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December 18, 2003

03-12-24P009338PAIN

Linda S. Kahl, Ph.D.
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Kahl,

We are hereby submitting, in triplicate, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' phospholipase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding a phospholipase A1 from *Fusarium venenatum*. The phospholipase enzyme preparation is intended for use in the dairy industry.

Please contact me by direct telephone at 919 494-3151 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

Lori Gregg
Regulatory Specialist

Enclosures (3 binders)

Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Tel: 919-494-3000 *Fax: 919-494-3450* *Internet: www.novozymes.com*

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03-12-24A11:55 RCVD

December 17, 2003

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RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R.§ 170.36 (c)(1) Novozymes North America Inc. hereby claims that phospolipase preparations produced by submerged fermentation of *Aspergillus oryzae* expressing the gene encoding a phospholipase A1 from *Fusarium venenatum* are Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

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

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

Novozymes North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

Phospholipase enzyme preparation from *Aspergillus oryzae* expressing the gene encoding a phospholipase A1from *Fusarium venenatum.*

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use.*

The phospholipase is intended for use in the dairy industry as a processing aid in the production of cheese. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

Proposed §170.36 (c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

17 December 2003
Date

John Carroll
Director, Regulatory Affairs

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Luna No. 2003-41536-01

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A phospholipase preparation produced by *Aspergillus oryzae* expressing the gene encoding a phospholipase A1 from *Fusarium venenatum*

Lori Gregg, Regulatory Affairs, Novozymes North America, Inc., USA
Peter Hvass, Regulatory Affairs, Novozymes A/S, Denmark

December 2003

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TABLE OF CONTENTS

		PAGE
1.	**GENERAL INTRODUCTION**	3
2.	**PRODUCTION MICROORGANISM**	4
2.1	Production Strain	4
2.2	Recipient Organism	4
2.3	Phospholipase Expression Plasmid	5
2.4	Stability of the Introduced Genetic Sequences	7
2.5	Antibiotic Resistance Gene	7
2.6	Absence of the Production Organism in Product	7
3.	**MANUFACTURING PROCESS**	7
3.1	Raw Materials	7
3.2	Fermentation Process	7
3.3	Recovery Process	8
3.4	Quality Control of Finished Product	9
4.	**ENZYME IDENTITY**	9
5.	**COMPOSITION AND SPECIFICATIONS**	9
5.1	Quantitative Composition	10
5.2	Specifications	10
6.	**APPLICATION**	11
6.1	Mode of Action	11
6.2	Use Levels	11
6.3	Enzyme Residues in the Final Food	11
7.	**SAFETY EVALUATION**	11
7.1	Safety of the Production Strain	11
7.2	Safety of the Donor Organism	16
7.3	Safety of the Phospholipase Enzyme	16
7.4	Safety of the Manufacturing Process	17
7.5	Safety Studies	18
7.6	Estimates of Human Consumption and Safety Margin	18
7.7	Results and Conclusion	20
8.	**LIST OF APPENDICES**	21
9.	**LIST OF REFERENCES**	22

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1. GENERAL INTRODUCTION

The subject of this notification is a phospholipase preparation produced by submerged fermentation of an *Aspergillus oryzae* microorganism carrying the gene coding for phospholipase from *Fusarium venenatum.* The Novozymes A/S trade name used for the phospholipase preparation is NOVOZYM® 46016. The enzyme preparation is intended to be used as a processing aid in the dairy industry mainly in the manufacture of cheese.

The enzyme is a phospholipase A1 (EC 3.1.1.32) which specifically acts on the fatty acid in position 1 of phospholipids. NOVOZYM 46016 is used in the Dairy industry as a processing aid during cheese production, where it is added to the cheesemilk. The modified phospholipids in the cheesemilk have improved emulsifying properties. Phospholipases including the A1 activity are found in most cells and tissues including animal tissues that are consumed by man (See section 7.3).

The information provided in the following sections is the basis for our determination of general recognition of safety of this phospholipase enzyme preparation produced by *A. oryzae* expressing a gene encoding a phospholipase from *F. venenatum.* Our safety evaluation in Section 7 includes an evaluation of the production strain including an evaluation of safe strain lineage, the donor strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

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 (Ref. 1, 2). The production organism for this phospholipase, *A. oryzae*, is discussed in Sections 2 and 7. The production organism is derived from an *A. oryzae* strain that has been used for many years to manufacture enzymes for use in food. Also, Novozymes has used A. oryzae strain 1560 for many years to develop improved strains by genetic modification. In section 7.1.1, we show the basis for a safe strain lineage for this production strain following the procedure outlined in Pariza and Johnson 2001 (Appendix 1).

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material (Ref. 3-8). The genetic modifications used to construct the production microorganism are well defined and are described in Section 2. The recipient strain, *A. oryzae* BECh2, is the same strain that was used in the construction of Novozymes' production strains for a modified lipase and a glucose oxidase, which are the subjects of GRN Nos. 103 and 106, respectively. Safety studies including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay, were performed on those preparations. Except for the coding sequence for the enzyme itself, the DNA that was introduced into the recipient strain to construct the phospholipase production strain is essentially the same as the introduced DNA in the GRN 103 production strain. Novozymes also has unpublished

toxicological data (comprising of a 13 week acute oral toxicity in rats, an Ames test, and an In vitro human lymphocyte cytogenetic assay) on a phytase preparation and a xylanase preparation produced by an A. oryzae strain constructed from A. oryzae BECh2. These studies support the view that strains derived from *A. oryzae* BECh2 can be safely used to manufacture food enzymes. Furthermore, Novozymes has published and unpublished toxicological data on related strains of *A.* oryzae used in the manufacture of food enzymes (see section 7.1.1). All of these studies confirm the safety of *A. oryzae* as a production organism for food enzymes.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *A. oryzae* strain, designated PFJo142, was constructed by plasmid transformation of the recipient strain, designated BECh 2 (see Section 2.2). This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (Ref. 9). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (Ref. 2) and several expert groups (Ref. 1, 3-8).

The phospholipase expression plasmid, pPFJo142 used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include: a gene encoding an *F. venenatum* phospholipase enzyme; an *Aspergillus nidulans* selectable marker gene, *amd*S (acetamidase) (Ref. 10); well-characterized noncoding regulatory sequences including the *A. niger* terminator (Ref. 11), the *A. niger* neutral amylase II (NA2) promoter (Ref. 12), and *A. nidulans* triose phosphate isomerase gene (Ref. 13); a 1163 bp fragment from the *Escherichia coli* cloning vector pUC19 (Ref. 14), and the *Saccharomyces cerevisiae* URA3 (Ref. 15) promoter (Pura3), coding sequence (URA3) and terminator (Tura3).

2.2 Recipient Organism

The recipient microorganism, designated BECh 2, used in the construction of the phospholipase production strain is an amylase negative, alkaline protease (alp) negative, neutral metalloprotease I (NpI) negative, cyclopiazonic acid deficient, kojic acid impaired derivative of the fully characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. The strain was obtained from the Institute for Fermentation, Osaka, Japan (IFO) and is designated strain IFO 4177 (synonym A1560). This classification of A1560 as *A. oryzae* has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland (Ref. 16).

The host strain, *A.oryzae* BECh 2, was constructed from A1560 through the following steps:

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1) **HowB101**, a *pyrG* mutant of *A. oryzae* A1560, was obtained by homologous recombination with linearized DNA from the 5' and 3' flanking sequences of the the *A. oryzae pyrG* gene.

2) **HowB424** was obtained by transformation of HowB101 with linear DNA, containing the entire *pyrG* gene inserted into the *A. oryzae* TAKA-amylase gene, followed by selection for uridine auxotrophy. HowB424 was selected because it showed no secretion of TAKA-amylase.

3) **HowB425**, a spontaneous *pyrG* mutant of HowB424, was isolated by plating of spores on agar plates containing 5-fluoro-orotic acid (5-FOA).

4) **JaL142** was obtained by transformation of HowB425 with linear DNA, containing the entire *pyrG* gene inserted into the *A. oryzae* alkaline protease gene, and selection for uridine auxotrophs.

5) **JaL151**, a spontaneous *pyrG* mutant of JaL142, was isolated by plating of spores on agar plates containing 5-fluoro-orotic acid (5-FOA).

6) **JaL228** obtained by transformation of JaL151 with linear DNA, containing the entire *pyrG* gene inserted into the *A. oryzae* neutral metalloprotease I gene, and selection for uridine auxotrophs.

