/kg body weight/day dose group thickened stomach walls were recorded at autopsy. It was concluded that administration of the tox-batch to CD rats resulted in only minor changes in the stomach of two males receiving 1000 mg/kg body weight/day (Jackson, 1994).

Subchronic 90-day oral toxicity in rats

Experimental design. In a subchronic 90-day toxicity study, the tox-batch was administered to SPF-bred CD rats by oral gavage. The study consisted of four groups, each comprising 20 males and 20 females. The tox-batch was prepared freshly each day immediately before dosing throughout the treatment period as a series of graded concentrations in purified water to provide the required doses at a constant volume-dosage. On the basis of the 14-day range-finding study doses of 0, 50, 200 and 1000 mg/kg body weight/day were selected.

Routine clinical observations, body weight and food consumption were measured throughout the study periods. During wk 13, blood was collected from each animal for clinical laboratory investigations. At the end of wk 13, all animals were autopsied and macroscopic observations and organ weights recorded according to the referred guidelines. Tissues collected from all animals of the controls and highest dose group, as well as all gross lesions of all animals (all dose groups), were processed and slides were examined.

Results. Accuracy, stability and homogeneity of the tox-batch in the vehicle were demonstrated by analyses of the lipase enzyme activity (van der Lecq, 1995). There were no changes in overall body weight gain, food consumption, food conversion efficiency, ophthalmoscopic examination, macroscopic examination, organ weights and microscopic examination that were considered to be an effect of treatment. In females of the 1000 mg/kg body weight/day group, total leucocyte and neutrophils counts were relatively high in comparison with the controls. In males of the highest dose group relatively low aspartate aminotransferase (AST) activities and marginally high urea concentrations were noted (Table 2). Furthermore, total plasma protein was somewhat high in both sexes receiving 1000 mg/kg body weight/day, although there was no corresponding effect in the albumin/globulin ratio or a consistent effect on globulin levels (data not shown). The changes in AST activities, urea concentration, plasma protein and leucocyte counts were within the normal ranges seen at these laboratories, and were not associated with *in vivo* observation nor with any macroscopic or microscopic examination; they were considered to be of no toxicological significance (Tables 2 and 3).

It was concluded that administration of the tox-batch at doses of 50, 200 and 1000 mg/kg body weight/day did not induce noticeable signs of toxicity. The NOAEL of the tox-batch in the subchronic study was therefore 1000 mg/kg body weight/day (Jackson, 1995).

Table 2. Haematological and biochemical results in the subchronic toxicity study of lipase tox-batch in rats

Blood parameters	Treatment (mg/kg body weight/day tox-batch)							
	Group 1 (control)		Group 2 (50 mg kg/day)		Group 3 (200 mg kg/day)		Group 4 (1000 mg kg/day)	
	M	F	M	F	M	F	M	F
Total WBC (1000/cm)	14.7	9 0	15.2	10 0	14 3	8.8	15 0	11 5[a]
	2.0	2.2	3 3	2 6	4 8	2.5	2 8	2.4
NEUTR 1	2 2	1 3	2.5	2 1	3 0	1 3	2 3	2 1[a]
	0.6	0.6	1.3	1 2	3 4	0.7	0 7	0 8
LYMPH 1	11.1	6 9	11.4	7 1	10 0	6 8	11 5	8 4
	1 4	1.6	2.2	1 9	1 3	2.2	2.4	2 3
EO. 1	0.3	0.2	0.2	0 2	0 2	0 1	0 3	0 2
	0 1	0.1	0 0	0 1	0.1	0.1	0 1	0.0
BASO. 1	0.1	0.0	0.0	0 0	0 0	0 0	0.1	0 0
	0 1	0.0	0.1	0 0	0 1	0 0	0.1	0.0
MONO. 1	0.8	0.5	0.8	0 5	0 7	0.4	0 7	0 6
	0 2	0 2	0 3	0.2	0.2	0.1	0.2	0 1
LU 1	0.3	0.2	0.4	0 2	0 3	0.2	0.3	0.2
	0 1	0.1	0.1	0 1	0.1	0 1	0 1	0.1
PT (Sec)	12.4	12.6	13 5[c]	12 8	13 1[a]	12.5	12.5	12.5
	0.6	0 5	0 8	1 0	0 7	0 6	0 5	0 5
ALT (IU/litre)	44	37	42	45	43	38	38	44
	7	5	5	14	8	16	7	18
AST (IU/litre)	95	71	88	91[b]	80	73	76[a]	81
	23	7	17	20	11	13	12	15
Urea (mg%)	29	34	30	37	30	35	35[c]	38
	4	2	3	4	3	6	5	6
Plasma protein total (g%)	6 1	6.3	6 3	6.4	6 1	6 2	6.4[a]	6.8[a]
	0.3	0 5	0 3	0 4	0.2	0 2	0 2	0 5

