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LAW OFFICES
KELLER AND HECKMAN LLP
1001 G STREET, N.W.
SUITE 500 WEST
WASHINGTON, D.C. 20001
TELEPHONE (202) 434-4100
FACSIMILE (202) 434-4646

25 RUE BLANCHE
B-1060 BRUSSELS
TELEPHONE 32(2) 541 05 70
FACSIMILE 32(2) 541 05 80

WWW.KHLAW.COM

JOSEPH E KELLER (1907-1994)
JEROME H HECKMAN
WILLIAM H BORGHESANI JR
WAYNE V BLACK
TERRENCE D JONES
MARTIN W BERCOVICI
JOHN S ELDRED
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JOHN B DUBECK
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FRANK J VITOLO
JENNIFER B BENNETT*
CAREN A.C GRAU
LUTHER L HAJEK*
SHANNON M HEIM*
CHRISTIAN C. SEMONSEN*

*NOT ADMITTED IN D C
◊RESIDENT BRUSSELS

SCIENTIFIC STAFF
DANIEL S DIXLER, PH D
HOLLY HUTMIRE FOLEY
CHARLES V BREDER, PH. D
ROBERT A. MATHEWS, PH. D., D.A.B.T
JANETTE HOUK, PH. D.
LESTER BORODINSKY, PH D
THOMAS C BROWN
MICHAEL T FLOOD, PH D
ANNA GERGELY, PH D.◊
STEFANIE M. CORBITT
RACHEL F. JOYNER
ELIZABETH A. HEGER
ROBERT J SCHEUPLEIN, PH D
ANDREW P JOVANOVICH, PH D., MBA
KAREN R OBENSHAIN

TELECOMMUNICATIONS ENGINEER
RANDALL D YOUNG

WRITER'S DIRECT ACCESS

January 31, 2001

(202) 434-4126
Joy@khlaw.com

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Hand Delivered

Dr. Alan M. Rulis
Office of Premarket Approval (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 *Penicillium camembertii*

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 G Amano 50 enzyme preparation, derived from *Penicillium camembertii* is generally recognized as safe (GRAS) when used as a processing aid in the derivation of dietary fatty acids from fats and oils. As a GRAS substance, Lipase G Amano 50 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

Enclosure
cc: Y. Hori, H.K. Watanabe

GRAS Notification for Lipase derived from *Penicillium camembertii*

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Appendix I: Manufacturing method for Lipase G Amano 50

Appendix II: Published article: Safety Evaluation of Lipase from *Penicillium camembertii*

1. Claim regarding GRAS status

Amano Enzyme Inc. hereby notifies the agency through its attorneys that its Lipase G Amano 50 enzyme preparation, derived from *P. camembertii*, 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 G Amano 50 is exempt from the premarket approval requirements of the Food, Drug, and Cosmetic Act.

January 30, 2001

Michael T. Flood, Ph.D.
Staff Scientist
Keller and Heckman, LLP

David R. Joy
Attorney for the Notifier
Keller and Heckman, LLP

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 *Penicillium camembertii*

iii. Conditions of use

Lipase G Amano 50 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 G Amano 50 selectively hydrolyzes mono- and di-glycerides 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. Fish oil and borage oil are expected to be used, among other fats and oils. 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.

iv. Basis for the GRAS determination

The GRAS determination for Lipase G Amano 50 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 *Penicillium camembertii*
ii.	Enzyme Commission number:	3.1.1.23
iii.	CAS number:	9040-75-9
iv.	Production organism:	*Penicillium camembertii*, Strain No. U-150
v.	Physical description:	Lipase G Amano 50 is a pale yellowish brown to pale brown powder.

vi. Method of manufacture:

The Lipase G Amano 50 enzyme is produced by the controlled fermentation of a selected strain of *Penicillium camembertti*. 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, crushed, and blended with dextrin to the desired enzyme activity. The TOS content of commercial Lipase G Amano 50 is 35.36%.[1] The manufacturing process is presented in greater detail in Appendix 1.

---

[1] Total Organic Solids (TOS) is defined as 100 - (A + W + D)% where A = ash content; W = water content; and D = content of diluents. The commercial preparation of Lipase G Amano 50 has a TOS value of 100 - (4.52 + 9.73 + 50.39)% = 35.36%.

vii. Specifications:

Specification	Limit
Lipase activity[2]	≥ 50,000 U/g
Arsenic[3]	≤ 2 ppm
Heavy metals[3]	≤ 30 ppm
Total viable count[4]	≤ 10,000/g
E. coli[5]	negative/25 g
Loss on drying (1g, 105°C, 4hrs.)[3]	≤ 8%
Side activity (lipolytic activity)[6]	≤ 5,000 U/g

---

[2] Vinyl laurate emulsion method. One unit of activity is defined as the quantity of enzyme that will liberate one μmole of fatty acid per minute under the conditions of the assay.