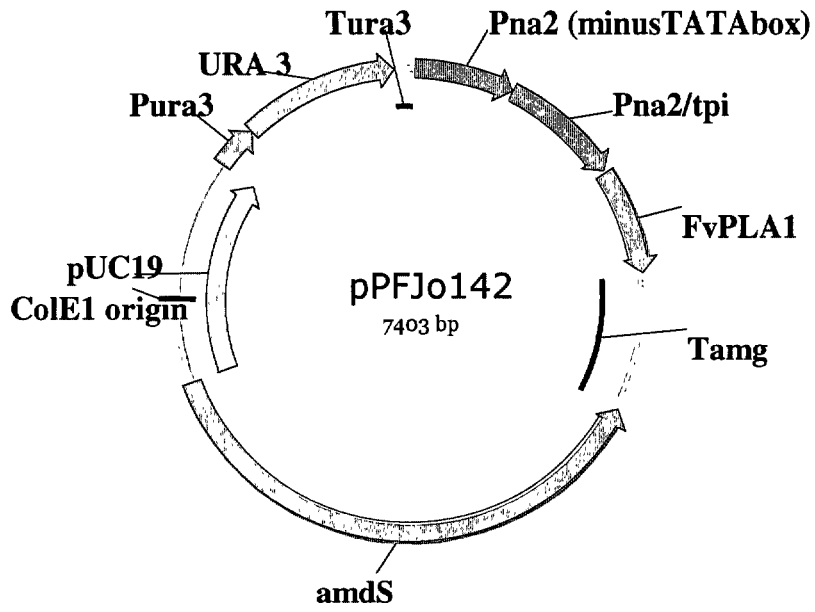
7) **BECh 1** obtained by γ irradiation of spores of JaL228 and screening of a mutant deficient in cyclopiazonic acid synthesis.

8) **BECh 2** obtained by U.V irradiation of spores of BECh 1 and screening of a mutant deficient in Kojic acid synthesis

Only *A. oryzae* DNA was transformed into the *A. oryzae* A1560 during the development of the host strain BECh2, so only homologous gene manipulations have been applied.

2.3 Phospholipase Expression Plasmid

The 7403 bp expression plasmid, pPFJo142, used to transform the *A. oryzae* host strain, BECh 2, contains the gene elements outlined below. This expression plasmid is essentially the same as the expression plasmid, pCaHj559, used in construction of the production strain for the modified lipase, which is the subject of GRN No. 103. The only difference is the use of the double promoter in pPFJo142 and the coding sequence of the enzyme itself.



Position (bp)	Size (bp)	Element	Origin
1-11	11	Linker	Synthetic
12-519	508	Pna2 (minusTATAbox)	*A. niger* BO1
520-525	6	Linker	Synthetic
526-1139	614	Pna2/TPI	*A. niger* BO1
1140-1148	9	Linker	Synthetic
1149-1700	552	FvPLA1	*F. venenatum*
1701-1708	8	Linker	Synthetic
1709-2407	699	Tamg	*A. niger* BO1
2408-5132	2725	amdS	*A. nidulans*
5133-6295	1163	pUC19	*E. coli*
6296-6518	223	Pura3	*S. cerevisiae*
6519-7319	801	URA3	*S. cerevisiae*
7320-7403	84	Tura3	*S. cerevisiae*

Pna2 (minusTATAbox) is the neutral amylase II promoter from *Aspergillus niger*, where the TATA box has been excluded.
Pna2/TPI is the neutral amylase II promoter from *Aspergillus niger*. The 5' nontranslated part of this promoter has been replaced with the 5' nontranslated part of the *Aspergillus nidulans* triose phosphate isomerase (TPI) promoter (position 1073-1139).
FvPLA1 is the phospholipase from *Fusarium venenatum*.
Tamg is the amyloglycosidase terminator of *Aspergillus niger*.
amdS is the acetamide gene (including promoter and terminator) from *A. nidulans*.
pUC19 is a fragment of the pUC19 vector including the origin of replication. The origin of replication initiation site (colE1 origin) is in position 5397.
Pura3, URA3 and Tura 3 are the *S. cerevisiae* URA3 promoter, coding sequence and terminator respectively.

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2.4 Stability of the Introduced Genetic Sequences

The presence of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism (Ref. 3-8). The transforming DNA is stably integrated into the *A. oryzae* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable (Ref. 9).

2.5 Antibiotic Resistance Gene

The introduced DNA does not contain antibiotic resistance genes.

2.6 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC (Ref. 3) is satisfactorily addressed.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for the phospholipase which follows standard industry practices (Ref. 17-19). The quality management system used in the manufacturing process for the phospholipase complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the phospholipase enzyme concentrate are standard ingredients used in the enzyme industry (Ref. 17-19). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

The maximum use level of antifoams in the phospholipase product is less than 1%.

3.2 Fermentation Process

The phospholipase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *A. oryzae* described in Section 2.

All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

3.2.1 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *A. oryzae,* described in section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

3.2.2 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermentor and the main fermentor before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1. Infection is observed in 2 or more samples by microscopy

2. Infection is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

3.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both the purification and the formulation processes.

3.3.1 Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1. Pretreatment - pH adjustment

2. Primary Separation - vacuum drum filtration

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3. Concentration - ultrafiltration and/or evaporation

4. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation

5. Preservation and Stabilization of the liquid enzyme concentrate

6. Final concentration – evaporation and/or ultrafiltration if enzyme concentration is too low to reach target yield

3.3.2 Formulation and Standardization Processes

The stabilized concentrate is blended with water, glycerol, sucrose and preserved with sodium benzoate and potassium sorbate. The product is standardized according to the product specification.

3.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in section 5.

4. ENZYME IDENTITY

Key enzyme and protein chemical characteristics of the phospholipase are given below:

Classification	Phospholipase A1 (generic name)
IUB nomenclature:	Phosphatidylcholine 1-acylhydrolase
IUB No.:	3.1.1.32
CAS No.:	9043-29-2
Specificity:	hydrolyses the sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipid and free fatty acid.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

The phospholipase enzyme preparation is presently available in a formula for use in dairy applications. The Novozymes A/S trade name used for the phospholipase preparation is NOVOZYM® 46016.

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5.1 Quantitative Composition

NOVOZYM 46016 has the following typical composition:

Enzyme solids (TOS)	approx.	1 %
Water	approx.	55 %
Glycerol	approx.	35 %
Sucrose	approx.	7.5 %
Ash (mainly Sodium chloride)	approx.	1 %
Sodium benzoate	approx.	0.2 %
Potassium sorbate	approx.	0.2 %

5.2 Specifications

NOVOZYM 46016 complies with the purity criteria recommended for enzyme preparations as described in Food Chemicals Codex, 4th edition, 3rd supplement, 2001 (Ref. 20). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (Ref 21).

The following Novozymes' specifications have been established for the phospholipase:

Enzyme activity	according to declaration
Heavy metals	not more than 30 ppm
Lead	not more than 5 ppm
Arsenic	not more than 3 ppm
Total viable count/g	not more than 1×10^4
Total coliforms/g	not more than 30
Enteropathogenic *E. coli*/25 g	negative by test
Salmonella/25 g	negative by test
Antibiotic activity	negative by test
Production organism	negative by test
Mycotoxins	negative by test

Antibiotic activity, and relevant mycotoxins are analyzed at regular intervals.

NOVOZYM 46016 has a typical activity of 1000 LEU/g. The enzyme activity (LEU) is measured relatively to a porcine pancreas phospholipase standard. The phospholipase hydrolyzes lecithin under constant pH and temperature. The activity is determined as the rate of titrant (0.1N NaOH) consumption during neutralization of the liberated fatty acid.

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6. APPLICATION

6.1 Mode of Action

The active enzyme in NOVOZYM 46016 is a phospholipase (EC 3.1.1.32), which specifically acts on the fatty acid in position 1 in phospholipid substrates. It catalyzes the hydrolysis of sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipid and free fatty acid.

NOVOZYM 46016 is used in the Dairy industry as a processing aid during cheese production, where it is added to the cheesemilk and allowed to act before the addition of coagulant. The modified phospholipids in the cheesemilk have improved emulsifying properties and as such result in better production efficiency by keeping more of the original milk components in the cheese and reducing the loss of fat and other solids into the whey stream.

6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP.

For cheese production, the recommended dosage of NOVOZYM 46016 is up to 5 g per kg milkfat corresponding to 5 LEU/g milkfat. If the cheesemilk contains 3.5 % fat this corresponds to 175 LEU/liter cheese milk or 17.5 g NOVOZYM 46016 per 100 liter cheesemilk.

6.3 Enzyme Residues in the Final Food

The enzyme is added to the cheesemilk and allowed to act before the addition of the coagulant. After the coagulation of the cheese, the majority of the enzyme is drained off with the whey stream, which is pasteurized causing an inactivation of the enzyme activity in the whey. A minor part of the enzyme remains in the cheese, where it has no effect due to physical hindrance in the solid cheese matrix and lack of substrate.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

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 (Ref. 1,2). 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 (Ref. 3). 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". *A. oryzae* meets these criteria for nontoxigenicity and nonpathogenicity. In addition, *A. oryzae* is not considered pathogenic by JECFA (Ref. 22).