Values are mean ± SD

[a,b,c]Student's t-test based on pooled error variance significant at 5%, 1% or 0 1% level.

WBC = total leucocyte count; NEUTR = neutrophils, LYMPH = lymphocytes; EO = eosinophils; BASO = Basophils; MONO = monocytes, LU = large unstained cells; PT = prothrombin time, ALT = alanine aminotransferase, AST = aspartate aminotransferase

Mutagenicity studies

Bacterial gene mutation test (Ames test, Salmonella typhimurium reverse mutation assay)

The tox-batch was examined for mutagenic activity in the Ames test using the histidine-requiring *S. typhimurium* mutant strains TA1535, TA1537, TA98 and TA100, and a liver fraction of Aroclor-induced rats for metabolic activation (S-9 mix), as described in detail by Ames *et al.* (1975) and Maron and Ames (1983). A total of five different mutagenicity assays was performed.

In the first mutagenicity assay the tox-batch was tested at five different concentrations ranging from 62 to 5000 μg per plate: a dose-related increase in the number of revertant colonies concomitant with an increase in the background lawn of bacterial growth was seen reaching a twofold increase in TA1537 S-9 and a nearly twofold increase in TA1535 − S-9 TA1535 + S-9 and TA98 − S-9. In TA1537 − S-9, TA98 + S-9, TA100 − S-9 and TA100 + S-9, a smaller increase in the number of revertant colonies per plate concomitant with an increase in the background lawn of bacterial growth was seen (Table 4).

To overcome growth enhancement and possible artefactual test results due to the proteinaceous nature of the tox-batch (Verhagen *et al.*, 1994), heat-inactivated tox-batch was tested at five different concentrations ranging from 85 to 6900 μg per plate in the second mutagenicity assay: again a dose-related increase in the number of revertant colonies

Table 3. Haematology and blood chemistry—background data for CD rats between 17 and 20 wk of age (April 1992–November 1994)

Parameter[a]	Sex	No.	Average age (wk)	Mean	SD	Normal min	Range max[b]
Total WBC (1000/cm)	Male	300	18	14 7	3.3	8.2	21.2
Total WBC (1000/cm)	Female	302	18	10.1	3.2	3.9	16.3
NEUTR (1)	Male	300	18	2.2	0.9	0 3	4 0
NEUTR (1)	Female	302	18	1.4	0 7	0 0[c]	2.8
ALT (IU/litre)	Male	305	18	41	18	5	76
ALT (IU/litre)	Female	305	18	40	16	8	72
AST (IU/litre)	Male	305	18	88	22	45	131
AST (IU/litre)	Female	305	18	87	29	30	145
Urea (mg%)	Male	305	18	26	5	16	36
Urea (mg%)	Female	305	18	34	8	18	50
Protein total (g%)	Male	300	18	6 4	0 4	5.5	7.2
Protein total (g%)	Female	305	18	6.6	0.5	5.7	7 5

[a]Abbreviations and units as in Table 2.

[b]Calculated as mean ± SD [t-value (for No. df)].

[c]Calculated value negative—data not normally distributed.

