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FOOD ADDITIVE SAFETY

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November 20, 2006

Via Federal Express

Dr. Laura M. Tarantino
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740-3835

Re: Submission of GRAS Notification for Lipase D Enzyme Preparation Derived from *Rhizopus oryzae*

Dear Dr. Tarantino:

Pursuant to proposed 21 C.F.R. § 170.36(c) and on behalf of our client, Amano Enzyme, Inc., of Nagoya, Japan, we hereby notify the agency of our determination on the basis of scientific procedures that Amano's Lipase D enzyme preparation is generally recognized as safe (GRAS) when used in the production of structured triglycerides as described in the enclosed notification. As with all GRAS substances, the described use of Lipase D is exempt from the premarket clearance requirement applicable to food additives under section 409 of the Food, Drug, and Cosmetic Act.

We trust you will find the enclosed notification acceptable. Should any questions arise during the review process, please do not hesitate to contact us, preferably by telephone, so that we may respond as quickly as possible.

Sincerely,

David R. Joy

cc: Mr. Shinya Hayashi

Encl: GRAS Notification in triplicate

GRAS Notification for Lipase derived from *Rhizopus oryzae*

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1. Claim Regarding GRAS Status

Amano Enzyme Inc. hereby notifies the agency through its attorneys that its Lipase D enzyme preparation, derived from *Rhizopus oryzae*, is GRAS when used in the production of structured triglycerides, as described in greater detail below. As such, Lipase D is exempt from the premarket approval requirements of the Food, Drug, and Cosmetic Act.

i. Name and Address of the Notifier

Mr. Shinya Hayashi
Amano Enzyme Inc.
2-7, 1-Chome, Nishiki
Naka-ku, Nagoya 460-8630
JAPAN

ii. Common or Usual Name of the Subject Substance

The proposed common or usual name of the notified substance is:

Lipase derived from *Rhizopus oryzae (R. oryzae)*

iii. Conditions of Use

Lipase D is intended for use in the production of a dietary triglyceride intended for use in infant formula. The scope of this GRAS Notification is limited to the GRAS status of the Lipase D enzyme preparation when used in this application. This GRAS status of the dietary triglyceride was reported to FDA in GRAS Notification No. 131. Lipase D may be used instead of the enzyme identified in that submission.

Lipase D selectively hydrolyzes triglycerides of fatty acids to release free fatty acids of interest. It may be used in combination with other enzymes. The starting material is a food-grade vegetable oil.

The triglycerides produced in this manner closely mimic the structure of the milk fat present in human breast milk. Specifically, they are characterized by the presence of a saturated fatty acid, such as palmitic acid, in the mid- or sn-2 position of the triglyceride.

The Lipase D enzyme preparation is immobilized during the production of the structured triglycerides, and residues of the enzyme in the finished structured triglycerides are undetectable. As described in Section 4 below, it is estimated conservatively that the

finished structured triglycerides contain no more than 40 ppm of residual Lipase D enzyme.

iv. Basis for the GRAS Determination

Amano's GRAS determination for Lipase D is based upon scientific procedures, as described in greater detail in Section 4 below.

v. Statement of Availability of Data and Information

The data and information that are the basis for Amano's GRAS determination are available for review and copying by FDA at the offices of Keller and Heckman, LLP, 1001 G Street, N.W., Washington, D.C. 20001. These documents will be sent to FDA upon request.

November 20, 2006

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2. Detailed Information About the Identity of the Notified Substance

i. Name: Lipase from *R. oryzae*

ii. Enzyme Commission Number: EC 3.1.1.3

iii. Production Organism: *Rhizopus oryzae*

iv. Physical Description: Lipase D is an off-white to tan powder that is soluble in water but insoluble in alcohol.

v. Chemical Abstracts Service Registry Number: 9001-62-1

vi. Method of Manufacture:

The Lipase D enzyme is produced by the controlled fermentation of a selected strain of *R. oryzae*. The production strain has not been subjected to recombinant DNA technology. The fermentation medium is prepared from food-grade ingredients. The lipase enzyme activity is separated from the production organism following fermentation by a series of

filtration steps followed by precipitation. The finished product is dried and crushed. The manufacturing process is presented diagramatically in Appendix 1.

vii. Specifications:

Lipase D enzyme preparation satisfies the purity criteria for Enzyme Preparations found in the 5th Edition of the *Food Chemicals Codex* including the General Requirements and the Additional Requirements described on pages 151-152.

3. Information Relevant to Self-limiting Levels of Use

Not applicable.

4. Detailed Summary of Basis for Notifier's GRAS Determination

Amano's GRAS determination is based upon a series of toxicological studies of the enzyme and of the production organism. These studies include a 90-day gavage study with rats, genotoxicity studies, and a pathogenicity study of the production organism, *R. oryzae*, using mice. A published article describing these studies in detail is presented as Appendix 2.

i. 90-Day Rat Gavage Study

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

No deaths occurred during the administration or recovery periods, and there were no clinical signs or ophthalmologic abnormalities. Mean food consumption and body weights did not differ significantly from the controls. There were no treatment-related changes in hematology parameters relative to the controls. A more complete discussion of the methods and observations recorded in this study is provided in the article presented as Appendix 2. The no-observed-adverse-effect level (NOAEL) was conservatively set at 1000 mg/kg body weight.

Note that the Lipase D test substance used in the 90-day study had roughly the same activity and total organic solids (TOS) content as the commercially marketed product. Lipase D is not sold in diluted form.

ii. Genotoxicity Studies

a. Reverse Mutation Test on Bacteria

Mutagenicity of Lipase D was evaluated in four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and in *E. coli* WP2*uvrA*. In a preliminary test, no growth inhibition was observed by Lipase D (activity 2,784,000 U/g) on any of the tester strains, with or without metabolic activation, at the highest level tested, 5000 µg/plate, the OECD recommended maximum test concentration for noncytotoxic substances. Therefore, in the final tests, 5000 µg/plate was selected as the maximum test dose, with lower doses decreasing in half multiples (0, 313, 625, 1250, and 2500 µg/plate). Plating was in triplicate at each dose, and two independent assays were conducted. Positive controls consisted of 2-(2-furyl)-3-(5-nitro-2-furyl)-3-(5-nitro-2-furyl)acrylamide for the TA98 and TA100 strains, 9-aminoacridine hydrochloride for the TA1537 strain, sodium azide for the TA1535 strain, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine for the *E. coli* strain, and 2-aminoanthracene for all tester strains in the presence of metabolic activation (S-9 mix).