Barbesgaard et al. reviewed the safety of *A. oryzae* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food grade enzymes (Ref. 23; Appendix 2). *A. oryzae* is accepted as a constituent of foods (Ref. 22). *A. oryzae* has been used to produce soy sauce in the United States since before 1958 (Ref. 3, 23). Therefore, food ingredients from *A. oryzae* meet the criterion of "common use in foods in the US before 1958" and can be considered "generally recognized as safe", GRAS (Ref.3). A GRAS petition, 3G0016, proposing affirmation that enzyme preparations from *A. oryzae* are GRAS for use in food was submitted to FDA and accepted for filing in 1973 (Ref. 24). Since that time, Novozymes and other companies have marketed enzyme preparations from *A. oryzae* in the US as GRAS substances. Carbohydrase and protease enzymes from *A.* oryzae are now the subject of GRAS notice No. 90. Therefore, enzyme preparations from *A. oryzae* are also considered GRAS (Ref. 2,3,22).

An evaluation of the genetically modified production microorganism for the phospholipase, embodying the concepts initially outlined by Pariza and Foster, 1983 and further developed by IFBC in 1990, the EU SCF in 1991, the OECD in 1992, ILSI Europe Novel Food Task Force in 1996, FAO/WHO in 1996, JECFA in 1998 and Pariza and Johnson, 2001 demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Section 2.

Because the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the phospholipase enzyme preparation derived from the genetically modified *A.oryzae* is considered safe (Ref. 3, 25).

7.1.1 Safe Strain Lineage

The safety of this *A. oryzae* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (Ref. 1, 3). The host strain, *A. oryzae* BECh2, has been thoroughly characterized as shown in sections 2.2. The introduced DNA is well-known and characterized in section 2.3. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (Ref. 1) have been met.

Novozymes' used the decision tree in Pariza and Johnson 2001 (Ref.1) as a basis for our safety assessment. The production strain is genetically modified by rDNA techniques as discussed in section 2. The expressed enzyme product is a phosholipase (See section 7.3). The enzyme preparation is free of transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is randomly integrated into the chromosome, however, the production strain is well characterized by qualified scientists and technicians. Novozymes has extensive experience working with *A. oryzae* production strains and has developed expertise in identifying and characterizing these strains in order to prevent contamination and ensure continuing acceptable, economic yields of a functional enzyme product. Research scientists, fermentation engineers, chemical operators, and quality control technicians follow standard aseptic microbiological procedures as well as specific Novozymes protocols for monitoring the biological activity, growth, and physiological characteristics of the production organism during strain improvement programs and during large scale industrial fermentations. In addition, the final commercial enzyme product must perform reproducibly, meet Novozymes' technical service department requirements, and consistently meet the needs of customers in the food industry. All of these periodic and continuous monitoring activities serve not only to guarantee customer satisfaction with Novozymes' enzyme products but also indicate that no unexpected secondary effects of the genetic modifications have been observed.

Finally, the production strain is derived from a safe lineage. Novozymes has used *A.oryzae* production strains for over 30 years. Table 2 below outlines some of Novozymes' products produced by *A. oryzae* production strains and the safety studies conducted on those products. We have published safety studies on two products produced from *A. oryzae* strains developed from *A. oryzae* A1560 (Ref. 26, 27). We have unpublished data on several other products produced by *A. oryzae*.

The recipient strain, *A. oryzae* BECh2, for this phospholipase is the same strain that was used in the construction of Novozymes' production strains for a modified lipase and a glucose oxidase, which are the subjects of GRN Nos. 103 and 106, respectively. Safety studies including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay, were performed on those preparations (Appendix 3 and 4). Except for the coding sequence for the enzyme itself, the DNA that was introduced into the recipient strain to construct the phospholipase production strain is essentially the same as the introduced DNA in the GRN 103 production strain. Novozymes also has unpublished toxicological data on a phytase preparation and a xylanase preparation produced by an A. oryzae strain constructed from A. oryzae BECh2. All of these studies concluded that the test preparations did not exhibit any toxic or mutagenic effect under the conditions of the test. These studies support the view, that strains derived form *A. oryzae* BECh2 can be used safely for the production of food enzymes. The same well known procedures have been used to construct this *A. oryzae* production strain. Therefore, we would not expect to see any negative results in similar toxicological studies.

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Table 2. Novozymes' products derived from *A.oryzae* strains

Enzyme	IUB no	Host strain[1]	Donor strain	Safety studies[2]	Published Safety Studies
Triacylglycerol lipase (GRN No. 43)	3.1.1.3	*Aspergillus oryzae* (A 1560)	*Humicola (now Thermomyces) lanuginosa*	Yes	Yes (Ref. 26)
Mucorpepsin (GRN No. 34)	3.4.23.23	*Aspergillus oryzae* (A 1560)	*Rhizomucor miehei*	Yes	No
Triacylglycerol lipase (GRASP 7G0323)	3.1.1.3	*Aspergillus oryzae* (A 1560)	*Rhizomucor miehei*	Yes	Yes (Ref. 27)
Triacylglycerol lipase (GRN No. 75)	3.1.1.3	*Aspergillus oryzae* (JaL 228)	*Fusarium oxysporum*	Yes	No
6-phytase	3.1.1.26	*Aspergillus oryzae* (JaL 228)	*Peniophora lycii*	Yes	No
Xylanase	3.2.1.8	*Aspergillus oryzae* (BECh2)	*Thermomyces lanuginosus*	Yes	No
Glucose oxidase (GRN No. 106)	1.1.3.4	*Aspergillus oryzae* (BECh2)	*Aspergillus niger*	Yes	No
Triacylglycerol lipase (GRN No. 103)	3.1.1.3	*Aspergillus oryzae* (BECh2)	*Thermomyces lanuginosus/ Fusarium oxysporum*	Yes	No

[1] Development of the host strain, A. oryzae BECh2, from the wild type:

A. oryzae (A 1560) — wild type strain
|
A. oryzae (JaL 228) — alp-, amy-, Npl-
|
A. oryzae (BECh 2) — alp-, amy-, Npl-, CPA-, KA deficient

Gene deletions:
Alp- : alkaline protease
Amy- : alpha-amylase
Npl- : neutral metallo protease I
CPA- : Cycloplazonic acid

Gene mutation:
KA- : Kojic acid

[2]At least the following: 13 week acute oral toxicity in rats; Ames test; In vitro human lymphocyte cytogenetic assay

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For this phospholipase, we have conducted an In vitro cytotoxicity test and an Ames test (as discussed in section 7.5). The conclusion of these tests is that the test preparation is considered non-cytotoxic and does not induce gene mutations in bacteria under the conditions of the study.

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson 2001 (Ref. 1) to evaluate the enzymes derived from *A. oryzae* production strains. The evaluations of two of the enzymes from the same host strain, BECh2, as that used in the construction of the production strain for this phospholipase enzyme have been documented in GRAS notifications to the FDA. Therefore, following the evaluation outlined in this section this production strain is considered to be derived from a safe lineage and is safe for use in the production of enzyme preparations for use in food.

7.1.2 Secondary Metabolites

A. oryzae is known to have the potential to produce the secondary metabolites, cyclopiazonic acid (CPA), 3 (β)-nitropropionic acid (NPA) and kojic acid (KA). Novozymes' has shown that the whole aflatoxin gene cluster and the genes involved in CPA biosynthesis have been deleted from this host strain, *A. oryzae* BECh2.

One batch of phospholipase was analyzed for secondary metabolites. The results are shown in Table 1.

Table 1. Secondary Metabolite Analyses

	Unit	PPW 22837
Aflatoxin B1	mg/kg	ND (<0.0002)
Ochratoxin A	mg/kg	ND (<0.001)
Sterigmatocystin	mg/kg	ND (<0.02)
T-2 toxin	mg/kg	ND (<0.02)
Zearalenon	mg/kg	ND (<0.02)
Cyclopiazonic acid	mg/kg	ND (<0.6)
Kojic Acid	mg/kg	ND (<6)
3-Nitropropionic acid	mg/kg	ND (<13)

ND = Not Detected (limit of detection is given in the brackets).

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Based on information presented in this section as well as safety assessments conducted on CPA, KA, and NPA (Ref. 28-30), it is concluded that the production strain, *A. oryzae* PFJo42, does not produce secondary metabolites of toxicological concern to humans.

7.2 Safety of the Donor Organism

The donor for the phospholipase enzyme is *Fusarium venenatum*. *F. venenatum* is a saprophytic fungus found in soil and is also known to occur on *Humulus lupulus, Solanum tuberosum, Spinacia oleracea, Triticum aestivum, Zea mays* (Ref. 31). *F. venenatum* is not considered a human pathogen (Ref. 32). *F. venenatum* is known to produce various secondary metabolites such as the trichothecenes, the culmorins, the enniatins and the fusarins.