Table 4 Bacterial mutagenicity assays with lipase tox-batch

Tox-batch treatment	Concentration (μg/plate)	Mean revertant colonies/plate with strain							
		TA1535		TA1537		TA98		TA100	
		− S-9	+ S-9[a]	− S-9	+ S-9	− S-9	+ S-9	− S-9	+ S-9
Solvent	0	32[b]	27	26	25	47	82	194	178
As such	62	38	27	24	29	51	76	176	182
As such	185	31	32	27	27	57	73	196	177
As such	556	38s	27s	29s	29s	59	87	185	179
As such	1667	45s	34s	31s	31s	78s	106s	206s	223s
As such	5000	59s	53m	33s	53m	92s	114m	257s	264m
Positive control[c]		501	571	1200	149	531	1192	627	168b
Boiled water	0	34	22	16	23	35	50	176	189
Heat inactivated	85p	48	24	19	20	41	49	179	173
Heat inactivated	256p	47	19	14	23	43	52	173	185
Heat inactivated	767p	44s	18s	19s	24s	40s	56s	147s	155s
Heat inactivated	2300p	58s	27s	30s	23s	56s	69s	178s	184s
Heat inactivated	6900p	83s	45s	44s	49s	63s	103s	241s	220s
Positive control[c]		479	424	1309	163	418	1504	577	1787
TCA	0	29	26	14	16	39	49	168	175
TCA extract	62	26	22s	11	21s	36s	50	182	169s
TCA extract	185	29s	17s	12	20s	40s	43	141s	160s
TCA extract	556	29s	20s	16	12s	43s	60s	188s	179s
TCA extract	1667	27s	19s	13s	11s	42s	54s	150s	162s
TCA extract	5000	39s	20s	12s	16s	47s	62s	201s	210s
Positive control[c]		451	377	1204	178	697	1418	577	1626
Ethanol	0	20	22	17	16	40	56	154	149
Ethanol extract	2469	26	23	13	13	40	62	153	158
Ethanol extract	7407	23	32	12	15	43	67	149	159
Ethanol extract	22,222	22	23	11	12s	41	67s	151	168s
Ethanol extract	66,667	28	23s	14	16s	63	64s	167	176s
Ethanol extract	200,000	22	32s	17	20s	44	60s	150s	170s
Positive control[c]		475	465	1180	180	713	1331	582	1740
DMSO	0	25	22	18	14	31	45	156	141
DMSO extract	2469	28	21s	16	12	36	54	152	141s
DMSO extract	7407	18	22s	18	12	33	43s	144	145s
DMSO extract	22,222	22	20s	19	13s	33	49s	156	173s
DMSO extract	66,667	22	18s	19	20s	37	47s	177	174s
DMSO extract	200,000	26	20s	19	14s	36s	48s	166s	190s
Positive control[c]		463	385	823	170	751	1651	542	1885

s = slightly more dense background lawn of bacterial growth than in concomitant control plates
m = more dense background lawn of bacterial growth than in concomitant control plates
p = precipitate

[a]The S-9 mix was checked for sterility and found to be sterile.
[b]Each experiment was carried out using triplicate plates.
[c]Positive controls: sodium azide: 1 μg/plate with strain TA1535 − S-9, TA100 − S-9; 9-aminoacridine: 80.0 μg/plate with strain TA1537 − S-9; 2-nitrofluorene: 2.0 μg/plate with strain TA98 − S-9; 2-aminoanthracene: 2.0 μg/plate with strain TA1535 + S-9, TA98 + S-9 and TA100 + S-9; 5 μg/plate with strain TA1537 + S-9

concomitant with an increase in the background lawn of bacterial growth was seen reaching a twofold increase in TA1535 − S-9, TA1535 + S-9, TA1537 − S-9, TA1537 + S-9, TA98 + S-9 and a nearly twofold increase in TA98 − S-9. In TA100 − S-9 and TA100 + S-9, a smaller increase in the number of revertant colonies per plate concomitant with an increase in the background lawn of bacterial growth was seen.

Again, to overcome possible growth-enhancing effects due to protein present on the test plates and thus possibly leading to false positive test results, it was decided to conduct the test while avoiding protein on the plates. For this purpose supernatants were tested after different methods of extraction as suggested by Battershill (1993):

—protein precipitation by adding strong trichloroacetic acid (TCA) solution. With this method possible water and/or acid-soluble mutagens are still present in the supernatant which is tested on the plates;

—extraction by organic solvents. Since proteins do not dissolve in organic solvents only mutagens (if present) soluble in organic solvents will be present in the supernatant, which is tested on the plates. For this purpose two organic solvents were used: ethanol and dimethyl sulfoxide (DMSO).

