GR

ORIGINAL SUBMISSION

000001

Kirkpatrick & Lockhart LLP

1800 Massachusetts Avenue, NW
Suite 200
Washington, DC 20036-1221
202 778 9000
www kl com

Gary L. Yingling
202.778.9124
Fax: 202.778.9100
gyingling@kl.com

February 6, 2004

George H. Pauli, Ph.D.
Acting Director
Office Of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Lipase Enzyme Preparation (Trade Name LysoMax™)

Dear Dr. Pauli:

On behalf of our client, Genencor International, Inc., we are submitting a notice of GRAS exemption for a Lipase Enzyme Preparation (trade name LysoMax™) derived from a safe strain of *Streptomyces violaceoruber*. Pursuant to proposed 21 C.F.R. § 170.36(b), we are submitting three copies of this notice.

If you have any questions regarding this submission, please do not hesitate to call me.

Sincerely,

Gary L. Yingling

FEB 9 2004

000002

DC-620249 v1 0306453.0100

Genencor International, Inc.®

925 Page Mill Road
Palo Alto, California 94304
650.846.7500
650.845.6500 fax
www.genencor.com

January 28, 2004

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: GRAS Notification - Exemption Claim for a Lipase Enzyme Preparation

Dear Sir or Madam:

Pursuant to proposed 21C.F.R. §§ 170.36(c)(1), 170.36(g)(2), and the Food and Drug Administration ("FDA") preamble discussion concerning the submission of a Generally Recognized As Safe ("GRAS") notification, Genencor International ("Genencor") is hereby providing FDA with notice that it has determined, based on publicly available information and scientific procedures, that a lipase enzyme preparation, a direct food ingredient, is Generally Recognized As Safe ("GRAS") and therefore is exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation.

Proposed §170.36(g)(2)(i): Name and address of the notifier.

Genencor International Inc.
925 Page Mill Road
Palo Alto, California 94304

Proposed §170.36(g)(2)(iii): The common or usual name of the substance (i.e., the notified substance).

The lipase enzyme preparation is the common or usual name for the substance for which this GRAS notification is made. Specifically, the lipase enzyme preparation is derived from *Streptomyces violaceoruber.*

Proposed §170.36(g)(2)(iv): Applicable conditions of use.

Genencor International, Inc.

925 Page Mill Road
Palo Alto, California 94304
650.846.7500
650.845.6500 fax
www.genencor.com

The lipase enzyme preparation is a direct food ingredient. The lipase enzyme preparation's primary functional use is as a processing aid for the hydrolysis of phospholipids, such as lecithin. The hydrolysis (or modification) of lecithin by this lipase enzyme preparation specifically cleaves the ester bond between glycerol and the fatty acid at the number 2 position of the glycerol backbone of lecithin, and thus improves the emulsifying ability of the lecithin

The lipase enzyme preparation is GRAS for use in food at levels not to exceed Good Manufacturing Practices ("GMPs"). Current GMPs results in typical usage level in lecithin modification of 0.5 – 5 units of enzyme per gram of lecithin.

Proposed § 170.36(g)(2)(v): Basis for GRAS determination.

This GRAS determination is based on publicly available information and scientific procedures.

Proposed § 170.36(g)(2)(vi): Availability of information.

The complete record that supports this GRAS determination is available for the FDA's review and copying at the above address or will be sent to FDA upon request.

Sincerely

Cynthia Z. Blumenthal
Specialist, Regulatory Affairs

A Lipase Enzyme Preparation Derived from *Streptomyces violaceoruber*

By
Genencor International Inc.
925 Page Mill Road
Palo Alto, CA 94304
USA

000005

TABLE OF CONTENTS

INTRODUCTION 4

SOURCE 5

ENZYME IDENTITY 5

A. CHEMICAL NAME 6

B. IUB Number 6

C. Common or Usual Name 6

D. CAS Registry Number 6

E. Properties 6

MANUFACTURING PROCESS (APPENDIX 1) 6

A. Raw Materials 6

B. Fermentation Process 6

C. Recovery Process 7

D. Formulation 7

SPECIFICATIONS (APPENDIX 2) 7

APPLICATION (APPENDIX 4) 7

A. Mode of Action 7

B. Foods in Which Used and Purpose of Use 7

C. Level of Use 8

SAFETY EVALUATION 8

A. Production Organism 8
1. Literature Review 9
2. *Streptomyces violaceoruber* Pathogenicity Assay (Appendix 5) 9
(University of South Alabama, August 1990) 9

000006

B. Enzyme 10
1. Literature 10
2. Past Uses in Food 11

C. Enzyme Product 12
1. Acute Oral LD_{50} in Rats (Appendix 6) 12
2. Reverse Mutation Assay with *Salmonella* (Appendix 7) 12
3. Forward Mutation Assay – Mouse Lymphoma L5178Y Cell Line (Appendix 8) 12
4. Unscheduled DNA Synthesis (Appendix 9) 13
5. Thirteen-week Oral (Gavage) Toxicity Study in the Rat (Appendix 10) 13
6. Conclusions of the Safety Testing 13
7. Allergenicity Discussion 14
8. Dietary Exposure Calculation 14
a) Residue in Food 14
b) Dietary Exposure 14
(1) Soy lecithin hydrolysis 14
(2) Egg Yolk Lecithin Hydrolysis 15
c) Summary of enzyme residue intake 15
d) Human Consumption Versus Level in 13-week Feeding Study 16

D. Manufacturing Process 16

CONCLUSION 16

REFERENCES 16

APPENDICES 19

000007

INTRODUCTION

The lipase enzyme preparation, marketed under the trade name of LysoMax™, is a microbial phospholipase A_2 enzyme preparation derived from a safe strain of *Streptomyces violaceoruber*, JAS-591. This lipase enzyme preparation has utility as a processing aid in the food industry for the hydrolysis of phospholipids, such as lecithin. Lecithin is used in a number of food applications as an emulsifier, but its utility is generally limited to those applications that are "fat-based". The hydrolysis (or modification) of lecithin by this lipase enzyme preparation, that specifically cleaves the ester bond between glycerol and the fatty acid at the number 2 position of the glycerol backbone of lecithin, improves the emulsifying ability of the lecithin. This lipase enzyme preparation can be used to produce modified lecithins, which are then added to foods, or can be incorporated directly into foods where the enzyme acts on the inherent phospholipids to improve the desired characteristics of the food ingredient.

The safety of the lipase enzyme preparation of this submission has been evaluated using the decision tree scheme of Pariza and Johnson (2001). These authors report that the safety of the production microorganism is the prime consideration when assessing the safety of an enzyme preparation intended for use in food. The production microorganism, a strain of *S. violaceoruber* (which has not been modified by rDNA techniques), was tested in mice and shown to be nonpathogenic and nontoxigenic under the conditions of the test. Enzyme test material from the production microorganism does not contain antibacterial activities.

Pariza and Johnson (2001) further state that, if the enzyme production microorganism is safe, then the only other source of possible contamination is the manufacturing process itself. The manufacturing process used to make this lipase enzyme preparation employs a pure culture submerged fermentation of a *S. violaceoruber* strain. Good manufacturing practices are used throughout the process that utilizes commonly accepted methods for enzyme manufacture and formulation. All raw materials used in the fermentation and recovery processes are food grade (or equivalent) and standard materials used by the enzyme industry. The lipase enzyme preparation meets the general and additional requirements for enzymes as outlined in the *Food Chemicals Codex* and by the Joint FAO/WHO Expert Committee on Food Additives.

Lipases have a long history of safe use in food processing. Microbial lipases have been used in food since at least 1952 (Beckhorn *et al.*, 1965). Many lipase enzyme preparations, including animal- and fungal-derived sources, are currently used around the world in a variety of applications, thus the safety of lipase use in the food industry is well established.

An extensive search of the literature keyed to the enzyme and the microorganism used for the production of the enzyme was performed. The search strategy

covered the period 1957 to the present and used synonyms for both the organism and the enzyme. A review of the literature search on the organism uncovered no reports that implicate *Streptomyces violaceoruber* in any way with a disease situation. The literature search for the enzyme was restricted to those articles which addressed health or safety concerns. This search generated about 2,500 citations, most of which dealt with the medical significance of human phospholipase A_2. Phospholipase A_2 enzymes are ubiquitous in all cells studied to date. Although some phospholipase A_2 enzymes may play a role in allergenicity or disease (Hoffman *et al.*, 2001; Okano *et al.*, 1999), no articles implicating the phospholipase A_2 of *S. violaceoruber* in pathogenicity or toxicity were found.

In assessing the safety of the lipase enzyme preparation, a number of safety studies were conducted, including: an acute oral LD_{50} in rats, a reverse mutation assay with *Salmonella*, a forward mutation assay using the mouse lymphoma L51784 cell line, an unscheduled DNA synthesis assay in rat liver cells, and a 13-week oral feeding study in rats. These studies did not find any treatment-related toxicity, nor induction of gene mutation or chromosomal aberrations in assays using the lipase test preparations derived from the production microorganism. Consumption calculations indicate that the potential human exposure to the lipase enzyme preparation from consumption of food made using this enzyme preparation is negligible.

The information in the following sections is the basis for our determination of the general recognition of safety of the lipase enzyme preparation. Our safety evaluation includes an evaluation of the production strain, the enzyme, and the manufacturing process as well as an evaluation of dietary exposure to the preparation. Based on a review of the information, Genencor International, Inc. concludes, through scientific procedures and analysis of publicly available information, that this lipase enzyme preparation is Generally Recognized As Safe (GRAS) under the intended conditions of use.

SOURCE

The lipase enzyme preparation is produced from the submerged fermentation of a nonpathogenic, nontoxigenic strain of *Streptomyces violaceoruber*. The strain used for production of the enzyme preparation is a derivative of *S. violaceoruber* strain ATCC14980, the type strain of the species. The production strain has not been modified using recombinant DNA techniques. What is now classified as *Streptomyces violaceoruber* was first described in 1916 by Waksman and Curtis and named *Actinomyces violaceus-ruber*. A detailed description of the species can be found in *Bergey's Manual of Systematic Bacteriology* (Locci, 1986).

ENZYME IDENTITY

A. Chemical Name

According to the Nomenclature Committee of the International Union of Biochemistry (IUB, 1992), phospholipase A_2 has the following designation: EC 3.1.1.4, phosphatidylcholine 2-acylhydrolase.

B. IUB Number

IUB 3.1.1.4

C. Common or Usual Name

Lipase, Lecithinase, Phospholipase A_2. Other names include: lecithinase A, phosphatidase, phosphatidolipase, phospholipase A.

D. CAS Registry Number

The CAS Registry Number for phospholipase A_2 is 9001-84-7.

E. Properties

Phospholipase A_2 is responsible for the following reaction:
Phosphatidylcholine + H_2O = 1-acylglycerophosphocholine + carboxylate.

MANUFACTURING PROCESS (Appendix 1)

The lipase enzyme preparation is manufactured according to procedures outlined in Pariza and Foster (1983) using standard microbial enzyme production technology (Aunstrup, 1979; Aunstrup et al., 1979; Enzyme Applications, 1994) and according to current good manufacturing practice (cGMP).

A. Raw Materials

The raw materials used in the manufacture of the lipase enzyme preparation are substances that are acceptable for general use in food.

B. Fermentation Process

The manufacturing process for the lipase enzyme preparation begins with a pure stock culture of a nonpathogenic and nontoxicogenic strain of *Streptomyces violaceoruber*, which is controlled for identity, absence of foreign microorganisms, and enzyme-generating ability. Environmental factors such as pH, temperature, and aeration are controlled. During growth, fermentors are routinely sampled and tested for possible contamination. Should evidence of a significant contamination exist, the batch is rejected.

C. Recovery Process

When fermentation is completed, the broth is clarified using various standard separation and clarification techniques, then concentrated to the desired activity level.

D. Formulation

Food approved stabilizers and/or preservatives that are suitable for general use in food are added to extend the shelf life of the lipase enzyme product. The enzyme preparation is formulated as a liquid product and is standardized according to the product specifications.

SPECIFICATIONS (Appendix 2)

Specifications for the lipase enzyme preparation meet or exceed the requirements for enzyme preparations as set forth in the *Foods Chemical Codex*, Fourth Edition, 1996 (National Academy of Sciences) and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001). The enzyme activity is expressed as unit/g of product (U/g). One unit is defined as the quantity of enzyme that hydrolyzes 1 micromole of substrate in one minute under specified assay conditions. The assay for phospholipase A_2 enzyme activity is included as Appendix 3.

APPLICATION (Appendix 4)

A. Mode of Action

The phospholipase A_2 of *S. violaceoruber* is responsible for the following reaction:

Phosphatidylcholine + H_2O = 1-acylglycerophosphocholine + carboxylate.