However, the pathogenic and toxigenic potential of the particular strain of *F. venenatum* used as donor for the phospholipase gene should not be of particular importance to the safety of our phospholipase enzyme preparation (Ref. 3). Pariza and Johnson (Ref.1) confirm that the safety of the production strain is the primary concern in evaluating enzyme safety. The primary issue in evaluating the safety of a production strain is its toxigenic potential. It is important to note that the only *F. venenatum* DNA introduced into the *Aspergillus oryzae* production organism is the protein coding sequence from the *F. venenatum* phospholipase gene contained in the phospholipase expression plasmid. No *F. venenatum* genetic sequences related to secondary metabolite production or pathogenic properties were introduced into the *A. oryzae* production microorganism. The steps used during strain construction ensure that the incorporated *F. venenatum* DNA is limited solely to the phospholipase coding sequence. Therefore, following the concepts outlined in Pariza and Johnson (2001) (Ref.1), we can conclude that all new DNA introduced into the *A. oryzae* production strain is safe and we have not changed the strain's toxigenic potential.

7.3 Safety of the Phospholipase Enzyme

A wide variety of enzymes are used in food processing (Ref 1). Enzyme proteins do not generally raise safety concerns (Ref. 2, 33, 34). Exceptions could include enzymes that produce substances that are not ordinarily digested and metabolized or that produce toxic substances (Ref.35). Pariza and Foster (Ref. 2) note that very few toxic agents have enzymatic properties. As indicated in section 4, the subject of this GRAS notification is a phospholipase A1 (PLA1), EC 3.1.1.32. Phospholipases are classified as hydrolases which are a group of enzymes that hydrolyze various bonds. Phospholipases, like other hydrolases used in food processing, breakdown substrates into smaller units that do not have toxic properties and that are readily metabolized by the human body. As noted above in Section 4, PLA1 enzymes hydrolyze the sn-1 ester bond of diacylphospholipids to form 2-acyl-1-

lysophospholipid and free fatty acid. The existence of enzymes that catalyze the release of fatty acids from phospholipids was proposed over 100 years ago (Ref. 36).

Phospholipase A1 is found in animal tissues (Ref. 36-38) and plant tissues (Ref. 39). It has been found in many cells and tissues of various organisms (Ref. 40) including animal pancreas and small intestines (Ref. 37, 38). Phospholipases are part of the digestive enzymes present in pancreatic juice of mammals including humans (Ref. 41). FDA concluded that phospholipase A2 should be digested like any other protein in food (Ref. 41). Because the PLA1 activity is also found in most cells and tissues including animal tissues that are consumed by man, we would also conclude that PLA1 should be digested like any other protein in food.

Animal derived lipases have been affirmed as GRAS substances (21 CFR§184.1415). These lipases are derived from porcine or bovine pancreas. Also animal derived phospholipase A2 has been safely used to manufacture enzyme modified lecithin (Ref. 41). It is very likely that these preparations contain PLA1 but it was not detected as some studies indicate that PLA1 activity can be difficult to distinguish from other lipolytic activities (Ref. 38).

It is known that some lipases have phospholipase activity (act upon the fatty acid in position 1 of a phospholipid) under certain conditions (Ref. 38, 40). *Staphylococcus hyicus* lipase (Ref. 42) and *Pseudomonas pseudoalcaligenes* lipase have high activity towards phospholipids (Ref. 43) as well as triglycerides. *Thermomyces lanuginosus* lipase have no activity towards phospholipids, however, it can obtain an phospholipid hydrolyzing activity if one to several amino acids near the active site are replaced by other amino acid (Ref. 44). *Rhizopus delemar* lipase has some activity against phospholipids. Immobilized *Rhizomucor miehei* (previously *Mucor miehei*) lipase hydrolyzed phosphatidylcholine in water-saturated hexane (Ref. 45) and synthesized sn1-acyl lysophosphatidic acid from phosphatidic acid and fatty acid in a solvent free system (Ref. 46).

Novozymes has two commercial lipases that exhibit this phospholipase activity under certain conditions. Novozymes' lipase preparation from *Aspergillus oryzae* carrying the gene encoding lipase from *Fusarium venenatum* (which is the subject of GRN 75) and our lipase preparation produced by *Aspergillus oryzae* expressing a gene encoding a modified/hybrid lipase from *Thermomyces lanuginosus/Fusarium oxysporum* (which is the subject of GRN 103) both show activity towards the fatty acid in position 1 of phospholipid substrates.

Based on the information above, Novozymes concludes that phospholipase A1 enzymes have a history of safe use in food.

7.4 Safety of the Manufacturing Process

The phospholipase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food

Chemicals Codex. As described in Section 3, the glucose oxidase 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 (Ref. 17-19).

7.5 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the phospholipase.

7.5.1 Description of Test Material

The safety studies described below were conducted on a liquid phospholipase enzyme concentrate that was prepared according to the description given in Section 3 except that stabilization and standardization were omitted.

7.5.2 Studies

The following studies were performed:

-Test for mutagenic activity (Ames test)
-*In vitro* Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture

These tests are described in Appendix 5. The conclusion of these tests is that the test preparation is considered non-cytotoxic and does not induce gene mutations in bacteria under the conditions of the study.

7.6 Estimates of Human Consumption and Safety Margin

As stated in section 6, NOVOZYM 46016 is used in the Dairy industry as a processing aid during cheese production. The enzyme activity is largely removed and heat inactivated during the cheese production process, however, in order to illustrate a "worst case" situation, the following calculation is made assuming that all enzyme activity is retained in the cheese.

NOVOZYM 46016 has a typical activity of 1000 LEU/g and an approximate content of 1% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

7.6.1 Estimates of human consumption

The average human intake of cheese is estimated using well-established statistics from various countries. Data from International Dairy Federation, Document 175, and EEC Dairy Facts & Figures 1988, indicate that the annual consumption per capita of rennet cheese is in the range of 10-20 kg.

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United Kingdom:

The Ministry of Agriculture, Fisheries and Food: 1987 Annual Report of the National Food Survey Committee, Household Food Consumption and Expenditure: Consumption of cheese per person per day is 17 g.

Denmark:

"Levnedsmiddelstyrelsen": Development of Food Consumption in Denmark, 1955-1990, Description of the Danish Diet based on food statistics and nutrition calculated data: Consumption of cheese per person per day is 40 g.

USA:

USDA Nationwide Food Consumption Survey 1977-1978: Consumption of cheese per person per day is 15 g.

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

a) The calculation is made assuming that all TOS originating from NOVOZYM 46016 remains in the cheese. NOVOZYM 46016 contains 1% TOS.

b) It is assumed that all cheese is produced using NOVOZYM 46016 as a processing aid during the production process, and that it is used at the highest recommended dosage.

The maximum recommended dosage of NOVOZYM 46016 is 5 g per kg milkfat, corresponding to 17.5 g NOVOZYM 46016 per 100 liter cheesemilk containing 3.5% fat. 100 liter cheesemilk typically gives 10 kg of cheese. Cheese therefore contains max. 0.0175 mg TOS per g.

Based on the highest average daily intake per person of cheese (40 g), this corresponds to an intake of 40 x 0.0175 = 0.7 mg TOS per person per day. For an average person weighing 60 kg this corresponds to 1.2×10^{-5} g TOS/ kg body weight per day.

7.6.2 Safety margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 13 weeks oral toxicity studies in rats for similar enzyme preparations from *A. oryzae* BECh2 strains was the highest dosages possible, approximately 1 g TOS/kg/day.

The estimated human consumption is 1.2×10^{-5} g TOS/kg/day

The safety margin can thus be calculated to be: $1/1.2 \times 10^{-5} = 8.3 \times 10^{4}$

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7.7 Results and Conclusion

On the basis of the evaluation contained in Section 7, a review of the published literature, the history of use of *A. oryzae,* and the limited and well defined nature of the genetic modifications, the phospholipase enzyme preparation can be safely manufactured and used as a processing aid in the dairy industry as well as in other food or non-food applications.

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4. Summary of Toxicity Data (File 2002-03562-01) from GRN 103, A lipase preparation produced by *Aspergillus oryzae* expressing a gene encoding a modified/hybrid lipase from *Thermomyces lanuginosus/Fusarium oxysporum.*

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Appendix 1

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Appendix

3

Toxicology

Date: 15 Jan 2002
File: 2002-00377-02
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SUMMARY OF TOXICITY DATA

Gluzyme GMM from *Aspergillus niger* expressed in *Aspergillus oryzae*

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CONTENTS

PAGE

ABSTRACT 3

1. INTRODUCTION 3

2. TEST SUBSTANCE 3
   2.1 Characterisation 3
   2.2 Production micro-organism 4

3. STUDY SUMMARIES 4
   3.1 General toxicity 4
      3.1.1 13-weeks Subchronic Oral Toxicity in Rat 4
   3.2 Mutagenicity 5
      3.2.1 Assessment of gene mutations in bacteria 5
      3.2.2 *In vitro* cytogenetic test – cultured human lymphocytes 6
   3.3 Cytotoxicity 7
      3.3.1 Neutral Red Uptake 7

4. CONCLUSION 8

5. REFERENCES 9
   5.1 Studies 9
   5.2 Guidelines 9

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ABSTRACT

Gluzyme™ (Batch No. PPX 7029) is a liquid enzyme concentrate, in which the main activity is glucose oxidase (GOX) activity with a minor catalase side activity. It is produced by submerged fermentation of a strain of *Aspergillus oryzae*, containing the glucose oxidase gene originating from *Aspergillus niger*.