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

b. Chromosome Aberration Test

The potential of Lipase D to induce chromosome aberrations was tested using CHL/IU cells, derived from fibroblasts from the lungs of Chinese hamsters. Cultured cells were incubated in the presence of Lipase D for 6 h, with and without S-9 mix, and, in a separate test, for 24 and 48 h without S-9 mix. In the 6-h test, cells were washed with phosphate buffered saline, then cultured in fresh medium for a further 18 h.

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

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

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

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

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

Lipase D (activity 2,046,000 U/g) was tested for its potential to induce forward mutations at the thymidine kinase locus (TK+/-) in mouse lymphoma L5178 cells. In two independent tests, Lipase D was added to cell suspensions, at concentrations determined on the basis of a preliminary cytotoxicity test, and gently shaken for 3 h. Sterile deionized water served as the negative control. Methyl methanesulfonate (MMS) was the positive control in the absence of metabolic activation. 3-methylcholanthrene (MC) was the positive control with metabolic activation. After 3 h, the cells were washed and then placed in a growth medium for 48 h to allow expression of induced mutants. Samples were checked for growth at 24 and 48 h. At the end of this period, cells were assessed for viability by plating in triplicate onto agar plates, incubating for 12 days, and counting the colonies. Mutant frequencies were assessed in similar fashion except that the nutrient media contained trifluorothymidine (TFT), which only permitted growth of mutant TK-/- colonies.

A total of 4 independent tests were performed, 2 without S-9 activation and 2 with S-9 activation. In the first test without S-9, a dose-related increase in mutant frequencies was observed, but the increases were not statistically significant. In the second test, there was no dose-related increase in mutant frequency. Similarly, in the experiments with S-9, a dose-related increase in mutant frequencies was observed in the first test, but the increases were not statistically significant. There was no clear dose-related increase in the second test. In all cases, the positive controls, MMS and MC, produced a statistically significant increase in mutant frequencies. It is concluded that the tests did not demonstrate mutagenic potential for Lipase D.

iii. Pathogenicity Study

Although the production organism is not present in the Lipase D enzyme preparation, it is preferable not to use pathogenic organisms for the production of enzymes intended for use in food applications. The safety of the production organism was evaluated in a series of studies. This is a more extensive set of data than is strictly necessary to evaluate the safety of the enzyme preparation.

Spores of *R. oryzae* were injected once into the tail veins of 3 groups of 10 mice at doses of 1.3×10^4, 1.3×10^5, and 1.3×10^6 colony forming units (CFU) per body. A solution of 0.01% Tween 80 (polysorbate 80) in physiological saline solution injected into a fourth group of 10 mice served as the control. Animals were observed for mortality and clinical signs over a 14-day period. Body weights were measured on days 0, 3, 7, 10 and 14 after administration. The animals were sacrificed after 14 days. No death or changes in general conditions were found at the two lower doses. Four animals died upon administration of the 1.3×10^6 CFU dose. The six surviving animals demonstrated piloerection until day 7, after which no symptoms were noted.

Mean body weight gains were reduced at days 3, 7 and 10 in animals receiving the highest dose and on day 7 in animals receiving the intermediate dose. Viable spores were found in organs examined at all doses, except in the brains of animals receiving the lowest dose. Histopathological changes were observed in some of the organs in which viable spores were found, and fungal threads were found in the brain of one animal receiving the lowest dose.

In another study, spores were administered by gavage to 10 fasted mice at 1.3×10^7 CFU/body. Saline solution served as the control, administered to a second group of 10 fasted mice. In a third study, culture fluid in which spores had been incubated for over 3 days was administered to 2 groups of 10 fasted mice at 0 and 2.2×10^5 CFU/body. Sterilized culture fluid served as the control. Animals were observed in an identical manner to the injection study.

Culture fluid also was administered by gavage at 0 and 2.2×10^5 CFU/body to groups of 10 non-fasted mice daily for 28 days. Finally, three groups of 10 unfasted mice received untreated feed, feed mixed with 1.7×10^5 CFU/g spores and subsequently sterilized, or feed containing the same concentration of viable spores. The diet was replaced with fresh feed every day. In these experiments, body weights were measured on days 0, 3, 7, 10, 14, 17, 21, 24 and 28 days.

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

No deaths or adverse clinical signs were observed in any of the animals receiving *R. oryzae* orally. Viable spores were not found in organs from animals receiving the oral doses. Although occasional histopathological lesions were found in these organs, they could not be related to treatment with the fungi. Feed consumption in animals receiving

spores mixed with feed for 28 days did not differ significantly from that of the controls or the animals receiving sterilized spores. Mean total weight gain over 28 days relative to the controls was significantly lower in animals receiving spores mixed with feed (6.7 g versus 9.7 g) but did not differ significantly from animals receiving the sterilized spore-mixed feed (8.4 g), and was higher than the controls from the 28 day study in which culture fluid was administered (5.6 g).

Studies in which viable microorganisms are injected into a host animal may be useful in comparing relative toxicities of different microorganisms, but do not reflect true pathogenicity (See Pariza and Johnson[1]). Many otherwise harmless microorganisms produce opportunistic infections if they can penetrate normal host barriers, but a true pathogen will produce disease in non-compromised hosts. No adverse treatment-related effects were observed when viable spores of *R. oryzae* were administered orally. In any event, the production process of Lipase D ensures that viable microorganisms will not be present in the enzyme preparation.

iv. Mycotoxin and Antibiotic Analysis

Three batches of Lipase D tested negative for aflatoxins B1, B2, G1, and G2 (limit of detection (LOD): 0.5 ppb), ochratoxin A (LOD: 0.5 ppb), sterigmatocystin (LOD: 20 ppb), zearalenone (LOD: 50 ppb), and T-2 toxin (LOD: 200 ppb). Antibiotic activity was negative in the analyzed batches.

v. Safety Determination

To summarize, the NOAEL in the 90-day toxicity study in rats was conservatively set at 1000 mg/kg body weight per day for the Lipase D. Lipase D was found to be non-mutagenic, and the production organism was found not pathogenic when ingested orally.

The estimated daily intake for Lipase D resulting from the application covered by this notification is no more than 0.47 mg/kg body weight per day as discussed below. This compares favorably with the NOAEL of 1000 mg/kg body weight per day for Lipase D.

vi. Intake Estimate

We estimate that residues of denatured Lipase D in the interesterified oil will be present, if at all, at levels no higher than 40 parts per million (ppm).

The interesterified oil is produced using Lipase D according to the following basic steps.

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1 Pariza, Michael and Eric Johnson, "Evaluating the Safety of Microbial Enzyme Preparations Use in Food Processing: Update for a New Century," *Regulatory Toxicology and Pharmacology*, 33: 173-186, (2001).