B. Foods in Which Used and Purpose of Use

The lipase enzyme preparation is to be used to hydrolyze soy and egg yolk lecithins. Lecithin is a naturally occurring mixture of the phosphatides of choline, ethanolamine, and inositol, with smaller amounts of other lipids and is widely used in many categories of foods. The benefits of lecithin as an emulsifier in food processing are well known; however, the functionality of "unmodified" lecithin is limited to "fat-based" systems. In aqueous systems, i.e., baked goods, lecithin must be structurally altered, either chemically or enzymatically, to exhibit good emulsifying properties. Chemical modification can be costly and nonspecific, generating undesired hydrolysis products. The lipase enzyme preparation hydrolyzes the ester bond between glycerol and the fatty acid at the number 2 position of the glycerol backbone of lecithin, producing one

molecule of lysolecithin and one molecule of fatty acid from one molecule of lecithin. The resulting lysolecithin (or lysophosphatide) is a compound whose emulsifying capabilities in many foods are superior to that of the unmodified lecithin.

Modified lecithins have many uses in foods (Meinhold, 1991; van Nieuwenhuyzen, 1981), including, but not limited to, the bakery, confectionary, dairy foods, fats, and beverage products. In these products, the modified lecithin can act as an emulsifying agent, a mixing/blending aid, a release agent, an egg replacer, and as a flavor in food systems.

Egg yolks, one of the oldest emulsifiers used by man, contain the phospholipid lecithin. Van Dam (1977) discovered that lecithinase (phospholipase A_2) treatment of egg yolks resulted in production of an emulsion that had improved heat stability over unmodified egg yolks. Furthermore, phospholipase A_2 treatment caused a significant thickening of the egg yolk product (van Dam, 1978). Thus egg yolk treated with phospholipase A_2 is a more potent emulsifier and allows for a significant reduction in the amount of thickening agents needed in egg yolk-containing products such as mayonnaise and other spoonable emulsified dressings (Dutilh and Groger, 1981).

The lipase enzyme preparation can hydrolyze lecithin to lysolecithin under a wide range of conditions. The enzyme's activity rises with increasing temperature and is greatest at about 40 °C. Above 50 °C, thermal decay becomes increasingly significant as enzyme stability fails. The lipase enzyme preparation is active over a wide pH range, depending on the specific application. The optimum pH for activity is near 8.5. Dosage will vary depending on the application and desired degree of conversion.

C. Level of Use

The recommended level of use for this lipase enzyme preparation is 0.5-5 U/g of lecithin.

SAFETY EVALUATION

A. Production Organism

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983; Pariza and Johnson, 2001). If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (IFBC, 1990). Pariza and Foster (1983) define a nontoxigenic organism as "one which

does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *S. violaceoruber* meets these criteria for nontoxigenicity and nonpathogenicity.

1. **Literature Review**

An extensive search of the literature keyed to *S. violaceoruber* and covering the period from 1957 to the present was performed. A review of the literature search on the organism uncovered no reports that implicate *S. violaceoruber* in any way with a disease situation. Only one report associated *S. violaceoruber* with toxin production. It is an unsubstantiated article on the aflatoxins of *Actinomycetes* that described a strain of *S. violaceoruber* isolated from paddy (a major crop cereal) that was reported to produce a B2-type aflatoxin (Koul, 1987). No taxonomic information on the strain was given, nor was the strain identified with a strain number. In general, bacteria are not known to produce aflatoxins and this report does not seem to be valid.

Streptomycetes are well known for their ability to produce antibiotics. Several antibiotic substances were reported to be produced by strains of *S. violaceoruber* (Heneishi *et al.*, 1974; Snipes *et al.*, 1979; Eckardt *et al.*, 1982; Locci, 1986; Bode and Zeeck, 2000). However, these substances are of no clinical significance. LysoMax™ is periodically tested for antibacterial activities as part of the product specifications. So far, all lots tested showed negative results.

2. ***Streptomyces violaceoruber* Pathogenicity Assay (Appendix 5)**

(University of South Alabama, August 1990)

Viable cells of the production microorganism were given to groups of eight Balb/c mice each by either intraperitoneal injection or by gavage at doses ranging from 0.00025 to 5,000 mg/kg. Dry weights were used for dosage due to the filamentous nature of the organism. All animals were observed daily for signs of toxicity for three weeks. A necropsy was performed at the end of the investigation.

No animal died throughout the entire investigation. No overt signs of toxicity were observed and no abnormalities were seen at necropsy. Under the conditions of this assay, *Streptomyces violaceoruber* JAS-591 was non-pathogenic and non-toxic to mice.

B. Enzyme

1. Literature

The literature search for the enzyme covered the period 1957 to the present and used synonyms for the enzyme. The search was restricted to those articles which addressed health or safety concerns. About 2,500 citations were generated, most of which dealt with the medical significance of human phospholipase A_2.

Phospholipase A_2 was the first phospholipase to be recognized. The enzyme is ubiquitous in nature and occurs in virtually all types of cells that have been examined (van den Bosch, 1989). The most widely studied phospholipase A_2 enzymes have eukaryotic origins and include those from pancreatic juice or cobra venom that play a digestive role for their hosts. These and other phospholipase A_2 enzymes have been associated with several toxic (neurotoxic, mycotoxic, etc.), pathological (inflammation, hypersensitivity, etc.), or physiological (contraction, proliferation, etc.) processes (Bomalaski *et al.*, 1991; Chen *et al.*, 1990; Hoffman *et al.*, 2001; Okana *et al.*, 1999).

Several reports discuss the production of lecithinase (phospholipase A_2) and its relationship to toxicity in certain bacterial strains, in particular, some members of the genera *Clostridium* and *Bacillus*. For example, Skjelkvale *et al.* (1979) examined 174 strains of *Clostridium perfringens* from a variety of sources for their ability to produce enterotoxin *in vitro*. Five strains of lecithinase negative *C. perfringens* produced high levels of enterotoxins. These authors concluded that it was not possible to relate the enterotoxin production of a strain to its other biochemical characteristics. Schmiel *et al.* (1998) evaluated the potential role of phospholipase in pathogenesis of several isolates of *Yersinia enterocolitica*. Mice were infected with high doses of a phospholipase positive strain or a phospholipase negative strain. Findings suggested that the phospholipase A_2 of *Y. enterocolitica* has a role in pathogenesis for this species. A total of 96 *Bacillus* species were studied by Obi (1980) for lecithinase (phospholipase A_2) and toxin production. Lecithinase production and toxin production were found to be widespread among the species but were strain specific. With the exception of *B. pumilus* isolates, an absolute correlation between lecithinase and toxin production could not be established among tested isolates. The conclusion from these published papers is that not all phospholipase A_2 (lecithinases) are toxic and the presence of phospholipase A_2-producing capabilities by a bacterial strain is not necessarily indicative of the strain's toxicity (Lozano, 1978; Obi, 1980; Skjelkvale, *et al.*, 1979).

In one article, pancreatic phospholipase A_2 was used to ferment egg yolk and soy bean lecithin that was then used in animal feeding trials. The results of the study showed that the phospholipase-hydrolyzed material, and by incorporation, the phospholipase A_2, posed no toxicological hazards over the lecithin that was not treated by this enzyme (Dulith and Groger, 1981)

Lucas Meyer, Inc. filed a Premanufacture Notice (PMN) Number P93-333 with the U.S. Environmental Protection Agency (EPA) in December 1992 requesting that enzyme modified lecithin (lysolecithin) be allowed for use 1) as a pigment dispersing agent in paint, 2) as an emulsifier in cosmetics, and 3) in leather tanning. Modification of the lecithin would be performed using phospholipase A_2 derived from porcine pancreatic tissue. In the EPA's Consent Order, it is stated "Use of the PMN substance in leather tanning operations may pose an unreasonable risk to aquatic organisms. Use as a pigment dispersing agent in paints and use as an emulsifier in cosmetics are not expected to pose a significant risk to aquatic organisms due to no significant water releases". Although the toxicity of modified lecithin to aquatic organisms was not specifically studied, the EPA based its concern on test data from structurally similar cationic surfactants. There were no safety concerns with the phospholipase A_2 enzyme itself and the enzyme, being a protein, is biodegradable.

Only two articles on the phospholipase A_2 of *S. violaceoruber* were uncovered. Matoba *et al.* (2002) and Sugiyama *et al.* (2002) have characterized, cloned, and studied the structure of the *S. violaceoruber* phospholipase A_2. These authors report that the structure of the prokaryotic *S. violaceoruber* phospholipase A_2 is significantly different from those of eukaryotic phospholipase A_2 enzymes. The amino acid sequence comparisons showed that the prokaryotic phospholipase A_2 had only four cysteines versus the 12-16 cysteines found in eukaryotic phospholipases. Furthermore, the overall structure of the *S. violaceoruber* phospholipase A_2, which is composed of only five α-helices, is completely distinct from those of the eukaryotic phospholipase A_2 enzymes, which consist of β-sheets and α-helices.

2. Past Uses in Food

According to Pariza and Foster (1983) and Pariza and Johnson (2001), enzyme proteins themselves do not generally raise safety concerns. Microbial lipases have been used in food since at least 1952 (Beckhorn *et al.*, 1965). Numerous lipase enzyme preparations (from *Rhizopus oryzae* var., the edible fore stomach

tissue of calves, kids, or lambs and animal pancreatic tissue i.e., pancreatin), are considered to be Generally Recognized As Safe (GRAS) in the U.S. based on use prior to January 1, 1958 (21 C.F.R. §184.1415 and 184.1583) or are approved for use as a food additive (21 C.F.R. §173.140). The *S. violaceoruber* lipase is functionally similar to the lipase of pancreatin, a phospholipase A_2 that is derived from animal pancreatic tissue. Additional lipase enzyme preparations that are considered to be GRAS based on scientific procedures include those derived from *Rhizopus niveus* (21 C.F.R. §184.1420), *Mucor miehei* (54 *Federal Register* 9565), *Penicillium camembertii* (GRASN #68), *A. niger* (GRASN #111), *A. oryzae* (GRASN #113), and several preparations from genetically modified *A. oryzae* strains (GRAS Notices #43, #75, and #103). *S. violaceoruber* is also listed as a safe source of phospholipase A_2 in the Pariza and Johnson enzyme safety publication (2001).

C. Enzyme Product

1. Acute Oral LD_{50} in Rats (Appendix 6)
(Hazleton Wisconsin, No. HWI 01200944, March 28, 1991)

The enzyme preparation (Batch No. 757677) was given as a single limit test at 20g/kg body weight to a group of 10 rats per sex. All animals were observed for mortality and toxicity for 14 days after treatment.

There were no mortalities and the oral LD_{50} was determined as $\geq$ 20g/kg body weight (corresponding to a volume of 19.8 ml based on a density of 1.01 g/ml). Based on the LD_{50} value, the enzyme preparation could be classified as non-toxic by ingestion.

2. Reverse Mutation Assay with *Salmonella* (Appendix 7)
(Hazleton Wisconsin, No. 14647-0-401R, November 6, 1991)

The enzyme preparation was tested in this assay at doses ranging from 100 to 5000 μg/plate. However, during the dose range finding assay contamination of the test material was detected and the test material was sterile-filtered.

In the main assay, contamination was still present and the mutagenic potential of the enzyme preparation to induce reverse mutation could not be determined with this test sample.

3. Forward Mutation Assay – Mouse Lymphoma L5178Y Cell Line (Appendix 8)
(Hazleton Wisconsin, No. 14647-0-431R, October 9, 1991)

The objective of this assay is to evaluate the ability of the enzyme preparation to induce forward mutation at the TK locus in the

mouse lymphoma L5178K cell line. Phospholipase A_2 was cytotoxic in the presence of metabolic activation at 625 μg/ml and higher and in the absence of metabolic activation at 2500 μg/ml and higher. The assay was conducted with doses ranging from 62.5 to 2500 μg/ml.

Under the conditions of this assay, the enzyme preparation was negative in inducing forward mutation in both the presence and absence of metabolic activation.

4. **Unscheduled DNA Synthesis (Appendix 9)**
(Hazleton Wisconsin, No. 14647-0-447R, October 11, 1991)
In this assay, rat liver cells were used and the enzyme preparation was tested at doses ranging from 0.5 to 5000 μg/ml. High cytotoxicity was found at 500 μg/ml and higher, however. The analysis of nuclear labeling was thus conducted for treatments ranged from 10 to 500 μg/ml only.

Under the conditions of this experiment, the enzyme preparation did not induce unscheduled DNA synthesis.

5. **Thirteen-week Oral (Gavage) Toxicity Study in the Rat (Appendix 10)**
(MDS No. 75/005, November 19, 2003)
In this study, the enzyme preparation was administered daily for 13 consecutive weeks by the oral route (gavage) to Sprague-Dawley rats at the dose levels of 5.75, 11.5, and 23 mg of total protein/kg b.w./day. No signs of toxicity were found. The NOEL for the enzyme preparation was determined as 23 mg of total protein/kg/day which is equivalent to 54.8 mg TOS/kg/day.

