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August 31,2001

Hand Delivered

Dr. Alan Rulis
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

Re: **GRAS Notification for Lipase Derived from *Candida rugosa***

Dear Dr. Rulis:

Pursuant to proposed 21 C.F.R. § 170.36(c) and on behalf of our client, Amano Enzyme Inc., of Tokyo Japan, we hereby notify the agency of our determination on the basis of scientific procedures that Amano's Lipase AY enzyme preparation, derived from *Candida rugosa* is generally recognized as safe (GRAS) when used as a processing aid in the derivation of dietary ingredients from fats and oils. As a GRAS substance, Lipase AY 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.

Very truly yours,

David R. Joy

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4 2001
OFFICE OF
PREMARKET APPROVAL

Enclosure

cc: Y. Hori
H.K. Watanabe

GRAS Notification for Lipase derived from *Candida rugosa*

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1. Claim regarding GRAS status

Amano Enzyme Inc. hereby notifies the agency through its attorneys that its Lipase AY enzyme preparation, derived from C. *rugosa*, as described below, is GRAS when used in the production of fatty acids and glycerides for nutritional use from animal and vegetable oils. **As** such, Lipase AY is exempt from the premarket approval requirements of the Food, Drug, and Cosmetic Act.

August 31,2001

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i. Name and address of the notifier

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

ii. Common or usual name of the subject substance

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

lipase from *Candida rugosa*

iii. Conditions of use

Lipase AY is intended for use in the production of dietary ingredients derived from fats and oils including, but not limited to, gamma linolenic acid (GLA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA).

Lipase AY 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 an animal or vegetable oil. Shea oil, fish oil, borage oil, sunflower oil, safflower oil, and other triglycerides are used as starting materials. At the end of the purification steps, the dietary ingredients are expected to contain no more than 100 parts per million (ppm) residual enzyme. The dietary ingredients are either free fatty acids or glycerides that are rich in specific fatty acids.

Lipase AY is also used in the esterification of fatty acids to produce glycerides, which are used as dietary ingredients. In this case, the starting materials are free fatty acids and glycerol. As is the case with hydrolysis products, denatured enzyme is expected to be present in the finished dietary ingredient at levels below 100 ppm.

iv. Basis for the GRAS determination

The GRAS determination for Lipase AY 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.

2. Detailed Information About the Identity of the Notified Substance

i.	Name:	Lipase from *Candida rugosa*
ii.	Enzyme Commission number:	EC 3.1.1.3
iii.	Production organism:	*Candida rugosa*
iv.	Physical description:	Lipase AY is an off-white to tan powder that is soluble in water but insoluble in alcohol.

v. Method of manufacture:

The Lipase AY enzyme is produced by the controlled fermentation of a selected strain of *Candida rugosa*. 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 in greater detail in Appendix 1.

As indicated in Appendix 1, and as discussed in the published article presented:in Appendix 3, Lipase AY is available in two different forms, Lipase AY Concentrate, and the more dilute Lipase AY30. Lipase AY Concentrate is the subject of this GRAS Notification. We note, however, that the safety information and analysis presented herein would obviously be useful to support a conclusion that the more dilute Lipase AY30 is GRAS in food processing applications.

vi Specifications:

Attached as Appendix 2 is a copy of the *Food Chemicals Codex* existing monograph for lipase derived from *Candida rugosa*. Amano's Lipase AY enzyme preparation satisfies these purity criteria.

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 the production organism. These studies include a 90-day gavage study with rats, a mutagenicity study using bacteria (Ames assay), and a pathogenicity study of the production organism, *C. rugosa,* using mice.