Thus, in the third mutagenicity assay, an extract of the tox-batch was tested after protein precipitation/acidic aqueous extraction by strong TCA. In this assay the highest of five dose levels tested was an extract of 5 mg tox-batch per plate. In this assay no reproducible increase in the number of revertant colonies was seen (Table 4). In the fourth and fifth mutagenicity assay, extracts of the tox-batch were made by mixing the tox-batch and an organic solvent (ethanol or DMSO) in a ratio of 2:10 (w/v); in these assays the highest of five dose levels tested was an extract of 200 mg tox-batch per plate. In both the fourth and the fifth mutagenicity assay no significant increase in the number of revertant colonies was seen. However, a slightly elevated number of revertants per

plate and/or increase in the background lawn of bacterial growth was seen in several instances, and these are likely to have arisen from single amino acids and/or smaller polypeptides (Verhagen *et al.*, 1994) still present in the supernatants (i.e. in the organic fraction).

In general, the picture was as follows: If protein was present on the plates (as in the first and second mutagenicity assay) a dose-related increase in the number of revertant colonies per plate was seen (in many instances reaching a twofold or greater increase over the background spontaneous mutant frequency) concomitant with an increase in the background lawn of bacterial growth. Alternatively, when extracts of the tox-batch were tested no twofold or greater increase over the background spontaneous mutant frequency was seen at dose levels of up to 5 mg per plate (TCA extract of tox-batch) or 200 mg per plate (ethanol and DMSO extracts of the tox-batch) if only the supernatant but not protein was present on the plates (as in the third, fourth and fifth mutagenicity assay). Thus the presence of protein on the plates determines whether or not a positive response is seen; these results are indicative of artefactual test results and do not suggest mutagenicity of the tox-batch (Table 4).

In all assays negative and positive controls were run simultaneously with the test substance. The positive controls gave the expected strong increase in the number of His^- revertants in both the absence and the presence of the S-9 mix.

It was concluded that the results obtained with the tox-batch in *S. typhimurium* strains TA1535, TA1537, TA98 and TA100 in both the absence and the presence of the S-9 mix under the conditions employed in this test do not indicate mutagenicity of the tox-batch (Verhagen, 1994).

Mouse micronucleus test. The effect of the tox-batch on chromosome structure in bone marrow cells was investigated following acute oral administration in mice. Chromosome damage was measured indirectly by counting micronuclei. A preliminary toxicity test was conducted, using doses of 625, 1250, 2500 and 5000 mg/kg body weight. Subsequently, male and female mice were given a single dose of the tox-batch at 1250, 2500 or 5000 mg/kg body weight. In all cases the tox-batch was dosed orally, suspended in sterile water (purified by reverse osmosis). Concurrent vehicle and positive control groups of mice were similarly dosed with sterile water or chlorambucil (30 mg/kg), respectively. Five males and five females from each group were killed 24 hr after treatment; further lots of five males and five females given the tox-batch at 5000 mg/kg body weight or the vehicle control were killed 48 and 72 hr after treatment. Bone marrow smears on glass slides were made from each animal. These slides were then stained and prepared for examination. A total of at least 2000 erythrocytes per animal were examined for the presence of micronuclei, using the light microscope. Calculated values of micronuclei per 1000 polychromatic erythrocytes were analysed statistically using the Mann–Whitney *U* test. The ratio of polychromatic: mature cells was also calculated for each group, as an indicator of gross toxicity.

No real indication of bone marrow toxicity, as evidenced by depression of bone marrow proliferation, was noted in any group treated with the tox-batch. In addition, no adverse reactions to treatment were recorded for any animal treated with the tox-batch. Frequencies of micronucleated polychromatic erythrocytes in animals killed 24, 48 or 72 hr after administration of the tox-batch were similar to those in concurrent vehicle controls. This lack of treatment-related effect was apparent in both sexes, and was confirmed by statistical analysis. Statistically significant increases over controls were, however, seen in positive control group animals given chlorambucil at 30 mg/kg ($P < 0.01$), indicating that the test procedure was very sensitive to the chromosome damaging action of chlorambucil.

It was concluded that, under the conditions of this test, there was no evidence of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice 24, 48 or 72 hr after oral administration of the tox-batch (Edwards, 1994).

DISCUSSION

Several safety studies were performed to investigate the safety for use of the tox-batch of lipase enzyme from *R. oryzae*.