6. **Conclusions of the Safety Testing**
The host microorganism, *Streptomyces violaceoruber* JAS-591, was neither pathogenic nor toxic by two routes of administration in mice.

The lipase enzyme preparation is classified as non-toxic by ingestion as substantiated by an oral LD_{50} (lethal dose) $\geq$ 20 g/kg body weight and a NOEL of 23 mg total protein/kg/day in laboratory animals. It was not mutagenic in the mouse lymphoma L5178K cell line and did not induce DNA damage in rat liver cells. Although the results from the Ames assay could not be ascertained due to the test article's interference with the selective conditions of this assay, there is no evidence to suggest that this lipase enzyme preparation

would induce point mutation in light of the two negative mutagenic assays.

7. **Allergenicity Discussion**

According to Pariza and Foster (1983), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. Lipases have been used extensively in food processes for approximately 50 years and have generated no known safety concerns.

8. **Dietary Exposure Calculation**

a) **Residue in Food**

Pariza and Foster (1983) recommend the use of the Total Organic Solids (TOS) concept when determining a reliable estimate of enzyme use and consumption. Estimates of residue in food are expressed as grams TOS per unit of consumed food. These estimates are used further to estimate consumer exposure data. TOS is composed of residues from the fermentation medium and metabolic end products from the production microorganism, including the functional material, the phospholipase A_2 enzyme. Use of TOS excludes consideration of ash.

The TOS of the enzyme preparation is 1.0×10^{-4} g/U of phospholipase A_2.

b) **Dietary Exposure**

(1) **Soy lecithin hydrolysis**

The potential soy lecithin consumption in the United States in 1990 was about 99 million pounds. Although not all lecithin would be treated with phospholipase A_2, as a "worst case" scenario the maximum consumption of enzyme-treated soy lecithin would be about 0.39 lb (170 grams) per person per year.

The maximum dose of the enzyme preparation in soy lecithin is 5U/g lecithin. Therefore, the maximum residue introduced is:

1.0×10^{-4} gram TOS/U phospholipase A_2 X 5 U/g lecithin = 5.0×10^{-4} gram TOS/g lecithin

Assuming that 100% of the enzyme remains (worst case scenario), then the human yearly consumption is:

5.0×10^{-4} gram TOS/g lecithin x 170 grams lecithin = 8.5×10^{-2} gram TOS

The calculated human daily consumption is:

8.5×10^{-2} gram TOS : 365 days = 2.3×10^{-4} gram TOS/day

Using an average human body weight of 60 kg, the consumption of phospholipase A_2 TOS per kg body weight is:

2.3×10^{-4} gram TOS/day : 60 kg = 3.8×10^{-6} gram TOS/kg/day

(2) Egg Yolk Lecithin Hydrolysis

The maximum dose of phospholipase A_2 in egg lecithin is 5U/g egg yolk. Therefore, the maximum residue introduced is:

1.0×10^{-4} gram TOS/U phospholipase A_2 X 5 U/g egg yolk = 5.0×10^{-4} gram TOS/g egg yolk

United States citizens consumed 2.8 pounds (1,200 grams) of yolk from processed eggs in 1997 (Putham and Allshouse, 1999). Assuming that 100% of the enzyme remains (worst case scenario), then the human yearly consumption is:

5.0×10^{-4} gram TOS/g egg yolk x 1,200 grams egg yolk = 4.8×10^{-1} gram TOS/year

The calculated human daily consumption is:

4.8×10^{-1} gram TOS/year: 365 days = 1.3×10^{-3} gram TOS/day

Using an average human body weight of 60 kg, the consumption of Phospholipase A_2 TOS per kg body weight is:

1.3×10^{-3} gram TOS/day : 60 kg = 2.2×10^{-5} gram TOS/kg/day

c) **Summary of enzyme residue intake**

A "worst case" estimate of human exposure to the residues of the enzyme product (expressed as g of TOS per kg body weight per day) from all sources (soy lecithin and egg yolk)

is made using consumption and population estimates for the United States.

The estimate of exposure to the lipase enzyme residues, as grams of TOS per kg body weight per day, is

3.8×10^{-6} gram TOS/kg/day + 2.2×10^{-5} gram TOS/kg/day = 2.6×10^{-5} gram TOS/kg/day or 0.026 mg TOS/kg/day

Maximum Total Exposure =	0.026 mg TOS/kg/day

d) **Human Consumption Versus Level in 13-week Feeding Study**

In the 13-week oral toxicity study, the NOEL of the enzyme preparation was established at 54.8 mg TOS/kg/day. This NOEL value would provide more than 2,000 margins of safety above the maximal total human daily exposure of 0.026 mg TOS/kg/day.

D. Manufacturing Process

The lipase enzyme preparation meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex*. The lipase enzyme preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (Aunstrup, 1979; Aunstrup et al., 1979, Enzyme Applications, 1994).

CONCLUSION

Genencor International, Inc. has evaluated the lipase enzyme preparation derived from *Streptomyces violaceoruber* according to the safety scheme of Pariza and Johnson (2001) and determined that this enzyme preparation is safe for use in the hydrolysis of phospholipids in food. This determination is supported by an extensive review of published and unpublished safety data available on the enzyme, the production organism, enzyme manufacturing processes, and the enzyme product.

REFERENCES

Aunstrup, K. 1979. Production, isolation, and economics of extracellular enzymes. In: Applied biochemistry and bioengineering, Vol. 2, enzyme

technology, *eds*. L. B. Wingard, E. Katchalski-Katzir, and L. Goldstein, pp. 28-68.

Aunstrup, K., O. Anderson, E. A. Falch, and T. K. Nielsen. 1979. Production of microbial enzymes. In: Microbial technology, 2nd ed., Vol. 1, *eds* H.J. Peppler and D. Perlman. Ch. 9, pp. 282-309.

Beckhorn, E. J., M. D. Labbee, and L. A. Underkofler. 1965. Production and use of microbial enzymes for food processing. J. Agric. Food Chem. 13:30-34.

Bode, H. B., and A. Zeeck. 2000. Structure and biosynthesis of kendomycin, a carbocyclic ansa-compound from *Streptomyces*. Perkin 1. (3):323-328.

Bomalaski, J. S., P. Lawton, and J. L. Browning. 1991. Human extracellular recombinant phospholipase A_2 induces an inflammatory response in rabbit joints. J. Immunol. 146:3904-3910.

Chen, S., S. Li, X. Fei, and Z. Wu. 1990. Phospholipase A2-induced lung edema and its mechanism in isolated perfused guinea pig lung. Inflammation 14:267-273.

Dutilh, C. E. and W. Groger. 1981. Improvement of product attributes of mayonnaise by enzymatic hydrolysis of egg yolk with phospholipase A-2 EC 3.1.1.4. J. Sci. Food Agric. 32: 451-458.

Enzyme applications. In: Encyclopedia of Chemical Technology, 4th ed. 1994. *ed*. J. I. Kroschwitz, Vol. 9, pp. 597-620.

Eckardt, K., D. Tresselt, W. Ihn, G. Bradler, and G. Reinhardt. 1982. Sarubicin B, a new quinine antibiotic, isolated from the fermentation broth of a *Streptomyces* strain. J. Antibiot. 35:1638-1640.

Heneishi, T., N. Kitahara, Y. Takiguchi, M. Arai, and S. Sugawara. 1974. New antibiotics, methylenomycins A and B. I. Producing organism, fermentation, and isolation, biological activities and physical and chemical properties. J. Antibiot. 27:386-392.

Hoffman, D. R., S. E. El-Choufani, M. M. Smith, and H. de Groot. 2001. Occupational allergy to bumblebees: Allergans of *Bombus terrestris*. J. Allergy Clin. Immunol. 108:855-860.

International Food Biotechnology Council (IFBC). 1990. Chapter 4: Safety evaluation of foods and food ingredients derived from microorganisms in biotechnologies and food: Assuring the safety of foods produced by genetic modification. Regul. Toxicol. Pharmacol. 12:S1-S196.

International Union of Biochemistry and Molecular Biology (IUBMB) Nomenclature Committee. 1992. Enzyme nomenclature-recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. Academic Press, Inc., San Diego. [Also see http://www.chem.qmw.ac.uk/iubmb/enzyme/]

Joint FAO/WHO Expert Committee of Food Additives (JECFA). 2001. General specifications and considerations for enzyme preparations used in food processing. FAO Food and Nutrition Paper 52, Add. 9. pp. 37-39.

Koul, U. K. 1987. Aflatoxins from actinomycetes. Curr. Sci. 56:1015-1017.

Locci, R. 1986. *Streptomyces* and related genera, p. 2451-2492. In: Bergey's manual of systematic bacteriology, vol. 4. *eds* S.T. Williams, M. E. Sharpe, and J. G. Holt. Williams and Wilkins, Baltimore.

Lozano, E. A. 1978. Comparison of some toxic lecithinases. Proc. Mont. Acad. Sci. 37:117-122.

Matoba, Y., Y. Katsube, and M. Sugiyama. 2002. The crystal structure of prokaryotic phospholipase A_2. J. Biol. Chem. 277:20059-20069.

Meinhold, N. M. 1991. Lecithin: Development and applications. Food Processing, May 1991, p. 130-134.

National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemicals Codex. 1996. *Food Chemicals Codex*, 4th ed. National Academy Press, Washington DC.

Obi, S. K. C. 1980. Lecithinase and toxin production in *Bacillus* spp. Zentralbl Bakteriol 1 Abt. Orig. A Med. Mikrobiol. Infektionskr. Parasitol. 246: 415-422.

Okano, M., K. Nishizaki, A. R. Satoskar, T. Yoshino, Y. Masuda, and D. A. Harn, Jr. 1999. Involvement of carbohydrate on phospholipase A_2, a bee-venom allergen, in *in vivo* antigen-specific IgE synthesis in mice. Allergy 54:811-818.

Pariza, M. W. and E. M. Foster. 1983. Determining the safety of enzymes used in food processing. J. Food Protect. 46:453-468.

Pariza, M. W. and E. A. Johnson. 2001. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul. Toxicol. Pharmacol. 33:173-186.

Putnam, J. J. and J. E. Allshouse. 1999. Food consumption, prices, and expenditures~ 1970-1997. Economics Research Service, U.S. Department of Agriculture. Statistical Bulletin No. 965.

Schmiel, D. H., E. Wagar, L. Karamanou, D. Weeks, and V. L. Miller. 1998. Phospholipase A_2 of *Yersinia enterocolitica* contributes to pathogenesis in a mouse model. Infect. Immun. 66:3941-3951.

Skjelkvale, R., M. F. Stringer and J. L. Smart. 1979. Enterotoxin production by lecithinase positive and lecithinase negative *Clostridium perfringens* isolated from food poisoning outbreaks and other sources. J. Appl. Bacteriol. 47: 329-340.

Snipes, C. E., C. Chang, and H. G. Floss. 1979. Biosynthesis of the antibiotic granaticin. J. Amer. Chem. Soc. 101:701-706.

Sugiyama, M., K. Obtani, M. Izuhara, T. Koike, K. Suzuki, S. Imamura, and H. Misaki. 2002. A novel prokaryotic phospholipase A_2: Characterization, gene cloning, and solution structure. J. Biol. Chem. 227:20051-20058.

Van Dam, A. F. 1977. Emulsions. U. S. Patent #4 034 124.

Van Dam, A. F. 1978. Oil-in-water emulsion and process for the preparation thereof. U. S. Patent #4 119 564.

Van den Bosch, H. 1989. Cellular Phospholipase A_2: Discussion Session Summary. Cell activities and signal initiation: Receptor and phospholipase control of inositol phosphate, PAF, and eicosanoid production, p. 317-321.

Van Nieuwenhuyzen, W. 1981. The industrial uses of special lecithins: A review. JAOCS, Oct. 1981, p. 886-888.

Waksman, S. A. and R. E. Curtis. 1916. The Actinomyces of the soil. Soil Sci. 1:99-134.