Toxicological tests were undertaken to evaluate the safety of this enzyme preparation with respect to general toxicity, cytotoxicty and mutagenicity. The main conclusions can be summarised as follows :

Oral administration to rats of up to 10 ml/kg body weight/day in males and 3 ml/kg body weight/day in females (equivalent to 1135 and 340 mg Total Organic Solids/kg/day, respectively) for 13 weeks did not revealed any significant toxic effects attributable to the test substance Glyzyme.

Gluzyme has shown no *in vitro* mutagenic activity either at the gene level in bacteria or chromosome level in human lymphocytes.

Gluzyme has shown no cytotoxic activity in the Neutral Red Uptake (NRU) assay applying the mouse fibroblast cell line L929 as test system.

It is overall concluded that Gluzyme can be considered safe for its intended use.

1. INTRODUCTION

In order to register Gluzyme for use in food processing, this glucose oxidase preparation was investigated with respect to general toxicity, cytotoxicity and mutagenicity. The results of these studies are summarized in this paper.

The studies were carried out at Inveresk (Eye, Scotland), Covance (Harrogate, North Yorkshire, England), and Novozymes A/S (DK-2880 Bagsværd, Denmark) during the period March 2001 to December 2001.

All studies, except NRU, were carried out in accordance with current guidelines of the Organisation for Economic Cooperation and Development (OECD) and they were conducted in compliance with the principles of Good Laboratory Practice (OECD, as revised in 1997).

2. TEST SUBSTANCE

2.1 Characterisation

Gluzyme, batch PPX 7029, which was applied in the present toxicological investigations, was a mixture of four separate fermentation sub-batches. They were fermented and recovered according to the same procedures as are used for the production of commercial Gluzyme preparations. The product complies with the Food and Agriculture Organization/World Health organization (FAO/WHO), Joint Expert Committee on Food Additives (JECFA) and Food Chemical Codex (FCC) recommended purity specifications for food grade enzymes.

The principal enzyme is a glucose oxidase (ß-D-Glucose : oxygen -1-oxidoreductase, E.C. 1.1.3.4). Glucose oxidase oxidizes ß-D-Glucose in the presence of oxygen to form gluconolactone and hydrogen peroxide. The enzyme activity is measured in an assay based on the oxidation of glucose under constant standard conditions. One glucose oxidase unit (GODUF) is the amount of

enzyme which produces 1 µmol hydrogen peroxide per minute under standard conditions using the FIA method.

The preparation appears as a brown liquid with a specific density of 1.061 g/ml. It is completely miscible with water. The molecular weight is 89 kD and the specific activity is 260 GODU(F)/mg enzyme protein.

This batch has been analyzed for chemical and microbial content; and all the analytical results were within the set limits.

The main enzyme activity was measured to be 4790 GODU(F)/g with an amount of 10.7 (w/w) % Total Organic Solids (TOS = 100% - % water - % ash). The pH was measured to 6.8.

Analyses of the antibiotic activity and microbial contamination showed that this batch complies with the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1992) and Food Chemical Codex (FCC IV, 1996).

2.2 Production micro-organism

Gluzyme is produced by a genetically modified strain of *Aspergillus oryzae*. The glucose oxidase gene originates from *Aspergillus niger.* This genetically modified production strain meets the criteria for a safe production micro-organism. It is constructed by common transformation procedures using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are not known to encode or express any harmful or toxic substances. The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure no unintended sequences were incorporated.

A. oryzae has a long history of safe use and it has been used since the beginning of the last century in the production of enzymes, and in the past decades as recombinant organism for production of a variety of bio-industrial products.

A. oryzae is generally regarded as non-pathogenic and non-toxigenic. Certain strains may produce one or more of the secondary metabolites cyclopiazonic acid, kojic acid and ß-nitropropionic acid. The toxicity of these metabolites is low to moderate and the production is tied both to strain specificity and culture conditions. The amounts of these metabolites produced during industrial fermentation processes are small, and there are no reports that their production has resulted in adverse effects on human health.

Gluzyme, batch PPX 7029, has been analysed for the presence of aflatoxin B_1, ochratoxin A, sterigmatocystin, T2 toxin and zearalenone as well as cyclopiazonic acid, kojic acid and ß-nitropropionic acid. None of these mycotoxins were detected in significant amounts. The present production strain does not contain any resistance markers and the test article does not contain the production strain.

3. STUDY SUMMARIES

3.1 General toxicity

3.1.1 13-weeks Subchronic Oral Toxicity in Rat

Inveresk Research Group, Inveresk Report Number No. 20161, Novozymes reference number 20016004 : Gluzyme, PPX 7029. 13-week Toxicity Study including Neurotoxicity Screening in Rats with administration by Gavage..

The study was performed in accordance with the OECD Guideline for Testing of Chemicals no 408 (adopted 1998).

Three groups of 10 male and 10 female (Low, Intermediate and High dose groups) Sprague-Dawley rats were dosed orally by gavage once daily at levels 1, 3 and 10 $ml.kg^{-1}.day^{-1}$ (equivalent to 5082, 15247 and 50822 $GODU\text{-}F.kg^{-1}.day^{-1}$ or 113.5, 340.6 and 1135.3 mg $TOS.kg^{-1}.day^{-1}$). A similar constituted group received the vehicle (sterile water for irrigation) and served as the control. The dose formulations for group 2 and 3 where prepared by diluting the undiluted test substance with vehicle to the appropriate concentration (w/v). The dose volume was 10 $ml.kg^{-1}.day^{-1}$. The rats were dosed for at least 91 days and until the day before necropsy.

No dose-related deaths occurred. There were no treatment related clinical observations and the male and female treated up to dosages of 10.0 ml/kg/day showed no evidence of neurotoxicity during the Functional Observation Battery procedures.

Bodyweight gain, food consumption and the efficiency of food utilisation were in males unaffected by the treatment. In the females however a very slight decrease in food consumption and an associated, non-statistically significant, but consistent decrease in body weight/body weight gain was observed in High dose females.

There was no treatment-related ocular. There was no treatment related alteration in the haematological or biochemical composition of the blood. Organ weights were unaffected by treatment. There were no findings at necropsy or after histopathological evaluation that were considered to be due to administration of Gluzyme.

The No Effect Level (NOEL) was considered to be 10 $ml.kg^{-1}.day^{-1}$ for males and 3 $ml.kg^{-1}.day^{-1}$ for females due to the slight decrease in food consumption and associated a very slight decrease in food consumption and associated, non-statistically significant, but consistent decrease in body weight/body weight gain in High dose females. 10 $ml.kg^{-1}.day^{-1}$ for males and 3 $ml.kg^{-1}.day^{-1}$ for females corresponds to 1135.3 and 340.6 mg TOS $kg^{-1}.day^{-1}$ equivalent to 50822 and 15247 and GODU-F $kg^{-1}.day^{-1}$, respectively.

3.2 Mutagenicity

3.2.1 Assessment of gene mutations in bacteria

Novozymes A/S Report No.: 20018029. (file nr. 2001-09756-01). Gluzyme (batch No. PPX 7029): Test for mutagenic activity with strains of Salmonella typhimurium and Escherichia coli. June 2001.

Gluzyme, batch PPX 7029, was examined in order to determine the ability to induce gene mutations in strains of *Salmonella typhimurium* and *Escherichia coli.*

Four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA) were applied in this study.

Crude enzyme preparations contain free amino acids such as histidine and tryptophan. Gluzyme™, batch No. PPX 7029, significantly supported growth of the histidine-requiring *S. typhimurium* strains by direct incorporation of the test substance on selective agar media. Therefore, in order to avoid the risk of artefacts due to growth stimulation, a "treat and plate" assay was applied with all Salmonella strains. Bacterial cultures were exposed to Gluzyme™, solvent and appropriate positive controls in phosphate buffered nutrient broth for three hours at 37°C. After this period, all nutrients originating from the test substance and broth were removed by centrifugation of the bacterial suspensions.

The growth stimulation of the tryptophan-requiring *E. coli* strain was only weak and insignificant. Therefore, this part of the study was conducted by direct plate incorporation.

Further Gluzyme causes pronounced cytotoxicity and dose related increases of induced mutations, when a plate incorporation assay as well as a liquid culture assay ("treat and plate") is applied. The principal enzyme activity of Gluzyme is a glucose oxidase. In the presence of glucose hydrogen peroxide is produced by the catalytic action of glucose oxidase. Hydrogen peroxide is a well known cytotoxic and mutagenic compound in vitro. Therefore, in this study the glucose oxidase was inactivated by heat treatment for 30 minutes at 60°C at pH 2 and subsequent adjustment to neutral pH.

The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 471 (1997) concerning the general specifications of the test. However, the exposure of the test bacteria in liquid culture ("treat and plate"), as applied in this study with strains of Salmonella, is not specifically described in any guidelines. The treat and plate assay is recommended by JMHW (1984) when highly toxic compounds, e.g. antibiotics, are being assayed for bacterial mutagenicity, and by UKEMS (1990) when feeding effects are suspected and if the test substance is of biological origin (e.g. foodstuff or biological fluid).