1. Lipase D is immobilized on an inert carrier. The weight percent of Lipase D immobilized on the carrier is between 1 and 4%.

2. The oils or oil and fatty acid mixture are passed over the immobilized Lipase D, and enzymatic interesterification takes place.

3. If fatty acids are used during the interesterification, they are removed by flash evaporation at temperatures up to 240 °C and at high vacuum.

4. The resultant interesterified oil is refined using normal oil refining techniques such as alkali neutralization, bleaching with bleaching earth and deodorizing.

A mass balance analysis shows that residues of Lipase D will not exceed 40 ppm in the finished triglyceride. Between 1 and 4 kg of Lipase D is immobilized on 100 kg of inert carrier. This amount of carrier plus immobilized enzyme represents typical conditions for interesterifying more than 100 tons of oil. If all of the enzyme were removed from the inert carrier during the interesterification, the maximum amount of enzyme would be 4 kg in 100 tons of oil (40 ppm). However, this is clearly an exaggerative estimate as the carrier remains active after processing 100 tons of oil, meaning that the Lipase D is not completely removed from the carrier during interesterification. Furthermore, the standard refining techniques used will reduce any Lipase D present in the interesterified oil.

Thus, we estimate that any residues of denatured Lipase D in the interesterified oil will be present, if at all, at a maximum of 40 parts per million.

The intended use for Lipase D covered by this application is for the interesterification of oils for use in infant formula. The fat content of infant formula is usually about 3.5 - 4.5 g/100mL.[2,3]

In addition, average daily infant formula consumption by healthy infants born at term is 750 mL for the first six months.[4] Assuming the higher fat content of 4.5 g/100mL and a daily intake of 750 mL, the total daily fat intake for formula-fed infants will generally be 34 g.[5] In its response letter to GRAS Notification No. 131, FDA notes that it estimated intake of high 2-palmitic vegetable oil in infants as 22 g/day at the mean and 35 g/day at the 90th percentile.

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2 Jensen, R.G. Lipids in human milk – Composition and Fat-Soluble Vitamins. In: Lebenthal E, ed. Textbook of Gastroenterology and Nutrition in Infancy. 2nd ed. New York: Raven Press, Ltd., 1989: 157-208. See Appendix 4.

3 Huisman, M., et. al., European Journal of Clinical Nutrition, 1996; 50: 255-60. See Appendix 5.

4 National Research Council. Recommended Dietary Allowances. 10th ed. Washington, DC: National Academy Press, 1989: 18. During the next six months, the average daily consumption is 600 mL. We chose to use the higher value in our calculations to illustrate the maximum daily exposure. See Appendix 6.

5 4.5 g/100mL x 750 mL = 34 g.

Using the agency's estimate of 35 g/day, and also assuming the upper limit of 40 ppm of residual Lipase D in the fat, the maximum daily intake of Lipase D would be 1.4 mg.[6] Thus, a small infant weighing 2.97 kg would have a maximum estimated daily intake of 0.47 mg/kg body weight Lipase D.[7,8]

We emphasize that this calculation provides an exaggerated estimate of the total amount of denatured enzyme present because it assumes that all of the enzyme is removed from the inert carrier during the interesterification, and that the refining techniques used will not reduce the amount of Lipase D present in the interesterified oil. Nevertheless, this exaggerated calculation results in a maximum potential level of dietary exposure over 2000 times below the NOAEL derived from the 90-day feeding study (0.47 mg/kg body weight per day versus 1000 mg/kg body weight per day).

vii. Additional Published Article on the Safety of Lipase Derived from *Rhizopus oryzae*

An additional peer reviewed study concerning the safety of lipase produced by a different strain of *R. oryzae* for use as a food additive was published in *Food and Chemical Toxicology.*[9] The enzyme was examined for acute, subacute, and subchronic oral toxicity, and mutagenic potential.[10] No evidence of subacute oral toxicity or mutagenic potential was found, and administration of the lipase at dosages of 50, 500, and 1000 mg/kg body weight per day did not induce noticeable signs of toxicity.[11] The NOAEL in the subchronic toxicity study was 1000 mg/kg body weight per day.

viii. Evaluation of Lipase D under the Pariza and Johnson Decision Tree

In a well-received article published in *Regulatory Toxicology and Pharmacology*, Michael Pariza and Eric Johnson present guidelines for evaluating the safety of production strain metabolites that are present in an enzyme preparation.[12] The authors consider the safety of the production strain the primary issue in evaluating enzyme safety, specifically the toxigenic potential of the production strain.[13]

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6 34 g x 40 mg/1000g = 1.4 mg

7 1.4 mg/day/infant x 1 infant/2.97 kg = 0.47 mg/day/kg body weight

8 A one-month old female infant in the 5th percentile weighs on average 2.97 kg. National Research Council. Recommended Dietary Allowances. 10th ed. Washington, DC: National Academy Press, 1989: 16-17. See Appendix 6.

9 Coenen, T.M.M., P. Aughton, and H. Verhagen, "Safety Evaluation of Lipase Derived from *Rhizopus oryzae*: Summary of Toxicological Data," Food and Chemical Toxicology, 35: 315-22 (1997). See Appendix 3.

10 Id.

11 Id.

12 See Pariza, Michael and Eric Johnson, "Evaluating the Safety of Microbial Enzyme Preparations Use in Food Processing: Update for a New Century," Regulatory Toxicology and Pharmacology, 33: 173-186, (2001).

13 Id.

Thus, the decision tree outlines twelve steps for determining the safety of the production strain, only some of which apply if the strain is not genetically modified.[14] Specifically, for non-genetically modified strains that have not previously been evaluated by this method, the questions focus on whether the strain is

(1) nonpathogenic[15]
(2) free of antibiotics
(3) free of oral toxins (or below levels of concern) known to be produced by other members of the same species, and
(4) shown to have a NOAEL sufficiently high to ensure safety.[16]

The authors conclude that separate mutagenicity testing is not necessary if this decision tree is used to evaluate the enzyme safety.

Because it is not genetically modified, the safety evaluation of Lipase D under the decision tree involves only the four questions set forth above. In particular, the production organism used to manufacture Lipase D is nonpathogenic, free of antibiotics, and free of oral toxins known to be produced by other members of the same species. Finally, as discussed above, the NOAEL for Lipase D was conservatively set at 1000 mg/kg body weight per day, which was 2000 times the estimated daily intake.