APPENDICES

Appendix 1. Flow Chart of the Manufacturing Process for LysoMax™

Appendix 2. LysoMax™ Product Specification

Appendix 3. Activity Assay Method for Phospholipase A_2 Enzyme

Appendix 4. Product Information of LysoMax™

Appendix 5. *Streptomyces violaceoruber* Pathogenicity Assay (University of South Alabama, August 1990)

Appendix 6. Summary of Acute Oral Toxicity Study in Rats (Hazleton Wisconsin, No. HWI 01200944, March 28, 1991)

Appendix 7. Summary of Reverse Mutation Assay with *Salmonella* (Hazleton Lab. No. 14647-0-401R, November 6, 1991)

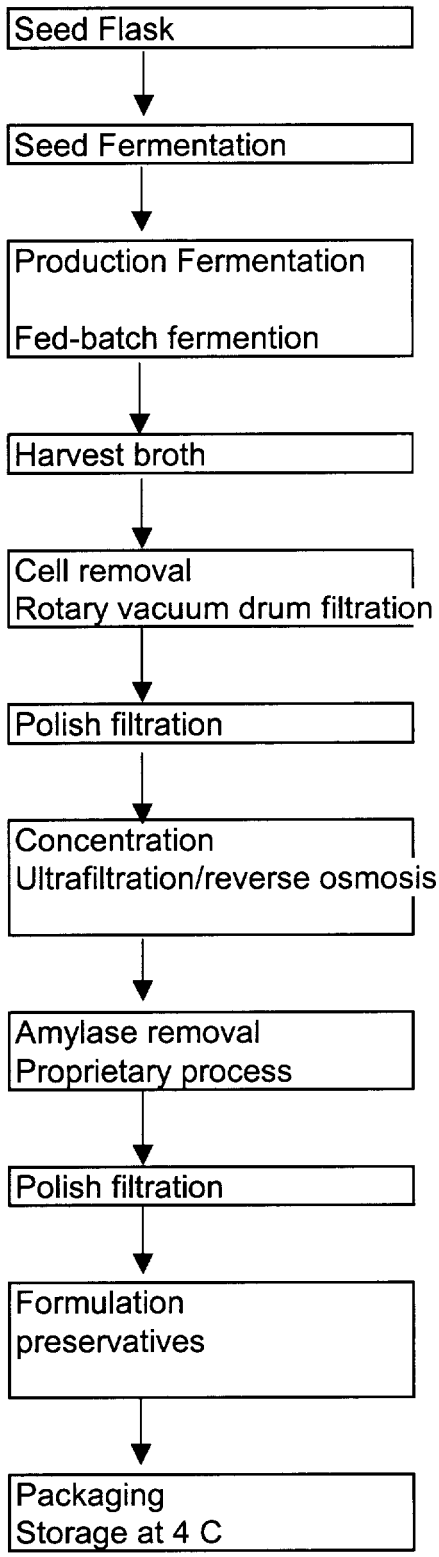
Appendix 8. Summary of Forward Mutation Assay – Mouse Lymphoma L5178Y Cell Line (Hazleton No. 14647-0-431R, October 9, 1991)

Appendix 9. Summary of Unscheduled DNA Synthesis Assay (Hazleton No. 14647-0-447R, October 11, 1991)

Appendix 10. Summary of Thirteen-week Oral (Gavage) Toxicity Study in Rats. (MDS No. 75/005, November 19, 2003)

1

LysoMax Production Process



2

Genencor International®

Genencor International Inc.
[illegible]eridian Centre Blvd.
Rochester, N.Y. 14618 - 3916
U.S.A

800.847.5311
716.244.4544 fax

Genencor International B.V.
P.O.Box 218
2300 AE Leiden
The Netherlands

31.71.5686.168
31.71.5686.169 fax

Genencor International Asia Pacific Pte Ltd
3 Killiney Road
#05- 02 Winsland House
Singapore 239519

65.838.7410
65.737.1218 fax

Genencor International Argentina, S.A.
Carlos Pellegrini 1149, Piso 13
(1009) Buenos Aires, Argentina

54.11.4129.2300
54.11.4326.3003 fax

Product Specification

Date Revised 03/14/2003

Product Name: **LysoMax**

Item Code: A14025

Assay	Unit	Low Spec	High Spec
ENZYME ACTIVITIES			
Phospholipase A2	U/g	400	600
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0	50000
Total Coliforms	CFU/ml	0	30
E. coli	/25ml		NEG
Salmonella	/25ml		NEG
Molds	CFU/ml	0	100
Yeasts	CFU/ml	0	100
[illegible]oduction Strain	/ml		NEG
Antibacterial Activity	/ml		NEG
PHYSICAL PROPERTIES			
pH		4.8	5.2
Specific Gravity		1.01	1.05
Appearance			Brown liquid
OTHER ASSAYS			
Heavy Metals as Pb	mg/kg	0	30
Arsenic	mg/kg	0	3
Cadmium	mg/kg	0.00	0.50
Mercury	mg/kg	0.00	0.50
Lead	mg/kg	0	5
Potassium Sorbate	% (w/w)	0.10	0.30
Sodium Benzoate	% (w/w)	1.3	1.7

This product complies with the current recommended purity specifications for food- grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).

3

Lab Method Phospholipase A2 Enzyme Activity

PHOSPHOLIPASE A_2 ENZYME ACTIVITY

PRINCIPLE

A commercially available enzymatic method is used to quantitate the fatty acids produced from phospholipids by the action of phospholipase A_2. Coenzyme A is acylated in the presence of free fatty acid and Acyl CoA Synthetase. Acyl CoA is then oxidized by Acyl CoA Oxidase, generating hydrogen peroxide, which permits oxidative condensation of 3-methyl-N-ethyl-N-ß-hydroxyethyl-aniline (MEHA) with 4-amino-antipyrine in the presence of peroxidase. The absorbance of the resulting purple adduct is measured spectrophotometrically at 550 nm. One unit of phospholipase A_2 is defined as the amount of enzyme that will form 1 μmole of free fatty acid/minute under the assay conditions.

SCOPE

This procedure can be used for phospholipase A_2 determination in fermentation broth, recovery process, and finished product samples.

APPARATUS

Water bath, one maintained at 37.0 ± 0.5° C.

Spectrophotometer, single or double beam digital instrument capable of operating in visible wavelength region (350-650 nm) and equipped with 1 cm pathlength cells.

Timer, electric, with 0.1-second precision over a 10,000 second range (Fisher Scientific #12-654-1 or equivalent).

Test tubes, 13 X 100 mm borosilicate glass, disposable.

Test tube mixer, Vortex-Genie (Fisher Scientific #12-812 or equivalent).

Micropipet, adjustable, 1.0 ml, to pipet 0.25, 0.50 and 1.0 ml samples .

Micropipets, adjustable, 100 µl or 200 µl, to pipet 25µl and 50 µl samples.

000030

REAGENTS

NEFA Kit (note 1)

Follow instructions for reagent preparation supplied with the kit.
Add 10 ml of Reagent A solution into Reagent A powder vial, mix to dissolve.
Add 20 ml of Reagent B solution into Reagent B powder vial, mix to dissolve.
Date bottles after preparation, kits and kit reagents must be kept refrigerated.

Stock Buffer solution, 1.0M Tris-HCl, pH 8.0

Dissolve 12.1g Trizma base (tris(hydroxymethyl)amino-methane) in approximately 60 ml of deionized water. Adjust pH to 8.0 with 1.0 M HCl. Dilute to final volume in a 100 ml volumetric flask. Store in refrigerator for up to two weeks.

Substrate Buffer, 0.1M Tris-HCl, pH 8.0

Dilute 1 ml of stock buffer in a 10 ml volumetric flask and fill to volume with deionized water. Make fresh daily.

Triton X-100 0.01%

Add 0.1 ml Triton X-100 to a 100 ml volumetric flask and dilute to volume.

Calcium Chloride, 0.1M

Dissolve 1.47 g calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in 100 ml of deionized water. Make fresh daily.

Stock Substrate, Lecithin suspension

In a sonicator tube place 1.0 g soybean lecithin paste (Sigma P-3644, 40% L-a-phosphatidylcholine), 16.0 ml of 0.01% Triton X-100, 2.0 ml of 0.1 M $CaCl_2$, and 2.0 of ml deionized water. Sonicate in an ice bath for 3-4 minutes using a 1 cm tip at 100 watts. (This is done to disperse the lecithin and reduce micelle size.) Prepare fresh daily; substrate spontaneously hydrolyzes with time.

Working Substrate

Dilute one part stock substrate with one part substrate buffer.

000031

PROCEDURE

1. Dilute samples to 0.5 - 1.0 PLA2 U/ml with deionized water.

2. Add 0.50 ml of Working Substrate to one set of 13 X 100 mm tubes. (Allow one extra substrate tube for water blank.) Incubate tubes in 37° C bath for 5 minutes.

3. Add 0.25 ml of Reagent A to another set of 13 X 100 mm tubes. Place this set of tubes in the 37° C bath at the 7-minute mark of the assay.

4. Add 50 µl of diluted enzyme or water to the substrate tubes in the 37° C water bath at 30 second intervals.

5. After 10 minutes (600 seconds) transfer 25 µl from each substrate tube to a Reagent A tube in 37° C water bath.

6. After 10 additional minutes (1200 seconds) add 0.50 ml of Reagent B sequentially to the tubes in the 37° C bath.

7. After 10 additional minutes (1800 seconds) remove tubes from bath and read absorbance at 550 nm in semi micro cuvette. Withdraw each tube approximately 15 seconds before the timepoint to vortex and fill the cuvette. Be sure to zero the spectrophotometer with deionized water first.

Standard Curve:

Prepare a standard curve using the 1 mM Oleic Acid in the NEFA C Kit. Run the diluted standards starting at Step 4 of the assay. (No incubation in substrate.)

Run a standard curve for each set of reagent bottles to determine the Average Calibration Factor. This Average Calibration Factor may be used for all assays done with that of set of reagent bottles within a 1 week period; beyond 1 week, a fresh calibration curve should be prepared.

Typical Standard Curve - Do Not use for Calculations

Std. Oleic Acid (ml)	Deionized Water (ml)	Oleic Acid (µmoles/ml)	ABS 550 nm	(1) Net ABS	(2) C.F.	(3) Average C.F.
1.00	0.00	1.0	.508	.464	.464	
0.80	0.20	0.8	.413	.369	.464	
0.60	0.40	0.6	.328	.284	.473	.464
0.40	0.60	0.4	.229	.185	.463	
0.20	0.80	0.2	.136	.092	.460	
0.00	1.00	(blank)	.044	------	------	

(1) Net ABS = (Sample ABS - Water Blank ABS)

(2) C.F. = Calibration Factor = $\frac{\text{Net ABS}}{\mu\text{moles/ml}}$

(3) Average the C.F. values of all oleic acid standards to obtain A.C.F. = Average Calibration Factor.

CALCULATIONS

$$\text{PLA2 U/g} = \frac{\Delta\,\text{ABS}}{\text{A.C.F.}} \times 1.1 \times \text{dilution factor}$$

[1]Do not use sample ABS above 0.500.

000033

4

LysoMax™

Microbial Phospholipase A2

Product Information

■ DESCRIPTION

LysoMax™ is a phospholipase A2, or lecithinase, produced by microbial fermentation. Like the similar enzyme extracted from animal sources, LysoMax™ lecithinase can be used to partially digest the phospholipids present in lecithin.

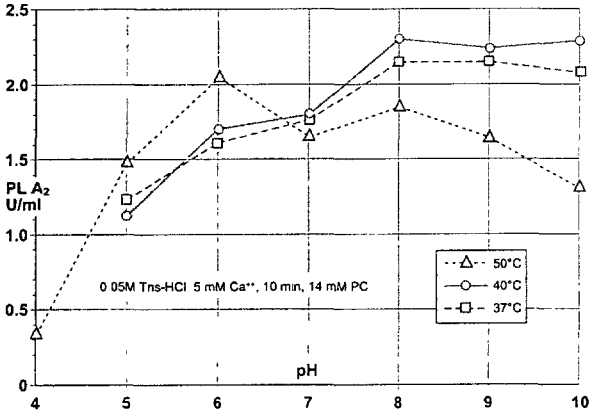
The lysophospholipids formed by treating lecithin with LysoMax™ lecithinase have emulsifying properties that are superior to lecithin in certain applications. (We also supply G-ZYME® G999 fungal lysophopholipase, that is used to further digest lysophosphoglyceride and remove this emulsifier.) Lecithin occurs naturally in many plant and animal sources; soy beans, corn and eggs are leading commercial sources. Lysolecithin also occurs naturally and is, for example, the predominant phospholipid in wheat starch.

Unlike the animal enzyme, LysoMax™ lecithinase is available with Kosher certification.

Effect of Temperature & pH

Assayed activity of LysoMax™ lecithinase increases with temperature and is maximal at about 40°C (see Figure 1). Above 50°C, thermal decay becomes increasingly significant as enzyme stability falls. LysoMax™ lecithinase is active over a wide pH range, with optimal pH near 8.5.

Figure 1: Effect of Temperature and pH on LysoMax™ lecithinase



Temperature and pH profiles, and the substrate preferences of LysoMax™ lecithinase are slightly different from the similar lecithinase from animal sources. However, LysoMax™ lecithinase may usually be substituted directly into existing applications with only minor adjustments. We can help you optimize your LysoMax™ lecithinase process.