The study was carried out with and without a metabolic activation system - a liver preparation from male rats pre-treated with Aroclor 1254, and the co-factors required for mixed function oxidase activity (S9 mix). The correct genotypes of all bacterial test strains used were checked.

Two independent and identical experiments were performed. All bacterial strains were exposed to serial dilutions of Gluzyme™, solvent (sterile deionised water), and positive controls. The final concentrations of the test article achieved were 5.0, 2.5, 1.25, 0.625, 0.313, and 0.156 mg (dry matter) per ml (*S. typhimurium)* or per plate (*E. coli*).

The number of revertants per plate was determined by triplicate plating at each dose on selective agar. The number of viable bacteria in each culture was determined by plate count.

No dose-related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to Gluzyme™, batch PPX 7029, either in the presence or absence of S-9 mix.

The sensitivity of the individual bacterial strains and the metabolising potential of the S-9 mix were confirmed in both studies by significant increases in number of revertant colonies induced by diagnostic mutagens under similar conditions.

It was concluded, that Gluzyme™, batch PPX 7029, did not induce gene mutations in bacteria in either the absence or presence of S-9 mix, when tested under the conditions employed in these studies.

3.2.2 *In vitro* cytogenetic test – cultured human lymphocytes

Covance Laboratories Limited Report No.1974/5-D6172. Novozymes reference No.20016017 Gluzyme: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocyte. October 2000.

The effects on chromosomal structure of exposure to Gluzyme™, batch PPX 7029, were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (1995).

Heparinised whole blood cultures from three female donors were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.

Two independent experiments were performed both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from animals induced with Aroclor.

Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls (–S-9: 4-Nitroquinoline 1-oxide, +S-9: Cyclophosphamide). Treatments with Gluzyme™ covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5376 µg/ml, which was in excess of the recommended maximum concentration for chromosome aberration studies according to current regulatory guidelines.

The lymphocyte cultures were exposed to the test substance for three hours and cells were harvested 17 hours later. In the second experiment treatment in the absence of S-9 was continuous for 20 hours. 9. The test article dose levels for chromosome analysis were selected by evaluating the effect of Gluzyme™ on mitotic index.

Chromosome aberrations were analysed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment, metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from Gluzyme™ treatments and negative controls. Slides were scored blind and aberrations classified according to the scheme described by ISCN (ISCN 1985).

The proportion of cells with structural aberrations in all cultures of the solvent controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with structural aberrations, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.

In the first experiment the highest concentration of Gluzyme™ chosen for analysis, 5376 µg/mL, produced approximately 32% and 0% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9, respectively. In the second experiment the highest concentration chosen for analysis, 2109 µg/mL and 5000 µg/mL, produced approximately 49% and 0% mitotic inhibition in the absence and presence of S-9, respectively.

Treatment of cultures with Gluzyme in the absence and presence of S-9 (both experiments) resulted in numbers of cells with structural aberrations which were similar to those observed in concurrent vehicle controls in the majority of cases. Exceptions to this were observed at the intermediate dose analysed (4301 mg/ml) for the pulse 3 hour +S-9 treatment in Experiment 1 and for the highest dose analysed (5376 mg/ml) for the pulse 3 hour -S-9 treatment in Experiment 1. In both cases one replicate culture had numbers of aberrant cells (excluding gaps) that exceeded the historical negative control (normal) range. However, these increases were small, were absent from the replicate cultures, not dose related and not reproduced between experiments. As such, these increases were considered spurious and of no biological importance.

Normal frequencies of cells with numerical aberrations were seen under all treatment conditions.

It was concluded that Gluzyme™, batch PPX 7029, under the conditions of test, did not induce chromosome aberrations in cultured human blood lymphocytes in either the absence or presence of S-9.

3.3 Cytotoxicity

3.3.1 Neutral Red Uptake

Novozymes A/S Study summary No.: 20018027. (file nr. 2001-16652-01). Gluzyme (batch No. PPX 7029): In vitro Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture. October2001.

The purpose of this study was to screen for the cytotoxic potential of the enzyme preparation.

The neutral red uptake assay is a quantitative, colorimetric method to measure the cell viability. Neutral red is actively taken up by the cells and retained in the lysosomes/endosomes. The amount of neutral red taken up by the cells after exposure to the test substance is an indication of the number of viable cells and thus provides a measure of general toxicity. The basis of the present test system is that a cytotoxic substance regardless of site or mechanism of action will interfere with the viability and growth of the continuously dividing fibroblasts and, thus, result in a reduction of the cell number. The degree of inhibition of growth, related to the concentration of the test substance, provides an indication of toxicity.

The test system L929 is an established mouse fibroblast cell line. It was selected for the ease with which these cells are maintained and grown as monolayer culture and it is commonly used as first order test system for general cytotoxicity.

L929 was grown in EMEM with 10% foetal calf serum (FCS). 96-well microplates were added 150 µl (5 x 105 cells per ml) cell culture per well. Plates were incubated for 24 hours at 37°C, establishing a near confluent monolayer. The test substance was dosed in five concentration (30,000, 10,000, 3,000, 1,000 and 300 µg neat test substance per ml growth medium (EMEM 10% FCS)) to accurately determine the cytotoxicity in this model. SDS in different concentrations (120, 100, 80 µg per ml growth medium) was used as concurrent positive controls. The exposure time was fixed to 24 hours.

The concentration of Gluzyme that was required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU_{50}). The NRU_{50} value for Gluzyme, batch PPX 7029, was estimated to be >30 mg/ml. The positive control, SDS, met the acceptance criteria of a valid test.

The results shown in this report indicate, that the sample of Gluzyme, batch PPX 7029, is non-cytotoxic *in vitro* in the Neutral Red Uptake assay applying the mouse fibroblast cell line L929 as test system.

The study was performed according to the Good Laboratory Practice Regulations described in OECD Principles of GLP, Dok. C (81) 30 (final), Paris, France (1981), but the summary report cited is a preliminary report before audit from the QA department has been finalized.

4. CONCLUSION

Based on the toxicological data summarised above and the fact that the production strain has a safe industrial history in use, it is concluded that Gluzyme can be considered safe for food processing.

5. REFERENCES

5.1 Studies

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Covance Laboratories Limited Report No.1974/5-D6172. Novozymes reference No.20016017 Gluzyme: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocyte. October 2000.

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5.2 Guidelines

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Appendix

24

Toxicology

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SUMMARY OF TOXICITY DATA

Lipase from *Thermomyces lanuginosus* expressed in *Aspergillus oryzae*

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CONTENTS

PAGE

1. Abstract 3

2. Test Substance 3

   2.1 Production of Test Substance 3
   2.2 Characterisation 3
   2.3 Production Microorganism 4

3. Toxicity Data 4

   3.1. Subacute Toxicity 4

   3.1.1 Toxicity Study by Oral Administration to CD Rats for 2 Weeks 4

   3.2 Subchronic Toxicity 5

   3.2.1 Toxicity Study by Oral Administration to CD rats for13 Weeks 5

   3.3 Mutagenic Potential 6

   3.3.1 Bacterial Mutation Assay (Ames Test) 6
   3.3.2 Chromosome Aberration Assay 6

4. Conclusion 7

5. References 8

   5.1 Study Reports 8
   5.2 Guidelines 8

Last page 8

1. ABSTRACT

Lipase, PPW 7023, is a liquid enzyme concentrate. It is produced by submerged fermentation of a strain of *Aspergillus oryzae*, containing the gene code of a protein-engineered variant of a fungal lipase originating from *Thermomyces lanuginosus*

In the following, toxicity studies carried out with Lipase are summarised.

All studies were carried out in accordance with current EU and OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were carried out at Huntingdon Life Sciences or at Covance Laboratories, England during the period January 2001 to November 2001.

The main conclusions of the safety studies can be summarised as follows:

Oral administration of Lipase, PPW 7023 to CD rats at dosages of up to 10.0 mL Lipase/kg bw/day (equivalent to 111615 LENU/kg bw/day or 193 KLU/kg bw/day or 1.020 g TOS/kg bw/day) for two weeks was well tolerated and produced no toxicological significant findings.

Oral administration of Lipase, PPW 7023 to CD rats at dosages up to 10.0 mL/kg body weight/day (equivalent to 193466 LU/kg/day, 111615 LENU/kg/day or 1.020 g TOS/kg/day) for 13 weeks was well-tolerated and did not produce any toxicological significant changes. Consequently, and this dose was considered to be the No-Observed-Adverse-Effect Level (NOAEL) in this study.

Lipase has shown no mutagenic activity in either Ames' test or the chromosome aberration test.