[3] Japanese Pharmacopeia method

[4] U.S. Pharmacopeia (XXI) method

[5] FDA - BAM; Bacteriological Analytical Manual 8th Edition (1998)

[6] Amano method

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, *P. camembertii*, using mice.

i. 90-Day Rat Gavage Study

A crude preparation of Lipase G Amano 50 (LGK) was produced by the commercial process without the final purification and dilution steps. LGK is a brown powder having a Lipase G activity of 100,222 U/g. The total organic solids (TOS) content of LGK was 75.76%.

Sprague-Dawley rats, Crj:CD strain, were obtained from Charles River (Japan). 100 rats of each sex were stratified by body weight, randomly assigned to treatment groups, and housed two rats of the same sex per cage. Commercial pelleted diet and tap water were available *ad libitum*. The test material was administered by gavage once each day for 90-92 days at levels of 0, 500, 1000, and 2000 mg/kg body weight as a suspension in distilled water. Control animals were administered distilled water alone. At week 13, 20 animals per sex per group were sacrificed, and 10 animals/sex in the high-dose group and control group were maintained untreated for another 31 days as a recovery group.

Body weight and food and water consumption were monitored throughout the study. A macroscopic examination was made on all rats daily. Other observations and measurements included urinalysis, auditory test, ophthalmological examination, haematology, clinical chemistry, absolute and relative organ weights, and histopathology. No animals died during the study, and no adverse effects attributed to administration of the test material were evident. The no-observable-adverse-effect level (NOAEL) was determined to be the high dose level, 2000 mg/kg body weight.

ii. Mutagenicity Studies

LGK was diluted with distilled water to yield solutions of 100,000, 50,000, 25,000, 12,500, 6250, or 3130 µg/ml and tested at a maximum concentration of 5000 µg/plate. *Salmonella typhimurium* strains TA1535 and TA100 (for base-pair substitutions) and TA1537 and TA98 (for frameshift mutations) and *Escherichia coli* strain WP2uvrA (for base-pair

substitutions) were used. These cultures were tested both with and without metabolic activation using S-9 fraction of the rat liver. Either with or without S-9 mix, LGK caused no increase in the number of revertants. All positive controls caused a distinct increase in the number of revertants.

iii. Pathogenicity Study

Although the production organism is not present in the Lipase G Amano 50 enzyme preparation, it is preferable not to use pathogenic organisms for the production of enzymes intended for use in food applications. Groups of 10 mice were inoculated with a suspension of 10^2, 10^4, or 10^6 viable spores of *Penicillium camembertii* injected into the tail vein. After a 14-day observation period, the mice were sacrificed, and the liver, kidney, and brain removed. A section from each of these organs was rubbed onto an agar plate, which was then incubated at 30°C for 48 hours. Organ sections were also prepared for histopathological examination. No fungal colonies were observed to grow on the plates after incubation. Histopathological changes were observed in liver and kidney sections but not in the brain sections. The fungal spores were distributed chiefly to the liver. It was concluded that host defense mechanisms probably inactivated the spores rapidly and, considering the absence of colony formation on the incubated plates, *Penicillium camembertii* is non-pathogenic.

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 lots of Lipase G Amano 50 have been analyzed for Aflatoxin B_1, Cyclopiazonic acid, Ochratoxin A, Penicillic acid, Roridin A, Zearalenone, Roquefortin, and Penitrem A. All analyses were negative at detection limits ranging from 0.5 ppb to 250 ppb.

v. Safety Determination

To summarize, the NOAEL in the 90-day gavage toxicity study in rats was 2000 mg/kg body weight per day for the Lipase G concentrate (75.76% TOS). There was no evidence of mutagenic potential, and the production organism was classified as non-pathogenic. To arrive at a safe level of intake for Lipase G Amano 50, we consider that the Lipase G concentrate (LGK) had a TOS content of 75.76%. Therefore, the NOAEL might be expressed as 1515 mg/kg b.w. TOS (2000 mg/kg b.w. x 75.76%).

The estimated daily intake for Lipase G TOS resulting from the application covered by this notification is no more than 1 mg per person per day, as discussed in greater detail below. This compares favorably with the NOAEL of 1515 mg/kg b.w. More specifically, the estimated 1 mg/day exposure corresponds to 0.017 mg/kg b.w. for a 60-kg adult. This is roughly 90,000 times lower than the NOAEL (1515 mg/kg b.w.).

vi. Intake Estimate

We estimate that residues of denatured Lipase G Amano 50 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.

The dietary ingredients produced with the aid of Lipase G Amano 50 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 10,000 mg every day of fatty acid supplements, all of which contains residual Lipase G at 100 ppm, this would result in daily exposure to Lipase G of 1 mg/day.