■ ENZYME CLASSIFICATION

Trivial Name:	lecithinase
Classification:	phospholipase A2
Source:	microbial fermentation
Systematic Name:	phosphotidylglyceride 2 acylhydrolase
IUB #:	3.1.1.4

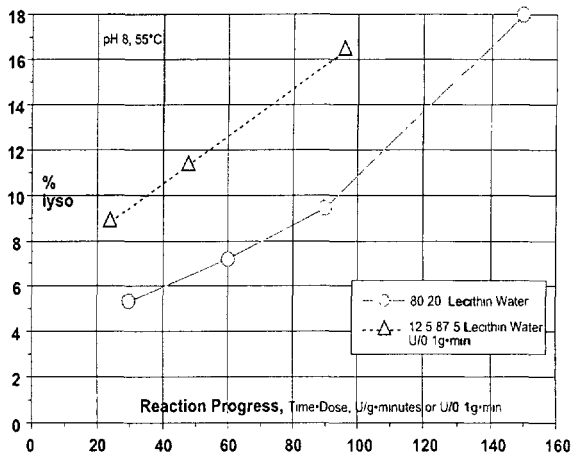
■ TYPICAL CHARACTERISTICS

Appearance:	brown liquid
Specific Gravity:	1. 05-1.15

■ APPLICATIONS

LysoMax™ lecithinase hydrolyzes lecithin to lysolecithin under a wide range of conditions. Dosage required will vary depending on application and desired degree of conversion (Figure 2).

Figure 2: Use of LysoMax™ lecithinase to modify Lecithin



Activity Determination

LysoMax™ lecithinase is incubated with a 2% lecithin solution at 37°C and pH 8.0 Released fatty acids are then determined One unit hydrolyzes 1 μ-mole of lecithin substrate in one minute under the specified assay conditions

PACKAGING

LysoMax™ lecithinase is available in 5 gallon pails.

STORAGE

LysoMax™ lecithinase should be shipped and stored under refrigeration when necessary to avoid exposure to temperatures above 25°C (77°F). Store LysoMax™ lecithinase at room temperature (20°C, 68°F) and use within two months; one month if temperatures exceed 20°C. Cold storage extends shelf life to at least one year.

SAFETY & ENZYME HANDLING

Inhalation of enzyme dust and mists should be avoided. In case of contact with the skin or eyes, promptly rinse with water for at least 15 minutes.

For detailed handling information, please refer to the appropriate Material Safety Data Sheet, the Enzyme Technical Association (ETA) handbook *Working Safely With Enzymes*, and the Association of Manufacturers of Fermentation Enzyme Products (Amfep) handbook *Guide to the Safe Handling of Microbial Enzyme Preparations*. All are available from Genencor International.

TECHNICAL SERVICE

Genencor International will work with customers to enhance processes and solve problems. Let us know what you need and we will assist you.

For more information:

USA and Canada
Genencor International, Inc.
200 Meridian Centre Blvd., Rochester, NY 14618 USA
Telephone: 1-800-847-5311 (USA)
Telephone: +1-585-256-5200
Telefax: +1-585-244-4544

Europe and Africa
Genencor International B.V.
P.O. Box 218, 2300 AE Leiden, The Netherlands
Telephone: +31-71-5686-168
Telefax: +31-71-5686-169

Latin America
Genencor International Argentina S.R.L.
Carlos Pellegrini 1149, Piso 13
1009 Buenos Aires, Argentina
Telephone: +54-11-4129-2300
Telefax: +54-11-4326-4411

Asia/Pacific
Genencor International Asia Pacific PTE, LTD.
3 Killiney Road
#05-02 Winsland House, Singapore 239519
Telephone: +65-6838-7410
Telefax: +65-6737-1218

Web Address
www.genencor.com

© Genencor International, Inc., 2001. G-ZYME, GENENCOR, GENENCOR INTERNATIONAL, and INNOVATIVE BY NATURE are registered trademarks of Genencor International, Inc. or its affiliated companies.

The information contained in this product literature is, to the best of our knowledge, true and accurate and the product as sold is in conformance with the specifications set forth herein as determined by the assay methods described. Due to conditions of use, technical errors or omissions, improper handling or storage beyond our control, Genencor International hereby DISCLAIMS ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING THE IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE Genencor International shall not be liable for any incidental, consequential, or special damages resulting in any way from the furnishing, performance, or use of this product literature or the product described herein

Nothing contained herein shall be construed as a representation by Genencor International that the use or resale of the product or processes described herein will not violate any rules or regulations or infringe upon patents or other intellectual property rights of third parties or that the recommendations and usage suggestions described constitute any authorization or inducement to infringe any such rights

REV0602 1765 LYSOMAX01 01

Printed on recycled paper

5

EVALUATION OF STREPTOMYCES CULTURE FOR MOUSE TOXICITY

ASSAYED FOR

ENZYME BIO-SYSTEMS LTD.

ATTENTION: MS. NANCY W. ZEMAN

MANAGER, R&D

3350 SALT CREEK LANE

ARLINGTON HEIGHTS, IL 60005

FROM

LEGIONELLA TESTING SERVICE

UNIVERSITY OF SOUTH ALABAMA

COLLEGE OF MEDICINE

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

MOBILE, ALABAMA 36688

[205] 460-6314
FAX [205] 460-7269

AUGUST 1, 1990

PROJECT FINAL REPORT

Evaluation of Streptomyces violaceoruber ATCC 14980 virulence potential in Balb/c mice by oral intubation and intraperitoneal [IP] injection.

Methods:

Male Balb/c mice (Charles River) were fed or injected to determine the virulence potential of Streptomyces violaceoruber ATCC 14980. Two routes of infection were used; A) intraperitoneal injection, and B) oral intubation. The organism was cultured 24-36 hours in broth medium specified in the order request letter [5/23/90] at 32°C with agitation at ~ 200-240 rpm. Good growth was obtained. Serial dilutions of this culture were made and introduced into the mice by the IP or oral intubation routes. Due to the filamentous nature of the organism dry weights were obtained to determine challenge dosage. All mice were observed for signs of toxicity hourly for the first four hours following infection, then daily for three weeks. After three weeks mice were sacrificed and a necropsy performed on two from each test group.

Results: Test groups were as follows;

	Surviving mice / No. Challenged	
Challenge level (mg/kg)	Intraperitoneal	Oral
5000	8/8	8/8
2500	8/8	8/8
250	8/8	8/8
25	8/8	8/8
2.5	8/8	8/8
0.25	8/8	8/8
0.025	8/8	8/8
0.0025	8/8	8/8
0.00025	8/8	8/8
Controls:		
Suspension buffer	8/8	8/8
Sterile broth	8/8	8/8
Spent growth medium*	8/8	8/8

*(0.45μ filtered before injection)

Following infection all mice injected with the test organism showed slight distress evidenced by ruffled fur and the huddling together of cage occupants. These symptoms disappeared within four hours and were more pronounced in those mice receiving >250 mg/kg IP. No other morbidity was observed for the remainder of the holding period. No abnormalities were seen in the mice necropsied at sacrifice.

Conclusions: Under the conditions of test, Streptomyces violaceoruber ATCC 14980 exhibited no evidence of pathogenicity or toxicity for Balb/c mice.

6

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
3301 KINSMAN BOULEVARD
MADISON, WISCONSIN 53707

a CORNING Laboratory Services Company

Sponsor:

Enzyme Bio-Systems Ltd.
Arlington Heights, Illinois

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of Lecithinase Enzyme in Rats

Author:

Steven M. Glaza

Study Completion Date:

March 28, 1991

Performing Laboratory:

Hazleton Wisconsin, Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

HWI 01200944

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608-241-7227

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
3301 KINSMAN BOULEVARD
MADISON, WISCONSIN 53707

a CORNING Laboratory Services Company

HWI 01200944

QUALITY ASSURANCE STATEMENT

Acute Oral Toxicity Study of Lecithinase Enzyme in Rats

The report for this study has been reviewed by the Quality Assurance Unit of Hazleton Wisconsin (HWI) in accordance with the Good Laboratory Practice Regulations as set forth in 21 CFR 58.35 (b) (6) (7). The report identifies and/or describes the authorized methods and Standard Operating Procedures followed in the conduct of the study. The Quality Assurance Unit has also conducted the following inspections of the testing facilities used in the conduct of this study and has submitted written reports of these inspections to the Study Director. Written status reports of inspections and findings are submitted to Management according to HWI Standard Operating Procedures.

Date of Inspection	Type of Inspection	Date Issued to Study Director
12/20/90	Protocol Review	12/20/90
01/11/91	Necropsy	01/14/91
03/06/91	Data Review	03/06/91
03/06/91	Report Review	03/06/91
03/28/91	Report Review	03/28/91

Rebecca S. Nelson
Representative, Quality Assurance Unit

3/28/91
Date

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471
Fax 608-241-7227

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
3301 KINSMAN BOULEVARD
MADISON, WISCONSIN 53707

a CORNING Laboratory Services Company

HWI 01200944

KEY PERSONNEL

Acute Toxicology

Steven M. Glaza
Study Director
Section Supervisor

Steven R. Sorenson
Study Coordinator

Patricia Padgham
Group Leader

Sharen L. Howery
Report Coordinator

Quality Assurance

Deborah L. Keller
Manager

Laboratory Animal Veterinarian

Cindy J. Cary, DVM
Diplomate, ACLAM

Anatomical Pathology

Thomas E. Palmer, PhD
Anatomical Pathologist

Dennis Hoffman
Group Leader
Necropsy

Anne Mosher
Group Leader
Pathology Data

CONTENTS

	Page
Quality Assurance Statement	2
Key Personnel	3
Objective	4
Regulatory Compliance	4
Test Material	4
Test System	4
Procedures	5
Summary of Results	7
Individual Pathology Comments	8
Discussion	12
Signature	12
Reference	12
Pathology Report	13
Raw Data/Protocol Appendix	14

000043

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
3301 KINSMAN BOULEVARD
MADISON WISCONSIN 53707

OBJECTIVE

The objective of this study was to assess the acute oral toxicity produced when the test material is administered by the oral route (gavage) to rats.

REGULATORY COMPLIANCE

This study was conducted in accordance with the U.S. Food and Drug Administration's Good Laboratory Practice Regulations for Nonclinical Laboratory Studies, 21 CFR 58.

TEST MATERIAL

Identification

The test material was identified as Lecithinase Enzyme; Batch No. 757677 and described as a brown liquid.

Purity and Stability

Sponsor assumes responsibility for purity and stability determinations (including under test conditions).

Storage and Retention

The test material was stored frozen. Any unused material will be discarded according to Hazleton Wisconsin (HWI) Standard Operating Procedure (SOP).

Safety Precautions

Normal handling procedures were used according to HWI SOP.

TEST SYSTEM

Test Animal

Young adult albino rats, Crl:CD®BR, were procured, maintained individually in screen-bottom, stainless steel cages in temperature- and humidity-controlled quarters, provided continuous access to Rodent Chow® #5001, Purina Mills, Inc., and water, and held for an acclimation period of at least 7 days. Animal husbandry and housing at HWI comply with standards outlined in the

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608-241-7227

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
3301 KINSMAN BOULEVARD
MADISON, WISCONSIN 53707

"Guide for the Care and Use of Laboratory Animals".[1] If variations from the prescribed environmental conditions existed, they were documented and considered to have no effect on the study outcome. No contaminants were expected to have been present in the feed or water which would have interfered with or affected the results of the study.

Acclimated animals were maintained during the study in the same manner as for the acclimation period. Twenty male and 20 female rats, weighing from 202 to 250 g, were assigned to two treatment groups consisting of a test group and vehicle control group using a stratified body weight randomization program. Each group consisted of 10 male and 10 female rats. Animals were housed individually and identified by animal number and corresponding ear tag. Food and water were available *ad libitum* throughout the study, except for 17 to 20 hours before test material administration when food, but not water, was withheld.

Justification for Species Selection

Historically, rats have been used as a representative of a rodent species and are preferred by various regulatory agencies.

PROCEDURES

Preparation and Administration of Test Material

The frozen test material was thawed and brought to room temperature before determining the bulk density. To treat the test group at a level of 20.0 g/kg of body weight, the undiluted test material was administered at a volume of 19.80 mL/kg based upon an average bulk density of 1.01 g/mL. The vehicle control animals were treated with deionized water only at the same dose volume as the test group. An individual dose of the undiluted test material or deionized water was calculated for each respective animal based upon its fasted body weight and administered by gavage.

Reason for Route of Administration

Historically, the oral route has been the route of choice for administering a known amount of test material.

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608-241-7227

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
3301 KINSMAN BOULEVARD
MADISON, WISCONSIN 53707

Observations

Clinical signs and mortality checks were conducted at 1, 2.5 and 4 hours after test material administration. The animals were observed daily thereafter for 14 days for clinical signs and twice a day (morning and afternoon) for mortality. Qualitative food consumption and the general appearance of the feces were also noted on a daily basis.

Body weights were determined before test material administration (Day 0), at Day 7, and at termination of the experimental phase (Day 14).