Thus, the Lipase D production organism, *R. oryzae*, can be deemed safe under the approach suggested by Pariza and Johnson, which is reflective of current scientific thinking regarding the evaluation of microbial enzyme preparations used in food processing.

ix. Expert Consensus

The safety studies described in Section 4 for Lipase D have been published in a peer-reviewed scientific journal,[17] a copy of which is presented as Appendix 2. The authors concluded that Lipase D may be regarded as safe when used as a processing aid in the food industry. It is unlikely that an expert would disagree with this conclusion, given the lack of toxicity associated with enzymes, the low estimated dietary exposure associated with this application, the safety of the production organism, and the large margin of safety between the 90-day NOAEL for this particular enzyme and its estimated daily intake described above.

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14 Id.

15 For enzyme preparations, nonpathogenicity is established if the preparation does not contain live organisms.

16 Id.

17 Flood, Michael T. and Mitsuru Kondo, Safety evaluation of lipase produced from Rhizopus oryzae: summary of toxicology data, Regulatory Toxicology and Pharmacology, 37: 293-304 (2003). See Appendix 2.

Moreover, the expert consensus of the safety of Lipase D for its intended use is further supported by the general recognition of the safety of its source organism for use in food. *R. oryzae* has been used for centuries in the production of fermented foods, including tempeh, an Indonesian fermented soybean food. Some reports identify *R. oryzae* as the essential microorganism in the preparation of tempeh.[18] [19] Other reports state that a variety of *Rhizopus* species have been used in the manufacture of tempeh. Tempeh is produced by growing mold on soybeans. The beans are soaked in water, cooked, inoculated, and fermented. During fermentation, the soybeans become bound together in a solid mass. The raw tempeh is then sliced, cooked in soup, fried, or roasted. The fungus present in tempeh is consumed directly after cooking. Tempeh is consumed daily by millions of people in Indonesia and to a lesser extent in the United States and in Europe. Thus, it is clear from the long history of widespread consumption that there is a general recognition of the safety of *R. oryzae* for use in food.

In addition, the FDA has approved the use of carbohydrase derived from *R. oryzae* for use in the production of dextrose from starch (21 C.F.R. § 173.130).

Also, GRAS Affirmation Petition No. 3G0016, submitted to FDA in 1973 by the Ad Hoc Enzyme Technical Committee, now known as the Enzyme Technical Association, included a claim that carbohydrase derived from *R. oryzae* is GRAS, but FDA seems not to have reviewed this aspect of the Petition/Notification (see Agency Response Letter to GRN No. 90).

Furthermore, carbohydrase derived from *R. oryzae* was evaluated by JECFA in 1971 and was assigned an acceptable daily intake (ADI) "not limited."[20]

Thus, the expert consensus and the general recognition elements of GRAS status are satisfied by the fact that (1) our published and peer reviewed safety evaluation is consistent with current scientific thinking regarding the evaluation of microbial enzyme preparations used in food processing, and (2) the production organism, *R. oryzae*, has a long history of safe use and has been evaluated favorably by qualified experts.

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18 See, *e.g.*, Steinkraus, K.H., et al., 1960, Studies on tempeh – an Indonesian fermented soybean food, *Food Research* 26: 777-788, Appendix 7.

19 See, *e.g.*, Stahel, Gerold, 1946, Foods from fermented soybeans...as prepared in the Netherlands Indies, Pt. II – Tempe, a tropical staple, *Journal of the New York Botanical Garden*, Vol. 47 (1946), No. 564, pp. 285-296, Appendix 8.

20 Fifteenth Report of JECFA (Geneva, 16 - 24 June 1971), FAO Nutrition Meetings Report Series No. 50, WHO Technical Report Series No. 488.

List of Appendices

Appendix 1: Manufacturing Method for Lipase D

Appendix 2: Published article: Flood, Michael T. and Mitsuru Kondo, Safety evaluation of lipase produced from *Rhizopus oryzae*: summary of toxicology data, *Regulatory Toxicology and Pharmacology*, 37: 293-304 (2003).

Appendix 3: Published article: Coenen, T.M.M., P. Aughton, and H. Verhagen, "Safety Evaluation of Lipase Derived from *Rhizopus oryzae*: Summary of Toxicological Data," *Food and Chemical Toxicology*, 35: 315-22 (1997).

Appendix 4: Jensen, R.G. "Lipids in human milk – Composition and Fat-Soluble Vitamins," Lebenthal E, ed. *Textbook of Gastroenterology and Nutrition in Infancy*. 2nd ed. New York: Raven Press, Ltd., 157-208 (1989).

Appendix 5: Huisman, M., et. al., "Triglycerides, fatty acids, sterols, mono- and disaccharides and sugar alcohols in human milk and current types of infant formula milk," *European Journal of Clinical Nutrition*, 1996; 50: 255-60.

Appendix 6: National Research Council. *Recommended Dietary Allowances*. 10th ed. Washington, DC: National Academy Press, 16-18 (1989).

Appendix 7: Steinkraus, K.H., et al., 1960, Studies on tempeh – an Indonesian fermented soybean food, *Food Research* 26: 777-788

Appendix 8: Stahel, Gerold, 1946, Foods from fermented soybeans…as prepared in the Netherlands Indies, Pt. II – Tempe, a tropical staple, *Journal of the New York Botanical Garden*, Vol. 47 (1946), No. 564, pp. 285-296.

1

Manufacturing Process of Lipase D

Process	Condition
Slant culture of *Rhizopus oryzae*	(b) [redacted]
1st Seed culture with flask	
2nd Seed culture with tank	
Production culture with tank	
Filtration with press filter	
Concentration of culture filtrate with ultrafilter	
Precipitation by ammonium sulfate	
Extraction of Lipase D with water	
Concentration with ultrafilter	
Microfiltration	
Freezing Dry	
Crashing and Mixing	
Product (Lipase D)	

2

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Pages 000117 - 000128 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