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

Regarding the manufacturing information, dietary ingredients are produced using Lipase G Amano 50 according to the following basic steps.

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 G Amano 50 enzyme preparation is dissolved in water and added to the oil/water mixture. The amount of Lipase G Amano 50 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. Denatured enzyme is believed to remain in the glyceride fraction (at a maximum of 400 ppm) with essentially no enzyme present in the distilled fatty acids.

8. The glyceride fraction is further purified using standard oil refining techniques so that residues of the denatured enzyme are reduced to no more than 100 ppm.

As mentioned above, analyses of the finished product for total nitrogen content tends to confirm that the denatured enzyme is present, if at all, in the range of 100 ppm. A customer of Amano's has tested its dietary ingredients produced with Lipase G Amano 50 for nitrogen content with findings that range from 1.3 ppm to 2.9 ppm total nitrogen. Lipase G concentrate (LGK) has been analyzed for nitrogen and found to contain 25 mg/g total nitrogen (2.5%). On that basis, we can estimate that the TOS portion of Lipase G Amano 50 contains approximately 3.3% nitrogen (2.5% ÷ 75.76% TOS). Therefore, assuming conservatively that all nitrogen present in the dietary ingredients produced with the aid of Lipase G Amano 50 is attributable to residual denatured enzyme, the total quantity of denatured enzyme in the dietary ingredients should be no greater than 90 ppm (2.9 ppm nitrogen ÷ 3.3% nitrogen in Lipase G Amano 50 TOS). We provide this calculation to show that it tends to corroborate the estimate made by Amano's customer that its dietary ingredients produced with the aid of Lipase G Amano 50 will contain no more than 100 ppm denatured enzyme. This general range clearly corresponds to a safe level of exposure (1 mg/person per day or .017 mg/kg b.w. for a 60-kg adult), which is 90,000 times lower than the NOAEL (1515 mg/kg b.w. for TOS) in the 90-day rat study.

The safety analysis presented above focuses on the portion of Lipase G Amano 50 referred to as TOS (total organic solids). This includes the enzyme itself and other materials extracted from the production organism. Commercial Lipase G Amano 50 contains 35.36% TOS. The remaining portion of the product is predominantly dextrin, which is used as a diluent and is GRAS affirmed at 21 C.F.R. 184.1277.

vii. Expert Consensus

The above-described safety studies for Lipase G Amano 50 were published in a peer-reviewed scientific journal (M. Kondo, T. Ogawa et al., Safety Evaluation of Lipase G from *Penicillium Camembertii*, *Fd. Chem Toxic.*, Vol 32, No. 8, pp 685-696, 1994), a copy of which is presented as Appendix II. The six authors arrived at the conclusion that Lipase G 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 in the above-described application.

A literature search was conducted to determine whether there have been any published reports of health concerns associated with the production organism, *Penicillium camembertii*. One published article reports on a possible case of extrinsic allergic alveolitis caused by *Penicillium camembertii*. The subject was employed in a salami factory, working near an area where the salami are seasoned with fungal inocula. This report is not considered damaging to the GRAS status of Lipase G Amano 50 because the production organism is not present in the enzyme preparation. Furthermore, this incident was characterized as a possible allergic reaction caused by inhalation of airborne *P. camembertii*. The circumstances and route of exposure are far removed from an evaluation of the safety of exposure to low levels of an extracellular lipase enzyme preparation derived from *P. camembertii*. Finally, we note that other *Penicillium* species used in food applications are mentioned in the article as having been reported to induce occupational extrinsic allergic alveolitis.

Finally, *P. camembertii*, though not the U-150 strain, has been used in the past in the manufacture of French Camembert cheese. (We understand that *Penicillium candidum* is often used tody in place of *Penicillium camembertii* as a means of controlling moisture in or on the cheese.) As indicated above, *P. camembertii* is also used in the manufacture of salami.

PROCESS FLOW FOR LIPASE G PRODUCTION

	Volume	Conditions
Slant culture of *Penicillium camemberti*		At 25°C
↓		
1st seed culture with flask	3000ml	at 25°C for 69 hours 140rpm
↓		
2nd seed culture with tank fermenter	1000L	at 26°C for 49 hours 200rpm, 0.6vvm
↓		
Lipase G fermentation with tank fermenter	16000L	at 26°C for 66 hours 125rpm, 0.6vvm
↓		
Filtration with press filter		pH 6.5 to 7.0
↓		
Concentration of culture filtrate with ultrafilter	1700L	MW 6000 cut
↓		
Heat treatment		at 42°C for 30 mins and pH 4.9 to 5.1
↓		
Filtration with press filter		at pH 6.4 to 6.6
↓		
Filtration with ceramic filter	2500L	
↓		
Precipitation with addition of ethanol		at 1 to 5°C and pH 4.9 to 5.1
↓		
Centrifugation		
↓		
Drying (under vacuum) at 25°C maximum → Crushing →	Mixing (for adjustment of lipolytic activity by addition of dextrin)	
↓		
Product (Lipase G Amano 50) ←	Sieving (through 42 mesh screen)	