Pathology

At termination of the experimental phase, all animals were euthanatized, subjected to a gross necropsy examination, and all abnormalities were recorded. After necropsy, animals were discarded and no tissues were saved.

Statistical Analyses

No statistical analysis was performed.

Location of Raw Data and Final Report

The raw data and a copy of the final report will be retained in the archives of HWI.

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608 241 7227

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
3301 KINSMAN BOULEVARD
MADISON, WISCONSIN 53707

a CORNING Laboratory Services Company

HWI 01200944

SUMMARY OF RESULTS

Test Animal: Albino Rats - Crl:CD®BR
Source: Charles River Laboratories, Inc., Portage MI
Date Animals Received: 12/17/90

Temperature and Humidity of Animal Room: 70 to 82°F;
33 to 78% Relative Humidity

Start Date (In-life): 12/28/90 End Date (In-life): 01/11/91

Sex	Dose Level (g/kg)	Average Body Weights (g) Day 0	Day 7	Day 14	Mortality (Number Dead/Number Dosed)
		Vehicle Control Group			
Male	0.00	238	317	362	0/10
Female	0.00	222	251	268	0/10
		Test Group			
Male	20.00	241	322	369	0/10
Female	20.00	219	250	267	0/10

Clinical Signs

All animals in the test group and vehicle control group appeared clinically normal throughout the study.

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608-241-7327

7

HAZLETON
WASHINGTON

SECTION I. SUMMARY

INTRODUCTION AND CONCLUSIONS

HAZLETON
WASHINGTON

SUMMARY

A. Introduction

At the request of Enzyme Bio-Systems Ltd., Hazleton Washington, Inc. examined Lecithinase for mutagenic activity in the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) with a Confirmatory Assay. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strain TA100 and ten dose levels of test article ranging from 5,000 to 6.67 μg per plate, one plate per dose, both in the presence and absence of S9.

The tester strains used in this study were TA98, TA100, TA1535, TA1537 and TA1538. The assay was conducted using three plates per dose level both in the presence and absence of S9. Six dose levels of the test article were tested, from 5,000 to 100 μg per plate in both the presence and absence of S9.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) indicate that under the conditions of this study, Enzyme Bio-Systems Ltd.'s test article, Lecithinase, could not be adequately evaluated using this test system due to the test article's interference with the selective conditions of the assay.

HAZLETON
WASHINGTON
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

a CORNING Laboratory Services Company

November 6, 1991

Dr. Martin Teague
Enzyme Biosystems
3350 Salt Creek Lane
Suite 103
Arlington, IL 60005

RE: FINAL REPORT AND PROTOCOL AMENDMENT
Salmonella/Mammalia-Microsome Reverse Mutation Assay With a Confirmatory Assay
Protocol No.: 401R, Edition No.: 17
Genetics Assay No.: 14647
Test Material: Lecithinase

Dear Dr. Teague:

Enclosed please find one original and two (2) copies of the above referenced report. This material has been reviewed by our Quality Assurance unit to ensure compliance with GLP requirements.

If you have any further questions or comments concerning the above material, please do not hesitate to contact me.

Thank you for giving us this opportunity to work with you.

Sincerely,

HAZLETON WASHINGTON INC.

Timothy E. Lawlor, M.A.
Microbial Mutagenesis
Department of Molecular and
Cellular Toxicology

TEL/pah
Enclosures

000051

United States • Germany • Japan • France • United Kingdom
Phone 703-893-5400 Fax 301-230-2369 Telex 899436 HAZLABS VINA

HAZLETON
WASHINGTON

AMENDMENTS TO STUDY PROTOCOL

STUDY TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) With a Confirmatory Assay

LABORATORY: Hazleton Washington Inc. EFFECTIVE DATE: 07/09/91

PROTOCOL NO.: 401R, Edition 17 HWA ASSAY NO.: 14647-0-401R

Amendment #1. Part 2, Section III.A.5.c. Overlay Agar for Selection of Histidine Revertants

This section will be changed to the following:

Overlay Agar for Selection of Histidine Revertants for Vehicle Control and Test Article-Treated Plates

For the vehicle controls and the test article-treated plates, overlay (top) agar will be prepared with 1.14% agar (W/V) and 0.81% NaCl (W/V) and will be supplemented with 13.9 ml of 0.5 mM histidine/biotin solution per 100 ml agar. For an agar overlay, 1.5 ml of the supplemented top agar will be used. When S9 mix is required, 1.5 ml of the supplemented top agar will be used in the overlay. However, when S9 mix is not required, water will be added to the supplemented top agar (0.5 ml of water per 1.5 ml of supplemented top agar) and the resulting 2.0 ml of diluted supplemented top agar will be used for the overlay. This dilution ensures that the final top agar and amino acid supplement concentrations remain equal in both the presence and absence of S9.

Reason for the Amendment:

Due to the limited solubility of the test article, a plating aliquot of 1,000 µl per plate was necessary to achieve a maximum dose of 5,000 µg per plate. In order to accommodate a plating aliquot of 1,000 µl, it was necessary to concentrate the overlay agar.

Amendment #2. Part 2, Section III.A.5.d.

The following section will be added:

Overlay Agar for Selection of Histidine Revertants for Positive Control-Treated Plates

For the positive control-treated plates, overlay (top) agar was prepared with 0.7% agar (W/V) and 0.5% NaCl (W/V) and was supplemented with 10 ml of 0.5 mM histidine/biotin solution per 100 ml agar. For an

HAZLETON
WASHINGTON

AMENDMENTS TO STUDY PROTOCOL

STUDY TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) With a Confirmatory Assay

LABORATORY: Hazleton Washington Inc. EFFECTIVE DATE: 07/09/91

PROTOCOL NO.: 401R, Edition 17 HWA ASSAY NO.: 14647-0-401R

Amendment #2 (Continued)

agar overlay, 2.0 ml of the supplemented top agar is used. When S9 mix is required, 2.0 ml of the supplemented top agar is used in the overlay. However, when S9 is not required, water is added to the supplemented top agar (0.5 ml of water per 2 ml of supplemented top agar) and the resulting 2.5 ml of diluted supplemented top agar is used for the overlay. This dilution ensures that the final top agar and amino acid supplement concentrations remain the same both in the presence and absence of S9.

Reason for the Amendment:

To indicate the concentration of overlay agar to be used for the positive controls where the plating aliquot will be 50 µl.

Study Director:

11·6·91
Date

Timothy E. Lawlor, M.A.
Microbial/Mutagenesis
Department of Molecular and Cellular Toxicology

Laboratory Director:

11/6/91
Date

for Brian C. Myhr, Ph.D.
Associate Director
Department of Toxicology

HAZLETON
WASHINGTON
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

a CORNING Laboratory Services Company

MUTAGENICITY TEST ON

LECITHINASE

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST) WITH A CONFIRMATORY ASSAY

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Hazleton Washington, Inc.
5516 Nicholson Lane
Kensington, Maryland 20895

LABORATORY PROJECT ID

HWA Study No.: 14647-0-401R

SUBMITTED TO

Enzyme Bio-Systems, Ltd.
3350 Salt Creek Lane
Suite 103
Arlington, IL 60005

STUDY COMPLETION DATE

November 6, 1991

United States • Germany • Japan • France • United Kingdom
Phone 301-230-0001 Fax 301-230-2369 Telex 899436 HAZLABS VINA

HAZLETON
WASHINGTON

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) with a Confirmatory Assay

STUDY NO.: 14647-0-401R

PROTOCOL NO.: 401R EDITION NO.: 17

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection - Date	Findings Reported	Auditor
Characterization of Tester Strains - 07/26/91	07/26/91	D. Wallace
Characterization of Tester Strains - 07/31/91	07/31/91	D. Wallace
Counting of Colonies - 09/13/91	09/13/91	D. Wallace
Final Report Review - 11/05/91	11/05/91	M. Murphy

Quality Assurance Unit 11-6-91 Date Released

000055

HAZLETON
WASHINGTON

COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160) and the Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practice Standards, 59 NohSan Notification No. 3850, issued August 10, 1984. To the best of the signer's knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The stability of the test article under the conditions of administration was the responsibility of the Sponsor. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HWA Division of Molecular and Cellular Services, 5516 Nicholson Lane, Kensington, Maryland 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington, Vienna, Virginia.

SUBMITTED BY:

Study Director:

Timothy E. Lawlor, M.A.
Microbial Mutagenesis
Department of Molecular and Cellular Toxicology

11 6 91
Study Completion Date

Laboratory Director:

Brian C. Myhr, Ph.D.
Associate Director
Department of Toxicology

11/6/91
Date

For Sponsor:

Date

000056

HAZLETON
WASHINGTON

TABLE OF CONTENTS

		Page No.
I.	SUMMARY	5
II.	STUDY INFORMATION	7
III.	MATERIALS AND METHODS	9
IV.	RESULTS AND CONCLUSIONS	21
V.	DATA TABLES	24

000057

000058

8

HAZLETON
WASHINGTON
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

a CORNING Laboratory Services Company

October 9, 1991

Dr. Martin Teague
Enzyme Biosystems
3350 Salt Creek Lane
Suite 103
Arlington, IL 60005

RE: FINAL REPORT AND PROTOCOL AMENDMENT
Mouse Lymphoma Forward Mutation Assay
Protocol No.: 431R, Edition No.: 1
Genetics Assay No.: 14647
Test Material: Lecithinase

Dear. Dr. Teague:

Enclosed please find one original and two (2) copies of the above referenced report. This material has been reviewed by our Quality Assurance unit to ensure compliance with GLP requirements.

Also enclosed is protocol amendment.

If you have any further questions or comments concerning the above material, please do not hesitate to contact me.

Thank you for giving us this opportunity to work with you.

Sincerely,

HAZLETON WASHINGTON, INC.

Maria A. Cifone, Ph.D.
Cell Biologist
Department of Molecular and
Cellular Toxicology

MAC/pah
Enclosures

000059

United States • Germany • Japan • France • United Kingdom
Phone 703-893-5400 Fax 301-230-2369 Telex 899436 HAZLABS VINA

HAZLETON
WASHINGTON

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: MOUSE LYMPHOMA FORWARD MUTATION ASSAY

GENETICS PROTOCOL NO.: 431R, EDITION 1 ASSAY NO.: 14647

LABORATORY: HAZLETON WASHINGTON, INC. AMENDMENT NOS.: 1 & 2

EFFECTIVE DATE: JULY 9, 1991

The following changes were made to the study protocol:

1. Methylmethane sulfonate (MMS) positive controls at 10.0 nl/ml and 15 nl/ml will be included in the nonactivation mutation assay.

   Reason: The MMS positive control will be included to provide an internal control for evaluating the recovery of small colony mutants. MMS is known to produce a majority of small colony mutants. The historical data base with MMS is small and therefore will not be included with the historical control mutant frequency data. The change should have no adverse impact upon the integrity of the study.

2. The assay acceptance criteria for the background mutant frequency (average of vehicle control values) is changed from a range of 20×10^{-6} to 90×10^{-6} to a range of 40×10^{-6} to 120×10^{-6}.

   Reason: Since the assay has been recently optimized for the recovery of small colonies, the historical data demonstrate that the background mutant frequency should be revised upward. Future editions of this protocol will incorporate this change.

STUDY DIRECTOR'S SIGNATURE: DATE: 10/4/91

HWA MANAGEMENT'S SIGNATURE: DATE: 10/7/91

HAZLETON
WASHINGTON
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

a CORNING Laboratory Services Company

MUTAGENICITY TEST ON

LECITHINASE

IN THE L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY WITH INDEPENDENT REPEAT

FINAL REPORT

AUTHOR

MARIA A. CIFONE, PH.D.

PERFORMING LABORATORY

HAZLETON WASHINGTON, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LABORATORY PROJECT ID

HWA STUDY NO.: 14647-0-431R

SUBMITTED TO

ENZYME BIO-SYSTEMS, LTD.
3350 SALT CREEK LANE
SUITE 103
ARLINGTON, IL 60005

STUDY COMPLETION DATE

October 9, 1991

000061

United States • Germany • Japan • France • United Kingdom
Phone 301-230-0001 Fax 301-230-2369 Telex 899436 HAZ_ABS VINA

HAZLETON
WASHINGTON

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY WITH INDEPENDENT REPEAT

PROJECT NO.: 20989 ASSAY NO.: 14647

PROTOCOL NO.: 431R EDITION NO.: 1

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection/Date	Findings Reported	Auditor
Cloning/8-21-91	8-21-91	Wendell Yee
Final Report Review/ 10-9-91	10-9-91	Mary Murphy

Quality Assurance Unit

10-9-91
Date Released

000062

HAZLETON
WASHINGTON

COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR - 58, 40 CFR 792, and 40 CFR 160) the Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practice Standards, 59 NohSan Notification No. 3850, issued August 10, 1984 (effective October 1, 1984). To the best of the signers' knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HWA Division of Molecular and Cellular Services, 5516 Nicholson Lane, Kensington, Maryland, 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington, Inc., Vienna, Virginia. Copies of the raw data will be supplied to the sponsor upon request.