1. 90-Day Rat Gavage Study

Primary Test. Six groups of 10 male and 10 female Sprague-Dawley rats (Crj:CD) received a water suspension of the test substance once daily by gavage (Lipase AY concentrate, activity 79,700 unitslg).' Dosages were set at 0,625, 1250, and 2500 mg/kg body weight. An additional two groups received 0 ("recovery control") or 2000 mg/kg body weight ("recovery") throughout the **13** weeks and were examined during a 30-day recovery period. Animals were observed for clinical signs. Body weights and feed

1 One Lipase **AY** unit of activity is defined as the amount of enzyme preparation that can release one micromole equivalent of fatty acids per minute under assay conditions.

consumption were recorded twice weekly. Other observations and measurements included urinalysis, auditory test, ophthalmological examination, hematolgy, clinical chemistry, absolute and relative organ weights, and histopathology. The no-observable-adverse-effect level (NOAEL) was determined to be the high dose level, 2500 mg/kg body weight.

Repeat Test. Due to a dose-dependent increase in potassium (K^+) excretion observed in both sexes, the test was repeated with males only. Two groups of 6 males received 0 or 2500 mg/kg body weight of the test substance for 90 days and were examined during a 60-day recovery period. The repeat test demonstrated that ingested potassium was excreted during the first four hours. After that time, K^+ levels did not differ significantly from corresponding control levels. Based on all of these results, the highest dose of 2500 mg/kg body weight represents a no-adverse-observed effect level.

ii. Mutagenicity Studies

a. Reverse Mutation Test on Bacteria

Mutagenicity was evaluated in four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and a strain of *E. **coli*** (WP2 *urvA*) in accordance with the OECD guidelines. The test substance was Lipase AY concentrate, having an activity of 153,000 units/g. No concentration-related increases in revertant colonies/plate were observed in any salmonella strain or in the E. coli strain.

b. Chromosome Aberration Test

The potential of Lipase AY to induce chromosome aberrations was tested using CHL/IU cells, derived from fibroblasts of the lungs of Chinese hamsters. The test was carried out in accordance with the OECD guidelines. No significant increase in the frequencies of polyploid cells or structural aberrations was observed in cells treated with Lipase AY concentrate.

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

The ability of Lipase AY to induce a mutation at the TK locus of mouse lymphoma cells was evaluated in duplicate assays. A dose-related increase in cytotoxicity (decrease in relative total growth, RTG) was observed in the absence of metabolic activation. In the presence of S-9 mix, a decrease in RTG was noted at the highest dose, but there was no clear dose response. None of the cultures treated with Lipase AY and cloned in the presence or absence of metabolic activation exhibited a mutant frequency statistically different from that of the controls.

iii. Pathogenicity Study

Although the production organism is not present in the Lipase AY 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 by injecting spores of *C. rugosa* into the tail vein of mice. The animals were observed daily for 14 days. At the end of this time, the mice were sacrificed, and independent sections of the brain, liver, and kidney were examined for histopathology and viable *C. rugosa*. No clinical signs were observed during the 14-week observation period. No histopathological abnormalities were apparent, and no living *C. rugosa* was found in any of the organs examined.

iv. Mycotoxin and Antibiotic Analysis

The enzyme preparation has been analyzed for antibiotic activity with negative results and for a wide variety of mycotoxins, none of which were present at detectable levels. More specifically, several batches of Lipase AY have been analyzed for aflatoxins B_1, B_2, G_1, and G_2; ochratoxin A; sterigrnatocystin; zearalenone; and T-2 toxin.

v. Safety Determination

To summarize, the NOAEL in the 90-day toxicity study in rats was 2500 mg/kg body weight per day for the Lipase AY concentrate. Lipase AY concentrate was found to be non-mutagenic, and the production organism was classified as non-pathogenic.

The estimated daily intake for Lipase AY resulting from the application covered by this notification is roughly 1 mg/person/day and no more than 4.5 mg/person/day, as discussed in greater detail below. This compares favorably with the NOAEL of 2500 mg/kg b.w. for Lipase AY.

vi. Intake Estimate

We estimate that residues of denatured Lipase AY in the dietary fatty acids and glycerol esters of fatty acids, if at all present, will be in the range of 100 parts per million, as discussed below. An upper limit of approximately 450 ppm residual Lipase AY may be calculated on the basis of a nitrogen analysis, as discussed below.