SUBMISSION END

Reference List for Industry Submission, GRN 000216

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000019 - 000030	Flood, Michael T.; Kondo, Mitsuru	Safety evaluation of lipase produced from Rhizopus oryzae: summary of toxicological data	2003	Regulatory Toxicology and Pharmacology	Volume 37, pgs 293-304
000032 - 000039	Coenen, T.M.M.; Aughton, P.; Verhagen, H.	Safety Evaluation of Lipase Derived from Rhizopus oryzae: Summary of Toxicological Data	1997	Food and Chemical Toxicology	Volume 35, pgs 315-322
000041 - 000092	Jensen, Robert G.	Lipids in Human Milk -- Composition and Fat-Soluble Vitarnins	1989	Textbook of Gastroenterology and Nutrition in Infancy, Second Edition	pgs 157-208
000094 - 000099	Huisman, M.; van Beusekom, CM; Lanting, CI; Nijeboer, HJ; Muskiet, FAJ; Boersma, ER	Triglycerides, fatty acids, sterols, mono- and disaccharides and sugar alcohols in human milk and current types of infant formula milk	1996	European Journal of Clinical Nutrition	Volume 50, pgs 255-260
000101	NA	Nutrient Allowances For Infants	1989	Recommended Dietary Allowances, Tenth Edition	pg 18
000102	NA	Nutrient Allowances For Infants	1989	Recommended Dietary Allowances, Tenth Edition	pg 17
000104 - 000115	Steinkraus, K.H.; Hwa, Yap Bwee; Van Buren, J.P.; Provvidenti, M.I.; Hand, D.B.	Studies on Tempeh - An Indonesian Fermented Soybean Food	1960	Food Research	Volume 26, pgs 777 - 788
000117 - 000128	Stahel, Gerold	Foods from Fermented Soybeans...As Prepared in the Netherlands Indies: II -- Tempe, A Tropical Style	December 1946	Journal of The New York Botanical Garden	Volume 47, Number 564, pgs 285-296

NA- Not applicable

Binion Williams, Felicia B

From: Joy, David R [Joy@khlaw com]
t: Wednesday, February 07, 2007 5 19 PM
To: Binion Williams, Felicia B
Cc: Shinya Hyashi, Flood, Michael T
Subject: RE GRAS Notice 000216
Attachments: response letter pdf, Attachment I pdf, Attachment 2 pdf

AM

Dear Dr Williams,

We've prepared the attached letter responding to the questions presented in your email below We will also send along a hard co of the letter by express mail

We'd be happy to answer any further questions

Best regards,

David R Joy
tel 202 434 4126 | fax 202 434 4646 | Joy@khlaw com
1001 G Street, N W , Suite 500 West | Washington, D C 20001

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From: Binion Williams, Felicia B [mailto:felicia.binionwilliams@fda.hhs.gov]
Sent: Thursday, January 25, 2007 10:34 AM
To: Joy, David R.
Cc: Binion Williams, Felicia B
Subject: GRAS Notice 000216
Importance: High

Dear Mr Joy,

During our telephone conversation on Tuesday, January 16, 2007, you requested that we follow-up with a written letter

The acknowledgement letter states that the subject of the notice is lipase enzyme preparation from *Rhizopus oryzae* However, based our preliminary review of GRAS Notice 000216 (GRN 216), we are identifying the subject of the GRAS notice as an immobilized lipase

1 Please provide clarification of the identity of the subject of the GRAS notice

2 Please provide composition and specifications for the final product as it will be marketed **Note:** This question has been modified from the previous e-mail

3 What is the immobilizing agent?

4 We understand that the middle dose level was chosen because there were no effects at this dose level Please provide recognition of the effects seen at the higher dose level What were the effects, do you think these effects were indicative of the

treatment, or that the effects could have been a byproduct of something else (study parameters, animal model, etc) Please prov
a comprehensive discussion

We believe that this information is readily available, we are requesting that you provide the additional information to us within 2 we' 's If you have further questions, please contact me

Sincerely,
Felicia B Williams

Felicia B Williams, Ph D
LCDR, USPHS
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
5100 Paint Branch Parkway
HFS-255
College Park, MD 20740
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Felicia BinionWilliams@fda.hhs.gov

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February 7, 2007

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(202) 434-4126
Joy@khlaw.com

Dr. Felicia B. Williams
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
5100 Paint Branch Parkway
HFS-255
College Park, Maryland 20740

Re: Additional Information for GRAS Notification No. 216

Dear Dr. Williams:

Thank you for your email of January 25 in which the agency requested additional information to support its review of the above-referenced GRAS Notification, which Keller and Heckman filed on behalf of our client, Amano Enzyme, Inc., of Japan. The agency's questions and Amano's responses are as follows.

1. *Please provide clarification of the identity of the subject of the GRAS notice*

We believe this question is aimed at clarifying whether the subject of the GRAS notice is the Lipase D enzyme itself or the immobilized enzyme (i.e., Lipase D in combination with an inert carrier). The notifier has claimed GRAS status only for the enzyme prior to immobilization.

We are providing some information below regarding the carrier on which the enzyme is immobilized. However, we stress that Amano's product is the enzyme itself, prior to immobilization Amano does not at any point handle or distribute the immobilized enzyme, and Amano does not wish to limit the types of carriers that can be used with its enzyme. GRN 216 asserts only that the enzyme itself is GRAS and is premised upon an understanding that any inert carrier used with the enzyme must conform to an applicable food additive regulation, be the subject of an effective Food Contact Notification, Threshold of Regulation determination, or must qualify as GRAS or otherwise comply with the requirements of the Federal Food, Drug, and Cosmetic Act and the applicable food additive regulations.

We also note that the dietary exposure estimate for Lipase D presented in the GRAS Notice is based upon a conservative and unrealistic assumption that all Lipase D immobilized on

the carrier will remain in the treated food Thus, the immobilization of Lipase D is not a critical aspect of the GRAS evaluation of the enzyme.

Despite these arguments, if FDA wishes to specify that GRN 216 applies only to immobilized Lipase D, Amano would not object to this, provided it remains clear that the enzyme can be used with any lawful and suitable carrier.

2. *Please provide composition and specifications for the final product as it will be marketed.*

Amano's specifications for its Lipase D enzyme are presented as Attachment 1 to this letter. As you know, the GRAS Notice states that Lipase D complies with the purity requirements for enzymes set forth in the 5th Edition of the *Food Chemicals Codex*. The attached set of specifications provides additional detail. Lipase D is distributed as a concentrated enzyme. There is no diluent added. As indicated in the manufacturing process diagram (Appendix 1 in the GRAS Notification), Lipase D is freeze dried; then crushed and mixed to obtain the final product. Please let us know if you require any additional information.

We note that the lipase activity is stated to be "not less than 8,000,000 u/g" in the attached specifications. This refers to lipase activity measured by Amano's own test method. There are multiple test methods for measuring lipase activity including a Japanese Industrial Standards (JIS) test method, which produces different values. Please let us know if you require additional information in this regard.

3. *What is the immobilizing agent?*

Again, the specific immobilizing agent currently in use is not within the scope of the GRAS Notice. We emphasize that GRN 216 claims Lipase D to be GRAS when used as described in the Notification with any suitable and lawful immobilizing agent

However, Amano's customer has permitted us to disclose that the enzyme is currently immobilized on porous polypropylene granules. The polypropylene is food-grade and is certified by its supplier to comply with 21 C.F.R. 177.1520 (olefin polymers). Before application of Lipase D to the polypropylene granules, the granules are wetted with a solution of water and surface-active agents that are either GRAS or cleared at 21 C.F.R. 178.3400 (emulsifiers and/or surface-active agents in articles intended to contact food). Lipase D is then loaded onto the polypropylene granules and is adsorbed spontaneously. The granules are washed with water to remove all non-immobilized proteins and surfactant from the surface and pores. After washing, the treated granules are left to drain and then dried.