SUBMITTED BY:

LABORATORY SUPERVISOR:

Fariba Oveisitork, B.S. Research Assistant	10/9/91 Date

STUDY DIRECTOR:

Maria A. Cifone, Ph.D. Cell Biologist	10/9/91 Study Completion Date

HWA MANAGEMENT:

Brian C. Myhr, Ph.D. Associate Director Department of Molecular and Cellular Services	10/9/91 Date

SPONSOR'S REPRESENTATIVE:

	Date

000063

HAZLETON
WASHINGTON

TABLE OF CONTENTS

QUALITY ASSURANCE STATEMENT 2

COMPLIANCE AND CERTIFICATION STATEMENT 3

ABSTRACT 5

I. STUDY IDENTIFICATION 6
- A. Sponsor
- B. Test Article
- C. Type of Assay
- D. Protocol Number
- E. Study Dates
- F. Supervisory Personnel

II. OBJECTIVE 6

III. RATIONALE 7

IV. MATERIALS 7
- A. Indicator Cells
- B. Media
- C. Control Compounds
- D. S9 Metabolic Activation System

V. EXPERIMENTAL DESIGN 9
- A. Solvent Selection and Dosing Procedure
- B. Dose Selection
- C. Mutagenicity Testing
- D. Data Presentation
- E. Assay Acceptance Criteria
- F. Assay Evaluation Criteria

VI. RESULTS AND DISCUSSION 17
- A. Test Material Handling
- B. Rangefinding Cytotoxicity Assay
- C. Mutation Assay

VII. CONCLUSIONS 21

VIII. REFERENCES 21

IX. EXPERIMENTAL DATA TABLES 22

APPENDIX A HISTORICAL MOUSE LYMPHOMA FORWARD MUTATION ASSAY CONTROL MUTANT FREQUENCY DATA 30

HAZLETON
WASHINGTON

ABSTRACT

The objective of this in vitro assay was to evaluate the ability of Lecithinase to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. The test material formed a suspension in Fischer's medium at 5000 μg/ml. The 5000 μg/ml stock was filter sterilized. The sterile filtrate was used in the cytotoxicity and mutation assays. In the preliminary cytotoxicity assay, cells were exposed to the test material for four hours in the presence and absence of rat liver S9 metabolic activation. Under nonactivation conditions, Lecithinase was highly toxic at 2500 μg/ml and lethal at 5000 μg/ml. In the presence of S9 metabolic activation, a shift in toxicity was observed. Treatment at 313 μg/ml was moderately toxic and at the next highest dose level (625 μg/ml) and above the test material was lethal.

Three acceptable nonactivation and S9 metabolic activation mutation assays were performed using single cultures per dose level. The test material produced dose-related increases in toxicity in all mutation trials. In the three nonactivation trials, no significant increases were observed except for one small increase at excessively high toxicity. A similar effect was observed in the presence of metabolic activation. One small increase was observed at very high toxicity. These increases were not repeatable and were considered spurious. Therefore, the test material, Lecithinase, was evaluated as negative for inducing forward mutations at the TK locus in L5178Y mouse lymphoma cells under the nonactivation and S9 metabolic activation conditions used in this study.

9

HAZLETON
WASHINGTON
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

a CORNING Laboratory Services Company

October 11, 1991

Dr. Martin Teague
Enzyme Biosystems
3350 Salt Creek Lane
Suite 103
Arlington, IL 60005

RE: FINAL REPORT
Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures
With a Confirmatory Assay
Protocol No.: 447R, Edition No.: 4
Genetics Assay No.: 14647
Test Material: Lecithinase

Dear Dr. Teague:

Enclosed please find one original and two (2) copies of the above referenced report. This material has been reviewed by our Quality Assurance unit to ensure compliance with GLP requirements.

If you have any further questions or comments concerning the above material, please do not hesitate to contact me.

Thank you for giving us this opportunity to work with you.

Sincerely,

HAZLETON WASHINGTON, INC.

Marie E. McKeon, M. Phil.
Cell Biologist
Department of Molecular and
Cellular Toxicology

MEM/pah
Enclosures

000067

United States • Germany • Japan • France • United Kingdom
Phone 703-893-5400 Fax 301-230-2369 Telex 899436 HAZLABS VINA

HAZLETON
WASHINGTON
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

GENOTOXICITY TEST ON

LECITHINASE

IN THE ASSAY FOR UNSCHEDULED DNA SYNTHESIS IN RAT LIVER PRIMARY CELL CULTURES WITH A CONFIRMATORY TRIAL

FINAL REPORT

AUTHOR

Marie E. McKeon, M. Phil.

PERFORMING LABORATORY

Hazleton Washington Inc.
5516 Nicholson Lane
Kensington, Maryland 20895

LABORATORY PROJECT ID

HWA Study No.: 14647-0-447R

SUBMITTED TO

Enzyme Biosystems Ltd.
3350 Salt Creek Lane
Suite 103
Arlington Heights, Illinois 60005

STUDY COMPLETION DATE

October 11, 1991

000068

HAZLETON
WASHINGTON

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Genotoxicity Test on Lecithinase in the Assay for Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures With a Confirmatory Assay

PROJECT NO.: 20991 ASSAY NO.: 14647

PROTOCOL NO.: 447R EDITION NO.: 4

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection/Date	Findings Reported	Auditor
Scoring 8/8/91	8/8/91	L. D. Griffith
Draft Report Review 10/3/91	10/3/91	D. S. Wallace
Final Report Review 10/10/91	10/10/91	M. Murphy

Quality Assurance Unit 10-11-91 Date Released

000069

HAZLETON
WASHINGTON

COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160) and in compliance with the Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practice Standards, 59 NohSan Notification No. 3850, issued August 10, 1984 (effective October 1, 1984). To the best of the signers' knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of the HWA Division of Molecular and Cellular Toxicology, 5516 Nicholson Lane, Kensington, Maryland 20895. After two years, these records will be transferred to permanent archives at Hazleton Washington Inc., Vienna, Virginia. Copies of the raw data will be supplied to the sponsor upon request.

SUBMITTED BY:

Andrea L. Ham, B.S.
Supervisor

Date

Study Director:

Marie E. McKeon, M. Phil.
Cell Biologist
Department of Molecular and Cellular Services

10/11/91
Study Completion Date

Testing Facitlity Management:

Brian C. Myhr, Ph. D.
Associate Director of Toxicology
Department of Molecular and Cellular Services

10/11/91
Date

Sponsor Representative:

Date

000070

HAZLETON
WASHINGTON

TABLE OF CONTENTS

		Page No.
ABSTRACT		6
I.	SPONSOR	7
II.	MATERIAL TESTED	7
	A. Genetics Assay No.	
	B. Identification	
	C. Physical Description	
	D. Date Received	
III.	TYPE OF ASSAY	7
IV.	PROTOCOL NUMBER	7
V.	STUDY DATES	7
	A. Study Initiation Date	
	B. Experimental Start Date	
	C. Experimental Termination Date	
VI.	SUPERVISORY PERSONNEL	7
	A. Study Director	
	B. Supervisor	
VII.	OBJECTIVE	7
VIII.	RATIONALE	8
IX.	MATERIALS	8
	A. Indicator Cells	
	B. Medium	
	C. Controls	
X.	EXPERIMENT DESIGN	9
	A. Dosing Procedure	
	B. Dose Selection	
	C. UDS Assay	
	D. Assay Acceptance Criteria	
	E. Assay Evaluation Criteria	
XI.	INTERPRETATION OF RESULTS	14
XII.	CONCLUSIONS	17
XIII.	REFERENCES	18

HAZLETON
WASHINGTON

TABLE OF CONTENTS (CONTINUED)

XIV. EXPERIMENT DATA TABLES 19

XV. APPENDIX: HISTORICAL CONTROLS 22

HAZLETON
WASHINGTON

ABSTRACT

In the Assay for Unscheduled DNA Synthesis (UDS) in Rat Liver Primary Cell Cultures, the test material, lecithinase, did not induce significant increases in UDS in two independent trials. In each trial described in this report, freshly prepared rat hepatocytes were exposed to lecithinase at concentrations ranging from 5000 to 0.500 μg/ml in the presence of 10 μCi/ml ^{3}HTdr (45.5 Ci/mmole). In Trial 1, fifteen treatments from 5000 to 0.500 μg/ml were initiated. The test material was soluble in media at all concentrations tested and any insoluble material, observed during solubilization of the test material, was removed by filtration. Six treatments from 5000 to 500 μg/ml were not analyzed for nuclear labeling due to high toxicity. Six treatments from 250 to 5.00 μg/ml covered a good range of toxicity (72.3% to 103.2% survival) and were selected for analysis of nuclear labeling. None of the criteria used to indicate UDS was approached by the chemical treatments in Trial 1 and no dose-related response was observed. A second trial was initiated to confirm these results.

Based upon cytotoxicity information obtained in Trial 1, ten dose levels from 1000 to 1.00 μg/ml were initiated in Trial 2. Treatment at 1000 μg/ml was not analyzed due to high toxicity. Six treatments from 500 to 10.0 μg/ml covered a good range of toxicity (53.9% to 107.2% survival) and were selected for analysis of nuclear labeling. None of the criteria used to indicate UDS was approached by the chemical treatment in Trial 2. The data confirmed the results from Trial 1 and lecithinase was evaluated as inactive in both trials of the Rat Primary Hepatocyte UDS Assay.

10

STUDY SPONSOR:
Genencor International, Inc.
925 Page Mill Road
Palo Alto, California 94304
United States of America.

STUDY TITLE:
Phospholipase A2 - 13 week oral (gavage) toxicity study in the rat.

MDS PHARMA SERVICES STUDY NUMBER:
75/005.

DATE:
19 November 2003.

TABLE OF CONTENTS

Pages

GLP COMPLIANCE STATEMENT **4**

PATHOLOGIST'S AUTHENTICATION **5**

PEER REVIEWING PATHOLOGIST'S AUTHENTICATION **6**

QUALITY ASSURANCE **7**

1. SUMMARY **9**

2. INTRODUCTION **11**

3. TEST ITEM AND VEHICLE INFORMATION **14**
- 3.1. TEST ITEM 14
- 3.2. VEHICLE 14
- 3.3. TEST ITEM PREPARATION 15
- 3.4. ANALYSES 16

4. METHODS AND EXPERIMENTAL DESIGN **17**
- 4.1. TEST SYSTEM 17
- 4.2. ANIMAL HUSBANDRY 17
- 4.3. PRE-TREATMENT PROCEDURES 18
- 4.4. EXPERIMENTAL DESIGN 19
- 4.5. ADMINISTRATION OF THE TEST ITEM OR VEHICLE 20

5. OBSERVATIONS **21**
- 5.1. MORBIDITY/MORTALITY 21
- 5.2. CLINICAL SIGNS 21
- 5.3. BEHAVIOURAL AND FUNCTIONAL TESTS 21
- 5.4. OPHTHALMOLOGY 22
- 5.5. BODY WEIGHT 23
- 5.6. FOOD CONSUMPTION 23
- 5.7. CLINICAL LABORATORY DETERMINATIONS 23

6. PATHOLOGY **26**
- 6.1. NECROPSY 26
- 6.2. ORGAN WEIGHTS 26
- 6.3. TISSUES FOR COLLECTION 27
- 6.4. HISTOPATHOLOGY 29

7. PRESENTATION OF RESULTS AND ANALYSIS OF DATA **30**

8. ARCHIVES **31**

9. STUDY TIMETABLE **32**

10. STUDY PLAN ADHERENCE **33**

11. RESULTS **34**
11.1. MORTALITY 34
11.2. CLINICAL SIGNS 34
11.3. BEHAVIOURAL AND FUNCTIONAL TESTS 34
11.4. OPHTHALMOLOGY 34
11.5. BODY WEIGHT 34
11.6. FOOD CONSUMPTION 35
11.7. HAEMATOLOGY 35
11.8. SERUM CLINICAL CHEMISTRY 35
11.9. URINE ANALYSIS 35
11.10. PATHOLOGY 36

12. CONCLUSION **37**

13. TABLES AND FIGURES **38**
Table 1: CLINICAL SIGNS 39
Table 2: OPEN FIELD TEST 42
Table 3: BODY WEIGHTS 45
Figures 1 and 2: MEAN BODY WEIGHTS 50
Table 4: BODY WEIGHT GAINS 53
Table 5: FOOD CONSUMPTION 56
Figures 3 and 4: MEAN FOOD CONSUMPTION 61
Table 6: HAEMATOLOGY 64
Table 7: SERUM CLINICAL CHEMISTRY 71
Table 8: URINE ANALYSIS 76

14. APPENDICES **79**
Appendix 1: OPEN FIELD TEST 80
Appendix 2: FUNCTIONAL TESTS 89
Appendix 3: OPHTHALMOLOGY 92
Appendix 4: BODY WEIGHTS 94
Appendix 5: FOOD CONSUMPTION 103
Appendix 6: HAEMATOLOGY 112
Appendix 7: SERUM CLINICAL CHEMISTRY 125
Appendix 8: URINE ANALYSIS 134
Appendix 9: PATHOLOGY TABLES 140

15. ADDENDA **278**
Addendum 1: STUDY PLAN NO. 75/005-D 279
Addendum 2: CERTIFICATE OF ANALYSIS 296
Addendum 3: BACKGROUND CONTROL DATA 300

GLP COMPLIANCE STATEMENT

I, the undersigned, hereby declare that, unless otherwise stated, the work described in this report was performed in accordance with the following:

- "Good Laboratory Practice" described in the U.S. Federal Register (Food and Drug Administration) dated 22 December 1978 with any applicable amendments.
- "OECD Principles of Good Laboratory Practice" concerning Mutual Acceptance of Data in the Assessment of Chemicals dated 26 November 1997 (C (97) 186 Final).
- "Good Laboratory Practice Standards" described in the U.S. Federal Register (Environmental Protection Agency - [FIFRA] or [TSCA] -) dated 29 November 1983 with any applicable amendments.
  FIFRA --> Federal Insecticide, Fungicide and Rodenticide Act.
  TSCA --> Toxic Substances Control Act.
- "Principles of Good Laboratory Practice " described in the French Official Journal of 23 March 2000.