The dietary ingredients produced with the aid of Lipase AY include gamma linolenic acid (GLA), conjugated linoleic acid (CLA), docohexanoic acid (DHA), and eicosapentaenoic

acid (EPA). Typical daily doses of these dietary ingredients are 1000 - 6000 mg/day. Considering that some consumers may take more than one type of fatty acid supplement per day, we assume conservatively that 10,000 mg per day represents a high level of intake.

If a heavy consumer takes 10,000mg every day of fatty acid supplements, all of which contain residual Lipase **AY** at 100 ppm, this would result in daily exposure to Lipase AY of 1 mg/day. If residual Lipase **AY** is present in the dietary ingredients at 450 ppm, that would result in maximum daily exposure to Lipase **AY** of 4.5 mg/day.

We estimate that the fatty acid dietary ingredients may contain roughly 100 ppm and no more than 450 ppm residual Lipase **AY** on the basis of (1) manufacturing information and (2) analyses of the ingredients for total nitrogen content.

Lipase AY is used in two ways in the manufacture of dietary ingredients. It is used to hydrolyze edible oils into fatty acids, and it is used to esterify fatty acids with glycerol. Both of these processes are described below.

a. Hydrolysis

1. **A** given quantity of triglyceride, such as shea oil, fish oil, borage oil, or other triglyceride starting material is used.

2. To this is added a quantity of water, ranging from 10% to 100% of the weight of the oil.

3. Lipase **AY** enzyme preparation is dissolved in water and added to the oil/water mixture. The amount of Lipase AY used ranges up to 1% of the weight of the oil.

4. The enzymatic hydrolysis is stopped by heating the oil/water mixture to denature the enzyme.

5. The water phase, which now contains glycerol resulting from the hydrolysis, settles and is drained off. Upwards of 90% of the denatured enzyme is contained in the water phase. This leaves a small amount of denatured enzyme in the oil phase, no more than 0.1% by weight.

6. Oil washing steps reduce the concentration of denatured enzyme remaining in the oil phase by a factor of at least 5, leaving no more than 0.02% by weight.

7. The oil phase is dried and then separated by distillation into free fatty acids and glycerides with essentially no enzyme present in the distilled fatty acids.

8. The distilled fatty acids are further processed into dietary ingredients.

9. The glyceride fraction is refined and further processed into dietary ingredients. The refining steps remove potentially present enzyme residues.

b. Esterification

1. **A** given quantity of fatty acids and glycerol are used as starting materials for the esterification.

2. Lipase **AY** is dissolved in water and added to the fatty acid/glycerol mixture. The amount of lipase **AY** ranges up to 1% of the weight of the fatty acids.

3. The esterification reaction is stopped by heating the oil/water mixture to denature the enzyme.

4. The denatured enzyme is filtered of using hyflo filter aid until a clear product is achieved.

5. The oil is dried and then separated by distillation into free fatty acid and glycerides.

6. The glycerides are bleached with bleaching earth and filtered again with hyflo and trisyl, removing remaining denatured enzyme. After this, the glycerides are filtered over a filter with a very small pore size (maximum 10 pm).

7. After filtration, the glyceride product is steamed at temperatures higher than 135 °C, certainly denaturing any enzyme still present.

8. After steaming, the product again is filtered over a filter with a very small pore size (maximum 10 pm) before the product is tapped in drums under nitrogen.

The cycle of filtration (4 times in total) will guarantee the level of denatured Lipase **AY** enzyme to be very low. Levels below 100 ppm are expected.