4. We understand that the middle dose level was chosen because there were no effects at this dose level Please provide recognition of the effects seen at the higher dose level. What were the effects, do you think these effects were indicative of the treatment, or that the effects could have been a byproduct of something else (study parameters, animal model, etc.). Please provide a comprehensive discussion.

Michael Flood of Keller and Heckman's scientific staff helped prepare the following response.

Two findings in the 90-day rat study may have been treatment-related: a decrease in urinary pH, and an increase in renal tubular basophilia. For the reasons explained below, however, it is our judgment, as well as the judgment of the study's investigators, that these findings are not relevant to the toxicity of Lipase D.

I. Decrease in Urinary pH

Mean pH of the groups receiving 2000 mg/kg bw Lipase D were lower than those of the controls. Mean urinary pH of the control and the 2000 mg/kg male groups were 8.2 and 7.7, respectively ($p<0.05$). Corresponding values for females were 7.8 and 6.8 ($p<0.01$). However, there was no clear dose response, and the mean urinary pH of the 4-week recovery groups (control and 2000 mg/kg bw) did not differ significantly.

Ingestion of large amounts of enzyme protein (up to 1 gram per day) could be expected to result in some change in mean pH, because at least some of the protein will be absorbed. Whether or not the observed decrease in pH was incidental to the test substance, we agree with Stevens and Mylecraine that urinary pH is

> [g]enerally meaningless unless outside of the range of 6.0-8.0. However, slight differences between treated and untreated animals may result from excretion of basic or acidic metabolites.[1]

Therefore, even if the observed decrease in pH is not incidental, the no-observed-adverse-effect level (NOAEL), as opposed to the no-observed-effect level (NOEL), should be the highest administered dose, 2000 mg/kg.

---

[1] Stevens, K.R. and Mylecraine, L. "Issues in Chronic Toxicology." Chapter 18, page 688, in *Principles and Methods of Toxicology, Third Edition*, edited by A. Wallace Hayes. Raven Press, Ltd., New York 1994.

II. Renal Tubular Basophilia

"Slight or mild" tubular basophilia was observed in 3 out of 12 control males, 3 out of 12 males in the 500 mg/kg bw dose group, 4 out of 12 males in the 1000 mg/kg bw dose group, and 6 out of 12 males in the 2000 mg/kg bw dose group. The finding was only observed in one female in the highest dose group. These findings may well be incidental. According to Peter *et al.* (1986), renal tubular basophilia is a relatively frequent spontaneous finding in young control rats.[2] Its incidence is higher in males than in females. It is believed that these tubular changes are early stages in chronic progressive nephrosis because they are frequently seen in strains where a high incidence of chronic progressive nephrosis occurs in older animals. The Sprague-Dawley strain is considered one of the most susceptible strains to chronic progressive nephrosis.

The complete summary tables of histopathological findings in male animals, including recovery animals, are provided as Attachment 2. It can be seen that, although there is a slight increase in the number of animals having "slight" tubular basophilia, there is no net increase in animals having "mild" basophilia.[3] In other words, the severity of the lesion did not increase with dose. Additionally, basophilia was not observed in any of the 6 recovery animals treated at 2000 mg/kg bw and sacrificed 28 days after completion of the main study.

Given that renal tubular basophilia is a common finding in young Sprague-Dawley rats, there was only a slight observed increase with increasing dose, and the severity of the lesions did not differ from those in the controls, we agree with the study's investigators, who conclude that the findings appear to be incidental.

Our overall conclusion is that the NOAEL for the 13-week study should be the highest administered dose, 2000 mg/kg bw/day. In this regard, we now believe that the NOAEL of 1000 mg/kg bw, suggested in the published article by Flood and Kondo, should be listed as a NOEL.[4] However, given the very low potential dietary exposure to this enzyme this distinction should not be important.

---

[2] Peter, C.P., Burek, J.D. and van Zwieten, M.J (1986). Spontaneous nephropathies in rats. *Toxicologic Pathology* **14(1)**, 91-99.

[3] On a scale of 0-4, 0 is no finding, 1 is slight, 2 is mild, 3 is moderate, and 4 is severe.

[4] Flood, M.T. and Kondo, M. (2003). Safety evaluation of lipase produced from *Rhizopus oryzae*: summary of toxicological data. *Reg. Toxicol. Pharmacol.* **37,** 293-304.

* * *

We trust you will find this letter fully responsive to your request for additional information. If you have any further questions, please do not hesitate to contact me.

Sincerely,

(b)(6)

David R. Joy

cc: Shinya Hayashi, Amano Enzyme, Inc.

AMANO

Specifications of Lipase D Conc.

Test item	Test method	Specification
Lipase activity	Amano method, pH 7.0	Not less than 8,000,000 u/g
Loss on drying	1g, 105℃, 4 hrs.	Not more than 10%
Heavy metals	Japanese Pharmacopoeia	Not more than 40 ppm
Arsenic	Japanese Pharmacopoeia	Not more than 3 ppm
Lead	Japanese Pharmacopoeia	Not more than 5 ppm
Total viable aerobic count	Japanese Pharmacopoeia	Not more than 50,000 /g
Coliforms	FDA BAM	Not more than 30/g
E. coli	Japanese Pharmacopoeia	Negative
Salmonella	Japanese Pharmacopoeia	Negative

Amano Enzyme Inc. 2-7 1-Chome Nishiki Naka-ku, Nagoya 460-8630 Japan Tel 052 211 3032 Fax 052 211 3054

Table 10-1 A 13-week oral toxicity study of Lipase D in rats with a recovery period of 4 weeks

Histopathological findings (13 weeks)

Male

Organs	Dose (mg/kg)	0							500							1000							2000						
	No. of animals	12							12							12							12						
-findings	Grade	0	1	2	3	4	P	TE	0	1	2	3	4	P	TE	0	1	2	3	4	P	TE	0	1	2	3	4	P	TE
Pancreas																													
-fibrosis, islet		12						12	11	1					12	12						12	10		2				12
Kidney																													
-basophilia, tubular		9	2	1				12	9	3					12	8	4					12	6	5	1				12
-eosinophilic body		8	2	2				12	12						12	12						12	6	3	3				12
-nephroblastoma		11					1	12	12						12	12						12	12						12
-pyelonephritis		11		1				12	12						12	12						12	12						12
Heart																													
-myocarditis, focal		10	1	1				12															11	1					12
Stomach																													
-erosion, glandular stomach		12						12										2				2	12						12
Prostate																													
-cell infiltration, interstitial		9	1	2				12															8		4				12
Skeletal muscle																													
-necrosis, focal		11		1				12															12						12