2. TEST SUBSTANCE

2.1 Production of Test Substance

Lipase, PPW 7023, is a liquid enzyme concentrate. The principal enzyme activity is a triacylglycerol acylhydrolase (EC 3.1.1.3), which cleaves the esterbonds in position 1 and 3 of triglycerides. It is a protein-engineered variant of a wild-type lipase from a strain of *Thermomyces lanuginosus*. This enzyme is expressed in a strain of *Aspergillus oryzae* and the product is produced by submerged fermentation and recovered by purification/ concentration of the fermented culture broth.
The Lipase batch, PPW 7023, used for the present toxicological programme, was a mixture of 3 identically produced sub-batches.

2.2 Characterisation

Batch PPW 7023, a dark brown liquid was used for all the studies.

Table 1. Characterisation of Lipase, PPW 7023

	Batch No. PPW 7023
Activity, LENU/g	10500
Activity KLU/g	18.2
Water (KF)	86.9%
Dry matter	13.1%
Ash (600°C)	3.5%
Total Organic Solids (TOS[1])	9.6%
Specific gravity (g/mL)	1.063

[1] %TOS = 100%- % water- % ash- % diluents

2.3 Production Microorganism

Lipase is produced by a strain of *Aspergillus oryzae*. This genetically modified production strain meets the criteria for a safe production microorganism. It is constructed by common transformation procedures using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are not known to encode or express any harmful or toxic substances. The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure no unintended sequences were incorporated.

A. oryzae is generally regarded as non-pathogenic and non-toxigenic. This species has long history of safe use and it has been used since the beginning of this century in the production of enzymes, and in the last decade as recombinant organism for production of a variety of bio-industrial products.
In the present host strain the ability to produce the secondary metabolites cyclopiazonic acid and Kojic acid has been deleted. Further analysis demonstrated the absence of the mycotoxins Aflatoxin B1, Sterigmatosystin, Ochratoxin A, Zearalenone, and T-2 toxin in each of the sub-batches which constitute PPW 7023.

The test substance does not contain this production strain. Absence of the production strain is part of the complete specification of the product.

3. TOXICITY DATA

3.1 Subacute Toxicity

3.1.1 Toxicity Study by Oral Administration to CD Rats for 2 Weeks

A subacute toxicity study in rats with a duration of two weeks served as a dose range finding study to the 13-week study.

Three groups each of five male and five female CD rats received Lipase, PPW 7023 orally by gavage, at dosages of 1.0, 3.3 or 10.0 mL/kg bw/day (equivalent to 11162, 36833 or 111615 LENU/kg bw/day, or 19, 64 or 193 KLU/kg bw/day or 0.102, 0.337 or 1.020 g TOS/kg bw/day) for two weeks. A similarly constituted group received the vehicle (purified water obtained by reverse osmosis) and served as the negative control.

At the end of 2 weeks treatment, all animals were killed and subjected to necropsy.

There were no signs related to treatment and no animals died prematurely. Bodyweight, food consumption and food conversion efficiency were unaffected by treatment with Lipase, PPW 7023. There were no treatment-related haematological and biochemical changes and no treatment-related organ weight or macroscopic changes after two weeks of treatment.

It was concluded that oral administration of Lipase, PPW 7023 to CD rats at dosages of up to 10.0 mL/kg bw/day for two weeks was well tolerated and produced no toxicological significant findings. Consequently, dosages up to 10.0 mL/kg bw/day (equivalent to 111615 LENU/kg bw/day or 193 KLU/kg bw/day or 1.020 g TOS/kg bw/day) are considered appropriate for use in a 13 week toxicity study in this strain of rat.

3.2. Subchronic Toxicity

3.2.1 Toxicity Study by Oral Administration to CD rats for 13 Weeks

The study was carried out in accordance with the OECD guideline 408 (as revised in 1997). It was conducted in accordance with Good Laboratory Practice.
Three groups of 10 male and 10 female CD rats were dosed Lipase, PPW 7023 by gavage at dosages of 1.0, 3.3 or 10.0 mL/kg bw/day. This corresponds to 19347, 63844 or 193466 LU/kg/day, 11162, 36833 or 111615 LENU/kg/day or 0.102, 0.337 or 1.020 g TOS/kg/day. A similar constituted group received the vehicle (purified water obtained by reverse osmosis) and served as the negative control.

Analysis of achieved concentration was performed on samples taken once during weeks 1 and 13. Achieved concentration was evaluated by measurement of enzyme activity, LU/g.

There were no signs related to treatment. Bodyweight gain, food consumption and food conversion efficiency were unaffected by treatment. There were no treatment-related ocular findings. The functional observation battery procedures did not indicate any changes that were attributable to treatment. There were no treatment-related haematological changes. Treatment-related changes in plasma during Week 13 were confined to slightly low cholesterol concentration in males receiving 10.0 ml/kg/day. The changes were not associated with any histopathological changes and were therefore not considered to be of toxicological significance. There were no treatment-related organ weight differences, macroscopic or histopathological changes after 13 weeks of treatment. The results from the content check analysis showed that the measured activity was as expected in week 1 and 13.

It was concluded that the oral administration of Lipase, PPW 7023 to CD rats at dosages up to 10.0 mL/kg body weight/day for 13 weeks was well-tolerated and did not produce any toxicological significant changes. Consequently, 10.0 mL/kg/day day (equivalent to 193466 LU/kg/day, 111615 LENU/kg/day or 1.020 g TOS/kg/day) was considered to be the No-Observed-Adverse-Effect Level (NOAEL) in this study.

3.3. Mutagenic Potential

3.3.1. Bacterial Mutation Assay (Ames Test)

Lipase was examined in a bacterial reverse mutation assay in accordance with OECD Guideline for Testing of Chemicals No. 471 (1997) in order to determine the ability to induce gene mutations in bacteria.

Four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA) were applied in this study.

Lipase (PPW 7023) significantly support growth of the histidine-requiring *S.typhimurium* strains by direct incorporation of the test substance on selective agar media. In order to avoid the risk of artefacts due to growth stimulation, a "treat and plate" assay was applied. Bacterial cultures were exposed to Lipase, solvent and appropriate positive controls in phosphate buffered nutrient broth for three hours at 37°C. After this period, all nutrients originating from the test substance and broth were removed by centrifugation of the bacterial suspensions.
The part of the study comprising the *E.coli* strain was conducted by direct plate incorporation.

Two independent mutation tests were performed in the presence and absence of an activating system derived from rat liver (S9 mix). All bacterial strains were exposed to serial dilutions of Lipase and the final concentrations of the test article achieved were 5.0, 2.5, 1.25, 0.625, 0.313, and 0.156 mg per ml (*S.typhimurium)* or per plate (*E.coli*).
The number of revertants per plate was determined by triplicate plating at each dose on selective agar. The number of viable bacteria in each culture was determined by plate count.
The sensitivity of the individual bacterial strains and the metabolising potential of the S-9 mix were confirmed in both studies by significant increases in number of revertant colonies induced by diagnostic mutagens under similar conditions.

No dose-related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to Lipase, either in the presence or absence of S-9 mix.

It was concluded, that Lipase, PPW 7023, did not induce gene mutations in bacteria in either the absence or presence of S-9 mix, when tested under the conditions employed in these studies.

3.4.2 Chromosome Aberration Assay

The effects on chromosomal structure of exposure to Lipase were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997).

Heparinized whole blood cultures, pooled from male donors, were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.
Two independent experiments were performed both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction

(S-9) from animals induced with Aroclor. Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls (–S-9: 4-Nitroquinoline 1-oxide, +S-9: Cyclophosphamide). Treatments with Lipase covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5000 µg/mL. The lymphocyte cultures were exposed to the test substance for three hours and cells were harvested 17 hours later. The second experiment included a continuous exposure for 20 hours in the absence of S-9. The test article dose levels for chromosome analysis were selected by evaluating the effect of Lipase on mitotic index.
Chromosome aberrations were analysed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from Lipase treatments and negative controls. Slides were scored blind and aberrations recorded according to international classifications.

The proportion of cells with structural aberrations in all cultures of the solvent controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with structural aberrations, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed. In the first experiment the highest concentration of Lipase chosen for analysis, 5000 µg/mL, produced approximately 3% and 1% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9, respectively. In the second experiment the highest concentration chosen for analysis, 5000 µg/mL produced approximately 0% and 22% mitotic inhibition in the absence and presence of S-9, respectively.

Cells treated with Lipase, either in the absence and presence of S-9, had similar numbers of aberrations to those observed in concurrent solvent controls. The one exception to this was a marginal increase in only one of duplicate cultures observed at the highest dose level in the 1. experiment in the absence of S-9. This increase was small and neither reproduced between replicate cultures nor in the 2. experiment. Therefore it was considered spurious and of no biological significance. Beyond this no reproducible increases in aberration frequency that were significantly higher than those observed in the negative controls or that fell outside the historical negative control ranges.
Normal frequencies of cells with numerical aberrations were seen under all treatment conditions.

Lipase (Batch No. PPW 7023), under the conditions of test, did not induce chromosome aberrations in cultured human blood lymphocytes when tested to a concentration of 5000 µg/mL in either the absence or presence of S-9.