As mentioned above, analyses of the finished product for total nitrogen content tends to confirm that the denatured enzyme may be present at a maximum level of a few hundred ppm. A customer of Amano's has tested its dietary ingredients produced with Lipase **AY** for nitrogen content with findings that range from 2 ppm to 7 ppm total nitrogen. Lipase AY concentrate has been analyzed for nitrogen and found to contain 15.4 mg/g total nitrogen (1.5%). Assuming conservatively that all nitrogen present in the dietary

ingredients produced with the aid of Lipase AY is attributable to residual denatured enzyme, the total quantity of denatured enzyme in the dietary ingredients may be in the range of 450 ppm (7 ppm nitrogen ÷ 1.5% nitrogen in Lipase AY). We emphasize that this calculation likely provides an exaggerated estimate of the total amount of denatured enzyme present in the dietary ingredients processed with Lipase AY. This is because it attributes all nitrogen present in the dietary ingredients to Lipase AY. Nevertheless, this calculation represents a maximum upper level of residual Lipase AY potentially present in the dietary ingredients, and the resulting maximum potential level of dietary exposure is many times below the No Observed Adverse Effect Level (NOAEL) derived from the 90-day feeding study (4.5 mg/day compared to 2500 mg/kg body weight per day).

vii. Expert Consensus

The above-described safety studies for Lipase **AY** were published in a peer-reviewed scientific journal (Flood and Kondo, Safety Evaluation of Lipase Produced **from** *Candida rugosa:* Summary of Toxicology Data, *Regulatory* ***Toxicology*** *and Pharmacology* **33** 157-164 (2001)), a copy of which is presented as Appendix III. The authors arrived at the conclusion that Lipase AY 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 jntake in the above-described application.

As noted above, Lipase derived from *Candida rugosa* is the subject of a monograph in the fourth edition of the *Food Chemicals Codex* (Appendix 11). Typical applications identified include hydrolysis of lipids, manufacture of dairy products and confectionery goods, and development of flavor in processed foods. This provides strong evidence that Lipase derived from *Candidu rugosa* is widely known and recognized among qualified experts as a suitable processing aid in a variety of food applications.

Appendix I: Manufacturing method for Lipase AY

Process Flow for Lipase AY Production

Process	*Conditions*
1st Seed culture with flask	at 25. for 48 hr 140 rpm (shaking)
↓	
2nd Seed culture with jar-fermentor	at 25 for 24 hrs 250 rpm (agitation) 0.5 vvm (aeration)
↓	
Production Culture with tank-fermentor	at 25. for approx. 67 hrs 120 rpm (agitation) 0.4 vvm (aeration)
↓	
Filtration with press filter	at pH 5.2±0.1
↓	
Concentration of culture filtrate with ultrafilter (M.W. 6000)	at 15±1.
↓	
Filtration with press filter	at pH 6.0±0.1
↓	
Microfiltration with ceramic filter	
↓	
Precipitation by addition of ethanol	
↓	
Centrifugation	
↓	
Drying under vacuum	at 35 45. for 21.24 hr
↓	
Crushing with pin-mill, Sieving and Mixing	through 60 mesh screen
↓	
Dilution and Blend by addition of dextrin	mixing for 10 min
↓	
Sieving and Mixing	through 42 mesh screen
↓	
Product (Lipase AY30)	Product (Lipase **AY** Concentrate)

Appendix II: *Food Chemicals Codex* Monograph

Pages 000015 - 000018 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix III: Published article: Safety Evaluation of Lipase from *Candida rugosa*

Pages 000020 - 000027 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

End Submission

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Reference List for Industry Submission, GRN 000081

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000015 - 000018	NA	Monograph Specifications: Enzyme Preparations	NA	Food Chemicals Codex	Edition IV, pgs 129, 132-134
000020 - 000027	Flood, Michael T.; Kondo, Mitsuru	Safety Evaluation of Lipase Produced from Candida rugosa: Summary of Toxicological Data	April 12, 2001	Regulatory Toxicology and Pharmacology	Volume 33, pgs 157-164

NA- Not applicable