0 : No remarkable changes 1 : Slight 2 : Mild 3 : Moderate 4 : Severe P : Present TE : Total Examined

Table 10-3 *A 13-week oral toxicity study of Lipase D in rats with a recovery period of 4 weeks*

Histopathological findings (Recovery)

Male

Organs	Dose (mg/kg)	0							2000						
	No. of animals	6							6						
-findings	Grade	0	1	2	3	4	P	TE	0	1	2	3	4	P	TE
Heart															
-myocarditis, focal		5		1				6	6						6
Lung (Bronchus)															
-pneumonia, focal		5	1					6	6						6
Pancreas															
-basophilic foci		6						6	5					1	6
Pituitary															
-hyperplasia, anterior lobe, focal		6						6	5					1	6
Thyroid (Parathyroid)															
-C cell hyperplasia, focal		6						6	5					1	6
Kidney															
-eosinophilic body		6						6	5		1				6
-pyelonephritis		6						6	5		1				6
-dilatation, pelvic		5		1				6	6						6
Prostate															
-cell infiltration, interstitial		4	1	1				6	3	2	1				6

0 : No remarkable changes 1 : Slight 2 : Mild 3 : Moderate 4 : Severe P : Present TE : Total Examined

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David R. Joy
(202) 434-4126
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February 7, 2007

Dr. Felicia B. Williams
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
5100 Paint Branch Parkway
HFS-255
College Park, Maryland 20740

Re Additional Information for GRAS Notification No 216

Dear Dr. Williams:

Thank you for your email of January 25 in which the agency requested additional information to support its review of the above-referenced GRAS Notification, which Keller and Heckman filed on behalf of our client, Amano Enzyme, Inc., of Japan. The agency's questions and Amano's responses are as follows.

1 *Please provide clarification of the identity of the subject of the GRAS notice*

We believe this question is aimed at clarifying whether the subject of the GRAS notice is the Lipase D enzyme itself or the immobilized enzyme (i.e., Lipase D in combination with an inert carrier). The notifier has claimed GRAS status only for the enzyme prior to immobilization.

We are providing some information below regarding the carrier on which the enzyme is immobilized. However, we stress that Amano's product is the enzyme itself, prior to immobilization. Amano does not at any point handle or distribute the immobilized enzyme, and Amano does not wish to limit the types of carriers that can be used with its enzyme. GRN 216 asserts only that the enzyme itself is GRAS and is premised upon an understanding that any inert carrier used with the enzyme must conform to an applicable food additive regulation, be the subject of an effective Food Contact Notification, Threshold of Regulation determination, or must qualify as GRAS or otherwise comply with the requirements of the Federal Food, Drug, and Cosmetic Act and the applicable food additive regulations.

We also note that the dietary exposure estimate for Lipase D presented in the GRAS Notice is based upon a conservative and unrealistic assumption that all Lipase D immobilized on

the carrier will remain in the treated food. Thus, the immobilization of Lipase D is not a critical aspect of the GRAS evaluation of the enzyme.

Despite these arguments, if FDA wishes to specify that GRN 216 applies only to immobilized Lipase D, Amano would not object to this, provided it remains clear that the enzyme can be used with any lawful and suitable carrier.

2. *Please provide composition and specifications for the final product as it will be marketed.*

Amano's specifications for its Lipase D enzyme are presented as Attachment 1 to this letter. As you know, the GRAS Notice states that Lipase D complies with the purity requirements for enzymes set forth in the 5th Edition of the *Food Chemicals Codex*. The attached set of specifications provides additional detail. Lipase D is distributed as a concentrated enzyme There is no diluent added. As indicated in the manufacturing process diagram (Appendix 1 in the GRAS Notification), Lipase D is freeze dried; then crushed and mixed to obtain the final product. Please let us know if you require any additional information.

We note that the lipase activity is stated to be "not less than 8,000,000 u/g" in the attached specifications. This refers to lipase activity measured by Amano's own test method. There are multiple test methods for measuring lipase activity including a Japanese Industrial Standards (JIS) test method, which produces different values. Please let us know if you require additional information in this regard.

3. *What is the immobilizing agent?*

Again, the specific immobilizing agent currently in use is not within the scope of the GRAS Notice. We emphasize that GRN 216 claims Lipase D to be GRAS when used as described in the Notification with any suitable and lawful immobilizing agent.

However, Amano's customer has permitted us to disclose that the enzyme is currently immobilized on porous polypropylene granules The polypropylene is food-grade and is certified by its supplier to comply with 21 C.F.R. 177.1520 (olefin polymers). Before application of Lipase D to the polypropylene granules, the granules are wetted with a solution of water and surface-active agents that are either GRAS or cleared at 21 C.F.R. 178.3400 (emulsifiers and/or surface-active agents in articles intended to contact food). Lipase D is then loaded onto the polypropylene granules and is adsorbed spontaneously. The granules are washed with water to remove all non-immobilized proteins and surfactant from the surface and pores. After washing, the treated granules are left to drain and then dried

4 We understand that the middle dose level was chosen because there were no effects at this dose level Please provide recognition of the effects seen at the higher dose level What were the effects, do you think these effects were indicative of the treatment, or that the effects could have been a byproduct of something else (study parameters, animal model, etc). Please provide a comprehensive discussion.

Michael Flood of Keller and Heckman's scientific staff helped prepare the following response.

Two findings in the 90-day rat study may have been treatment-related: a decrease in urinary pH, and an increase in renal tubular basophilia. For the reasons explained below, however, it is our judgment, as well as the judgment of the study's investigators, that these findings are not relevant to the toxicity of Lipase D

I. Decrease in Urinary pH

Mean pH of the groups receiving 2000 mg/kg bw Lipase D were lower than those of the controls. Mean urinary pH of the control and the 2000 mg/kg male groups were 8.2 and 7.7, respectively ($p<0.05$). Corresponding values for females were 7.8 and 6.8 ($p<0.01$). However, there was no clear dose response, and the mean urinary pH of the 4-week recovery groups (control and 2000 mg/kg bw) did not differ significantly

Ingestion of large amounts of enzyme protein (up to 1 gram per day) could be expected to result in some change in mean pH, because at least some of the protein will be absorbed. Whether or not the observed decrease in pH was incidental to the test substance, we agree with Stevens and Mylecraine that urinary pH is

> [g]enerally meaningless unless outside of the range of 6 0-8 0. However, slight differences between treated and untreated animals may result from excretion of basic or acidic metabolites.[1]

Therefore, even if the observed decrease in pH is not incidental, the no-observed-adverse-effect level (NOAEL), as opposed to the no-observed-effect level (NOEL), should be the highest administered dose, 2000 mg/kg.