Lipase was not shown to induce chromosome aberrations in an *in vivo* test. To overcome growth enhancement and possible artefactual test results due to the proteinaceous nature of the tox-batch in the *in vitro* bacterial gene mutation test which could lead to false positive test results (Verhagen *et al.*, 1994), it was decided to conduct the test while avoiding protein on the plates. For this purpose supernatants were tested after different methods of extraction. As no twofold or greater increase over the background spontaneous mutant frequency was seen, it was concluded that the tox-batch was not mutagenic in this assay.

In the 14-day range-finding oral toxicity test thickened stomach walls were recorded at autopsy in two males of the highest dose group (1000 mg/kg body weight/day). This is not an uncommon finding in response to high concentrations of orally administered xenobiotics and may indicate mild irritation, although, in the absence of an effect in females and the majority of the males, the effect is considered to be of doubtful toxicological significance.

In the 90-day subchronic oral toxicity study administration of the tox-batch to CD rats at doses of up to 1000 mg/kg body weight/day resulted in a few minor changes in the chemical composition of the

blood. The higher white blood cell count in females, primarily reflecting a higher neutrophil count, was not associated with any other evidence of disease either by *in vivo* observation or at macroscopic or microscopic evaluation. No similar effect was noted in males. Fluctuations in leucocyte numbers are not uncommon in CD rats and this variation is considered fortuitous. Alanine aminotransferase (ALT) and AST are liver enzymes and serve no purpose in the plasma. High plasma levels of ALT and AST are indicative of hepatic changes and potentially of liver damage. There are, however, no toxicological implications for low AST activities in the plasma. Moreover, the effect was only apparent in a single sex. The test material is an enzyme and therefore the raised total protein levels in both sexes are probably a reflection of some absorption of the test material, or its components parts, for example peptides, and the higher urea levels in males probably reflect the excretion route. The degree of change is relatively small in both cases (within the normal range) and since they represent a normal, and predictable, physiological response to ingestion of high quantities of protein they are not considered to be of toxicological significance.

The changes in AST activities, urea concentration, plasma protein and leucocyte counts were therefore of no toxicological significance. It was concluded that administration of the tox batch at doses of 50, 200 and 1000 mg/kg body weight/day did not reveal any indications of toxicity.

Calculation of consumption of enzymes is based on the contents of TOS in the preparation, as the rest of the preparation (i.e. ash, water and diluents) is fully characterized and is well known as safe for consumption in the quantities involved. For the calculation of the safety margin, it is necessary to know the concentration of the enzyme-TOS in the final food products, the human consumption of the product and the NOAEL of the subchronic oral toxicity study in rats.

In the 90-day oral toxicity study no observed effects were seen in rats given 1000 mg/kg body weight/day (i.e. 658 mg TOS/kg body weight/day). This NOAEL is considerably higher than daily doses of the digestive aid products and is also considerably higher than the anticipated amounts used in baking, cheese, flavourings and fats, and oil modification, namely 20 mg lipase TOS eaten per day (equivalent to about 0.29 mg TOS/kg body weight/day). This results in a safety factor of at least 2000.

The overwhelming evidence points to the safety of the lipase enzyme preparations of *R. oryzae* organisms. Direct animal toxicity tests and routine ingestion by humans have not resulted in evidence of safety concerns. Literature surveys have not directly indicated that the lipase preparations or the organism are of a safety concern. It is therefore concluded that no safety concerns were identified in the studies conducted with this lipase preparation derived from *R. oryzae* and produced under controlled fermentation conditions.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES

January 14, 1998

John J. Bushnell
Vice President
Regulatory Affairs
Gist Brocades Int'l
700 American Avenue
King of Prussia, PA 19406

Re: GRAS Affirmation Petition 5G0413

Dear Mr. Bushnell:

This letter acknowledges receipt on January 8, 1998, of your amendment dated January 2, 1998, for the subject petition.

Because of the nature of the information you have submitted in support of your petition, we consider this to be a substantive amendment that requires an extended review period.

Sincerely yours,

Angela M. Evans
Division of Product Policy
Center for Food Safety and
Applied Nutrition

cc: HFS-200 HFS-215 HFS-206 HFS-226 HFS-246
HFS-247

f/t:ame:1-14-98

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