Pages 000014 - 000025 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

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LAW OFFICES
KELLER AND HECKMAN LLP
1001 G STREET, N.W.
SUITE 500 WEST
WASHINGTON, D.C. 20001
TELEPHONE (202) 434-4100
FACSIMILE (202) 434-4646

25 RUE BLANCHE
B-1060 BRUSSELS
TELEPHONE 32(2) 541 05 70
FACSIMILE 32(2) 541 05 80

WWW.KHLAW.COM

JOSEPH E KELLER (1907-1994)
JEROME H HECKMAN
WILLIAM H BORGHESANI, JR
WAYNE V BLACK
TERRENCE D JONES
MARTIN W BERCOVICI
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C DOUGLAS JARRETT
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JOAN SYLVAIN BAUGHAN◇
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THOMAS C BERGER
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KOMAL J HERSHBERG
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*NOT ADMITTED IN D.C.
◇RESIDENT BRUSSELS

SCIENTIFIC STAFF
DANIEL S DIXLER, PH D.
HOLLY HUTMIRE FOLEY
CHARLES V. BREDER, PH D
ROBERT A MATHEWS, PH D., D.A.BT
JANETTE HOUK, PH D
LESTER BORODINSKY, PH D
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ROBERT J SCHEUPLEIN, PH. D
ANDREW P JOVANOVICH, PH D., MBA
KAREN R OBENSHAIN

TELECOMMUNICATIONS ENGINEER
RANDALL D YOUNG

WRITER'S DIRECT ACCESS
(202) 434-4126
Joy@khlaw.com

February 28, 2001

Robert I. Merker
Office of Premarket Approval (HFS-206)
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 G Amano 50; Specifications

Dear Mr Merker:

Following up on my telephone message this afternoon, we are writing to confirm that the Lipase G Amano 50 enzyme preparation satisfies the general purity requirements for enzyme preparations established in the *Food Chemicals Codex* (FCC). As you know, Keller and Heckman submitted a GRAS Notification for a lipase enzyme preparation derived from *Penicillium camemberti* on behalf of our client, Amano Enzyme Inc., on January 31, 2001. You contacted us last week to ask whether the enzyme preparation satisfies the purity requirements of the FCC, notably the 5 mg/kg lead limit. Enclosed is a revised set of specifications for Lipase G Amano 50 showing compliance with the FCC limits, which you may treat as a supplement to the original GRAS Notification.

Please let us know if you have any further questions regarding the Notification.

Very truly yours,

David R. Joy

cc: Mr. Y. Hori
Mr. H. K. Watanabe

vii. Specifications:

Specification	Limit
Lipase activity[1]	≥ 50,000 U/g
Assay:	Not less than 85.0% and not more than 115.0% of the declared units of enzyme activity.
Arsenic[2]	Not more than 2 mg/kg
Heavy metals (as Pb)[3]	Not more than 30 mg/kg
Lead[4]	Not more than 5 mg/kg
Microbial Limits	
Coliforms[5]	Not more than 30 per g
Salmonella sp.[6]	Negative in 25 g
Loss on drying (1g, 105°C, 4hrs.)[2]	≤ 8%
Side activity (lipolytic activity)[7]	≤ 5,000 U/g

---

1 Vinyl laurate emulsion method. One unit of activity is defined as the quantity of enzyme that will liberate one μmole of fatty acid per minute under the conditions of the assay.

2 Japanese Pharmacopeia method

3 Prepare and test a 667-mg sample as directed in *Method II* under the *Heavy Metal Test*, Appendix IIIB, *Food Chemicals Codex IV,* using 20 µg of lead ion (Pb) in the control (*Solution A*).

4 A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, Appendix IIIB, *Food Chemicals Codex IV*, using 5 µg of lead ion (Pb) in the control.

5 *FDA Bacteriological Analytical Manual,* Eighth Edition, 1998.

6 *FDA Bacteriological Analytical Manual,* Eighth Edition, 1998.

7 Amano method

Reference List for Industry Submission, GRN 000068

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000014 - 000025	Kondo, M.; Ogawa, T.; Matsubara,Y.; Mizutani, A.; Murata, S.; Kitagawa, M.	Safety Evaluation of Lipase G From Penicillium Camembertii	1994	Food and Chemical Toxicology	Volume 32, Number 8, pgs 685-696

NA- Not applicable