---

[1] Stevens, K.R. and Mylecraine, L. "Issues in Chronic Toxicology." Chapter 18, page 688, in *Principles and Methods of Toxicology, Third Edition*, edited by A. Wallace Hayes. Raven Press, Ltd., New York 1994

II. Renal Tubular Basophilia

"Slight or mild" tubular basophilia was observed in 3 out of 12 control males, 3 out of 12 males in the 500 mg/kg bw dose group, 4 out of 12 males in the 1000 mg/kg bw dose group, and 6 out of 12 males in the 2000 mg/kg bw dose group. The finding was only observed in one female in the highest dose group. These findings may well be incidental. According to Peter *et al.* (1986), renal tubular basophilia is a relatively frequent spontaneous finding in young control rats.[2] Its incidence is higher in males than in females It is believed that these tubular changes are early stages in chronic progressive nephrosis because they are frequently seen in strains where a high incidence of chronic progressive nephrosis occurs in older animals. The Sprague-Dawley strain is considered one of the most susceptible strains to chronic progressive nephrosis.

The complete summary tables of histopathological findings in male animals, including recovery animals, are provided as Attachment 2. It can be seen that, although there is a slight increase in the number of animals having "slight" tubular basophilia, there is no net increase in animals having "mild" basophilia [3] In other words, the severity of the lesion did not increase with dose Additionally, basophilia was not observed in any of the 6 recovery animals treated at 2000 mg/kg bw and sacrificed 28 days after completion of the main study.

Given that renal tubular basophilia is a common finding in young Sprague-Dawley rats, there was only a slight observed increase with increasing dose, and the severity of the lesions did not differ from those in the controls, we agree with the study's investigators, who conclude that the findings appear to be incidental

Our overall conclusion is that the NOAEL for the 13-week study should be the highest administered dose, 2000 mg/kg bw/day. In this regard, we now believe that the NOAEL of 1000 mg/kg bw, suggested in the published article by Flood and Kondo, should be listed as a NOEL.[4] However, given the very low potential dietary exposure to this enzyme this distinction should not be important

---

[2] Peter, C.P., Burek, J.D. and van Zwieten, M.J (1986). Spontaneous nephropathies in rats. *Toxicologic Pathology* **14(1),** 91-99.

[3] On a scale of 0-4, 0 is no finding, 1 is slight, 2 is mild, 3 is moderate, and 4 is severe

[4] Flood, M.T. and Kondo, M. (2003). Safety evaluation of lipase produced from *Rhizopus oryzae*: summary of toxicological data. *Reg. Toxicol Pharmacol* **37,** 293-304.

* * *

We trust you will find this letter fully responsive to your request for additional information. If you have any further questions, please do not hesitate to contact me.

Sincerely,

(b)(6)

David R. Joy

cc: Shinya Hayashi, Amano Enzyme, Inc.

AMANO

Specifications of Lipase D Conc.

Test item	Test method	Specification
Lipase activity	Amano method, pH 7.0	Not less than 8,000,000 u/g
Loss on drying	1g, 105℃, 4 hrs.	Not more than 10%
Heavy metals	Japanese Pharmacopoeia	Not more than 40 ppm
Arsenic	Japanese Pharmacopoeia	Not more than 3 ppm
Lead	Japanese Pharmacopoeia	Not more than 5 ppm
Total viable aerobic count	Japanese Pharmacopoeia	Not more than 50,000 /g
Coliforms	FDA BAM	Not more than 30/g
E. coli	Japanese Pharmacopoeia	Negative
Salmonella	Japanese Pharmacopoeia	Negative

Amano Enzyme Inc. 2-7, 1-Chome, Nishiki Naka-ku, Nagoya 460-8630 Japan Tel. 052 211 3032 Fax. 052 211 3054

Table 10-1 A 13-week oral toxicity study of Lipase D in rats with a recovery period of 4 weeks

Histopathological findings (13 weeks)

Male

Organs	Dose (mg/kg)	0							500							1000							2000						
	No. of animals	12							12							12							12						
-findings	Grade	0	1	2	3	4	P	TE	0	1	2	3	4	P	TE	0	1	2	3	4	P	TE	0	1	2	3	4	P	TE
Pancreas																													
-fibrosis, islet		12						12	11	1					12	12						12	10		2				12
Kidney																													
-basophilia, tubular		9	2	1				12	9	3					12	8	4					12	8	5	1				12
-eosinophilic body		8	2	2				12	12						12	12						12	8	3	3				12
-*nephroblastoma*		11					1	12	12						12	12						12	12						12
-pyelonephritis		11		1				12	12						12	12						12	12						12
Heart																													
-myocarditis, focal		10	1	1				12															11	1					12
Stomach																													
-*erosion*, glandular stomach		12						12										2				2	12						12
Prostate																													
-cell infiltration, interstitial		9	1	2				12															8		4				12
Skeletal muscle																													
-necrosis, focal		11		1				12															12						12

0 : No remarkable changes 1 : Slight 2 : Mild 3 : Moderate 4 : Severe P : Present TE : Total Examined

Attachment 2

Table 10-3 A 13-week oral toxicity study of Lipase D in rats with a recovery period of 4 weeks

Histopathological findings (Recovery)

Male

Organs	Dose (mg/kg)	0							2000						
	No of animals	6							6						
-findings	Grade	0	1	2	3	4	P	TE	0	1	2	3	4	P	TE
Heart															
-myocarditis, focal		5		1				6	6						6
Lung (Bronchus)															
-pneumonia, focal		5	1					6	6						6
Pancreas															
-basophilic foci		6						6	5					1	6
Pituitary															
-hyperplasia, anterior lobe, focal		6						6	5					1	6
Thyroid (Parathyroid)															
-C cell hyperplasia, focal		6						6	5					1	6
Kidney															
-eosinophilic body		6						6	5		1				6
-pyelonephritis		6						6	5		1				6
-dilatation, pelvic		5		1				6	6						6
Prostate															
-cell infiltration, interstitial		4	1	1				6	3	2	1				6

0 : No remarkable changes 1 : Slight 2 : Mild 3 : Moderate 4 : Severe P : Present TE : Total Examined