This report is a true and accurate record of the results obtained.

Changes to the dispensary software used during this study were partially validated or tested and this demonstrated that the core functions were unaltered with respect to a previously validated version. For security any concentration calculations for this study were manually verified. For these reasons the data thus generated are considered satisfactory.

The results of test item formulation analysis are considered to be the reponsibility of the Study Sponsor, and are reported separately. A summary is included in Addendum 2.

Signature: _____

Name: H. Voute

Title: Study Director

Date: 19 November 2003

PATHOLOGIST'S AUTHENTICATION

I, the undersigned was responsible for the histopathology evaluation and reporting of the pathology results and am in agreement with the interpretation of the pathology results in the report.

Signature:	
Name:	Y. Guichard
Title:	Study Pathologist
Date:	19 November 2003

PEER REVIEWING PATHOLOGIST'S AUTHENTICATION

A peer review of the unaudited draft of histopathology data was conducted by the undersigned Pathologist.

Extent of Review : All tissues from animal nos. 31 and 75.

Individual differences were discussed and, where agreed, the data were modified. Even though there were some variations on individual animal observations, the study pathologist's conclusions were confirmed.

Signature: _____

Name: P. Liberge

Title: Study Peer Reviewing Pathologist

Date: 10 November 03

QUALITY ASSURANCE

TITLE: Phospholipase A2 - 13 week oral (gavage) toxicity study in the rat.

Inspection of the study plan was made in accordance with Standard Operating Procedure AQ PROT 1. Dates for inspection of any study plan amendments, in accordance with this SOP, are not quoted.

Dates (day - month - year)		
Inspection	Report to Study Director	Report to Management
24/04/03	24/04/03	24/04/03

Inspection(s) of data generated on this study was made in accordance with Standard Operating Procedure AQ-AUD 1.

Dates (day - month - year)		
Inspection	Report to Study Director	Report to Management
05/11/03	06/11/03	06/11/03

Inspection(s) of procedures on this study was made in accordance with Standard Operating Procedure AQ-INSP 1.

Dates (day - month - year)			
Inspected phase(s)	Inspection	Report to Study Director	Report to Management
Formulation	29/04/03	13/05/03	13/05/03
Administration	29/04/03	30/04/03	30/04/03
Necropsy	29/07/03	30/07/03	30/07/03
Body weighing	20/05/03	21/05/03	21/05/03
Clinical signs	17/06/03	23/06/03	23/06/03
Food consumption	08/07/03	08/07/03	08/07/03
Gripping reflex	24/07/03	25/07/03	25/07/03
Startle reflex	24/07/03	25/07/03	25/07/03
Open field	24/07/03	25/07/03	25/07/03

Other routine procedures used in this type of study were inspected regularly and reports made in accordance with Standard Operating Procedure AQ-INSP 1.

This report has been reviewed by the Quality Assurance Department, employing methods detailed in Standard Operating Procedure AQ-RAP 1. The reported methods and procedures were found to describe those used, and the results constituted an accurate representation of recorded data. Any data supplied by or under the responsibility of the Sponsor were not subjected to review.

L. Billand.
(QA Manager)

Date: 20 November 2003

1. SUMMARY

1.1. The objective of the study was to evaluate the toxicity of the test item Phospholipase A2 following daily oral (gavage) administration to the Sprague-Dawley rat for 13 consecutive weeks.

1.2. The study was conducted according to the following design:

Group/ Treatment	Dose volume (ml/kg/day)	Dose concentrations as total proteins in mg/ml of dosing solution	Dose levels expressed as total proteins received in mg/kg b.w.(2) /day	Dose levels expressed as TOS(1) received in mg/kg b.w. (2) /day	Number of animals	
					Males	Females
1. Control	5	0	0	0	10	10
2. Low dose	5	1.15	5.75	13.7	10	10
3. Intermediate dose	5	2.3	11.5	27.4	10	10
4. High dose	5	4.6	23	54.8	10	10

(1) TOS: Total Organic Solids.
(2) b.w: body weight.
Group 1 animals (control) received the vehicle (water for injection).

1.3. Morbidity/mortality checks were performed at least twice daily. Clinical examinations were performed daily. A full clinical examination was performed weekly.

Behavioural and functional tests were performed during week 13.

Ophthalmological examination was performed pretest and during week 13.

Individual body weights were recorded weekly. Food consumption was measured weekly for each animal.

Clinical laboratory determinations were performed after week 13.

All animals were killed after week 13 and selected organs were weighed. Tissue samples were fixed and preserved at necropsy for all animals. Selected tissues from group 1 and 4 animals killed after week13 were examined histopathologically.

1.4. Results

There was no mortality, no treatment-related effects on clinical signs, behavioural and functional tests, and ophthalmological examinations.

There were no treatment-related effects on body weight, food consumption, haematological, serum clinical chemistry or urinary parameters.

There were no organ weight changes, macroscopic or microscopic findings that were considered to be related to treatment.

1.5. Conclusion

In conclusion, under the experimental conditions of the study, the test material Phospholipase A2 administered daily for 13 consecutive weeks by the oral route (gavage) to Sprague-Dawley rats at the dose levels of 5.75, 11.5 and 23 mg of total proteins/kg b.w./day (corresponding to 13.7, 27.4 and 54.8 mg of TOS/kg. b.w./day, respectively) did not induce any signs of toxicity. The NOEL can thus be established at 23 mg of total proteins/kg b.w./day (54.8 mg of TOS/kg. b.w./day for this study.

SUBMISSION END

AM

Kirkpatrick & Lockhart LLP

1800 Massachusetts Avenue, NW
Suite 200
Washington, DC 20036-1221
202.778.9000
www.kl.com

May 26, 2004

Gary L. Yingling
202.778.9124
Fax: 202.778.9100
gyingling@kl.com

Mr. Robert I. Merker
Office of Premarket Approval
Center for Food Safety & Applied Nutrition
US Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Letter from Genencor Responding to Questions on GRAS Notification

Dear Mr. Merker:

Attached is the response from Genencor International, Inc., to the questions FDA has asked as to GRAS Notification No. 145. If you have any questions, please call me.

Very truly yours,

Gary L. Yingling

Cc: Genencor International, Inc.

RECEIVED
MAY 28 2004
OFFICE OF
FOOD ADDITIVE SAFETY

000088

Genencor International, Inc.®

925 Page Mill Road
Palo Alto, California 94304
650.846.7500
650.845.6500 fax
www.genencor.com

Mr. Robert I. Merker
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

May 17, 2004

Re: GRN145: Phospholipase A2 Enzyme Preparation Derived from *Streptomyces violaceoruber*

Dear Mr. Merker:

This letter is in reference to Genencor International Inc.'s recent GRAS Notification submission (GRN145) and in response to questions regarding this submission in your telephone conversation with Mr. Gary Yingling on March 22, 2004. Below we are addressing specifically each of the three questions you have, forwarded to us by Mr. Yingling.

1. In the GRAS notice, there are references to toxicity studies. The discussion of these studies does not clearly identify the test substance used in the study. Was the lecithinase that is the subject of GRN145 actually used in those studies? What is the correct TOS?

Yes. The enzyme preparation that is the subject of GRN145 was used in all toxicity studies. This enzyme, phosphatidylcholine-2-acylhydrolase (IUB 3.1.1.4) derived from *Streptomyces violaceoruber* JAS-591, is commonly named lipase, lecithinase, and phospholipase A2. The three common names are used in different study reports and also throughout the submission. However, they are all synonymous and refer to the same enzyme which is the subject of GRN145.

The correct TOS is 1.0×10^{-4} g/U. This figure was used in the document to calculate the dietary exposure.

2. The GRAS notice does not state whether the processing aids used were those listed in the 1998 ETA's flocculants/defoamers list to the FDA. Are the processing

Genencor International, Inc.®

925 Page Mill Road
Palo Alto, California 94304
650.846.7500
650.845.6500 fax
www.genencor.com

aids listed on the 1998 ETA list or are they GRAS based on GRAS affirmation/notification or some other sources?

Any flocculants or defoamers used in the processing are on the 1998 ETA list.

3. The GRAS notice does not provide adequate discussion of allergenicity. Why is allergenicity not an issue and why is labeling not necessary?

Enzymes, as proteins, have the potential to cause allergic responses in sensitive individuals. However, the concern of allergenicity of enzymes used as processing aids has only been associated with occupational respiratory exposure to aerosolized enzymes, a worker safety issue. There have been no confirmed reports of allergies in consumers caused by ingestion of enzymes used in food processing (Pariza, M. W. and E. M. Foster. 1983. Determining the safety of enzymes used in food processing. J. Food Protect. 46:453-468), despite the vast variety of applications of these enzymes.

In addition to the safe history of use of enzymes used as processing aids in food and the absence of scientific evidence for the allergenicity of these enzymes when ingested as part of a food, enzymes are used in food processing as catalysts and thus used in very small quantities. In many cases, they are removed and/or denatured and do not become a component of the food ingredient or the final food, resulting in low exposure to the enzyme by consumers. For example, in starch processing, the processing aid enzymes are typically removed successfully during the process by repeated purification steps.

As has been well-documented, the vast majority of food-allergic consumers do not react adversely to ingestion of less than 1 mg of some major allergenic food proteins (Wensing, M. et al. 2002. The range of minimum provoking doses in hazelnut-allergic patients as determined by double-blind, placebo-controlled food challenges. Clin. Exp. Allergy 32:1757-1762; Morisset M. et al. 2003. Thresholds of clinical reactivity to milk, egg, peanut and sesame in immunoglobulin E-dependent allergies: evaluation by double-blind or single-blind placebo-controlled oral challenges. Clin. Exp. Allergy 33:1046-1051). Compared to this threshold dose for known, potent allergenic foods, the allergy risk due to consumption of enzymes with no known allergenic potential when used as processing aids is considered negligible since the use level is well below this reported level.

Finally, in their history of use in food applications, microbial lipases have generated no known allergy concerns. Specifically, phospholipase A2 derived from *S. violaceoruber* is not derived from, nor related to, known food allergens identified by the FDA. In GRN 145, we noted that the maximum potential human dietary exposure is calculated as 0.026 mg TOS/kg/day. Since 10% of TOS is the enzyme protein, the maximum total human dietary exposure is (assuming the average body weight of an adult is 60 kg):

Genencor International, Inc.®

925 Page Mill Road
Palo Alto, California 94304
650.846.7500
650.845.6500 fax
www.genencor.com

0.026 mg TOS/kg/day x 10% x 60 kg = 0.156 mg enzyme protein/day

This amount is well below the threshold of 1 mg food protein for adverse allergic responses in humans for known food allergens as discussed above.

Based on (1) the history of safe use of enzyme preparations including lipases in the food industry, (2) the absence of scientific evidence linking the phospholipase A2 from *S. violaceoruber* to know food allergens, and (3) the low estimated dietary exposure as a result of the enzyme preparation use as a processing aid, it is Genencor's view that the lipase enzyme preparation derived from *S. violaceoruber* JAS-591 poses a low risk of causing food allergy in consumers and, therefore, that allergy labeling is not necessary for this enzyme preparation.

We believe that the information provided herein is fully responsive to the questions you have asked. If you have questions regarding this information, please contact me at 650-846-7625 or email cblumenthal@genencor.com.

Sincerely

Cynthia Blumenthal
Specialist, Regulatory Affairs