4. CONCLUSION

The results of the toxicological studies outlined above did not reveal any adverse effect of Lipase, PPW 7023. Based on these toxicological data and the fact that the production strain reportedly has a history of safe use, it is our conclusion that there are no reasons for safety concerns when using this Lipase as a food additive. In our opinion Lipase can be considered generally recognized as safe.

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Appendix

5

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Safety & Toxicology

Date : 16 December 2003
Ref.: SGE
File : 2003-41475-01
Proj: NL2 00331

To : PHva, LRHe,
From : SGE
Copy : MSi/HRoh, QAD

Re : Phospholipase, batch PPW 22837
***In Vitro* Cytotoxicity Test:**
Neutral Red Uptake in L929 Monolayer Culture
Study No. 20038074
Study summary.

CONCLUSION

On the basis of the results of the present study, Phospholipase, batch PPW 22837, was considered non-cytotoxic.

BACKGROUND

The purpose of this study was to evaluate the cytotoxic potential of Phospholipase, batch PPW 22837. The study was conducted in compliance with the current GLP regulations

METHOD

The mouse fibroblast cell line L929 was used as a test system. Time of exposure for the test substance and positive control was 24 hours. The following concentrations were selected for Phospholipase, batch PPW 22837, for an accurate determination of cytotoxicity in this test model: 30 - 10 - 3 - 1 and 0.3 mg test substance per ml growth medium. SDS was used as a positive control and the following concentrations were selected: 120 – 100 and 80 µg/ml growth medium. The concentration of the test substance required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU_{50}).

RESULTS

The NRU_{50} value for the Phospholipase, batch PPW 22837, was estimated to be >30 mg/ml. The positive control, SDS, met the acceptance criteria of a valid test.

DISTRIBUTION

This report will be distributed to: PHva, MSi.

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MEMO

Safety and Toxicology

Date : 2003-11-28
File : 2003-35730-01
Ref.: PBjP

To : PHva, SGE
From : PBjP

Report : Phospholipase (Batch No. PPW 22837): Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*.
Study number: 20038075

PURPOSE

This report describes experiments, performed to assess the mutagenic activity of Phospholipase, PPW 22837 in amino acid dependent strains of *Salmonella typhimurium* and *Escherichia coli* capable of detecting both induced frame-shift (TA1537 and TA98) and base-pair substitution mutations (TA1535, TA100 and WP2uvrA).

GUIDELINES

The study was conducted in accordance with OECD Guideline for testing of chemicals, No. 471: Bacterial Reverse Mutation Assay" (July 1997), European commission Annex V. Test Method B14, and the Japanese guideline: "Concrete Operation Procedure of Mutagenicity Study Using Bacteria, Ministry of Labour, 1988", concerning the general specifications of the test. However, an additional confirmatory experiment was not included.
The exposure of test bacteria in liquid culture ("treat and plate"), as it is applied in this study with strains of Salmonella, is not specifically described in any guidelines.

The study was conducted in compliance with current GLP-regulations.

METHODS

The study was carried out as described in the standard operating procedures: TOX-SM-0809.01: Bakterielle mutagenicitets tests: *Salmonella typhimurium* his$^+$ reversions assay, "Treat and plate proceduren, and TOX-SM-0806.01: Bakterielle mutagenicitets tests: *Escherichia coli WP2* reversions assay, "Standard plade inkorporations metoden".

The mutagenic activity was examined in four histidine-dependent strains of *Salmonella typhimurium*, strains TA98, TA100, TA1535 and TA1537 using the "treat and plate" procedure and the tryptophan-dependent strain *Escherichia coli* WP2uvrA, using the direct plate incorporation method.

The study was conducted in the presence and absence of an activating system derived from rat liver (S9 mix). All tests included solvent (purified water) and positive controls with and without S9 mix.

Bacteria were exposed to 6 doses separated with bi-sections. The highest dose level applied was 5 mg per ml with *Salmonella* strains and 5 mg per plate with the *E.coli* strain.

RESULTS

Treatment of bacteria with Phospholipase in the absence and the presence of S9 did not result in significant dose related and reproducible increases in mutation levels compared to those seen in concurrent solvent controls. Based on the viable counts in the "treat and plate" assay the viability of test bacteria was reduced by the test substance in some of the test series with the Salmonella strains, most pronounced when the metabolising system was included. This effect was without any obvious dose relation and apparently without significant influence on the revertant colony counts.

CONCLUSION

It is concluded that Phospholipase, batch PPW 22837, did not induce gene mutations in bacteria in either the absence or presence of S9, when tested under the conditions employed in this study.

DISTRIBUTION

The report is in preparation.

Date	Peder Bjarne Pedersen Study director

SUBMISSION END

Luna No. 2004-18445-01

AM

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May 7, 2004

Richard E. Bonnette
Division of Biotechnology and
GRAS Notice Review, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

RE: GRAS Notice No. GRN 000142

Dear Mr. Bonnette,

In response to the phone conversation on March 19, 2004, Novozymes would like to take this opportunity to clarify some information on our phospholipase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding a phospholipase A1 from *Fusarium venenatum,* the subject of GRN No. 142.

In the phone conversation we discussed 3 areas as follows:

1) Are the antifoams and/or flocculants used in the manufacture of the enzyme preparation used in accordance with ETA's April 10, 1998 submission to FDA?
2) What is the rationale for classifying the enzyme as a processing aid? Does it have a functioning effect in the final food?
3) Has Novozymes' considered the allergenic potential of the enzyme?

Antifoams and Flocculants

The antifoams used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. There are no flocculants used in the manufacture of this enzyme preparation.

Enzyme Application and Function

As discussed in the GRAS notification and described specifically in Section 6, this phospholipase is used as a processing aid in the dairy industry, specifically during cheese production. The phospholipase is added to the milk to hydrolyze phospholipids in the milk. The modified phospholipids result in a milk with improved emulsion characteristics. The improved characteristics of the milk allow for a more

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efficient coagulation process when the clotting enzymes are added to the milk to set the milk into a semi-solid form ("curds"). Because the emulsion is more stable, more of the original milk components are kept in the cheese matrix as it coagulates, reducing the loss of fat and other solids in the whey stream.

Also as presented in Section 6.3 of the notification, the enzyme performs its function in the milk before the clotting enzymes are added to the cheese. The enzyme is not performing a function in the final cheese product, mainly because the enzyme mediated reaction quickly consumes nearly all of the available phospholipid substrate. The majority of the enzyme is then drained off with the whey stream, which is pasteurized causing an inactivation of the enzyme activity in the whey. Any enzyme that remains in the solid fraction still cannot perform a function because, as mentioned above, there is no substrate left for the enzyme to act upon. Also, even if any substrate remains, it is now unavailable to the enzyme because it is occluded by the solid cheese matrix.

Novozymes conducted analyses to confirm that the phospholipase reaction stops due to lack of substrate. The phospholipids in milk were measured. After addition of phospholipase, more than 95% of the target phospholipids were hydrolyzed within 20 minutes. There was no further reaction after 2.5 hours.

Because enzymes are specific, only a very small amount of enzyme is required to achieve the intended effect in the milk. Therefore, any phospholipase present in the final cheese product, would be present at very insignificant levels and would not perform a function in the final product. This is why we have described the use of the enzyme as a processing aid in the manufacture of cheese.

Consideration of the allergenic potential of the phospholipase enzyme when used as indicated

Enzymes are proteins and proteins can be potential allergens. However, only a small percentage of all dietary proteins are allergens. Novozymes is not aware of any allergic reactions caused by the ingestion of phospholipase. Novozymes' believes that, in general, the risk of allergenicity due to ingestion of enzymes is negligible based on the following considerations:

- the long history of safe use of a wide variety of enzymes in food and food processing
- the ubiquitous nature of enzymes
- the extremely low exposure potential
- the purpose

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As discussed in Section 7.3, enzyme proteins do not generally raise safety concerns. A wide variety of enzymes have been used safely for centuries in food processing. Enzymes, including phospholipase enzymes, are found in many cells and tissues of plants and animals including those that are consumed by man. They are ubiquitous and in this respect, common and ordinary. They are a normal part of the human diet.

Enzymes are a special type of protein with a highly specific catalytic function. They are essential to many biochemical reactions in microorganisms, plants, animals and humans. Due to the specific nature of enzymes, only very small amounts are required to achieve the desired effect in food. As with other enzymes, this phospholipase is used at a very low level in the processing of cheese. After coagulation, almost all of the enzyme is removed from the cheese in the whey fraction.

Based on the fact that:

- there are no known cases of allergic responses to phospholipase in food,
- known cases of allergic responses to food enzymes are very rare, and
- exposure to this enzyme protein is extremely low,

the allergy risk due to the ingestion of this phospholipase is negligible.

Please contact me by direct telephone at 919 494-3151 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

Lori Gregg
Regulatory Specialist

Reference List for Industry Submission, GRN 000142

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000030 - 000043	Pariza, Michael W.; Johnson, Eric A.	Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century	2001	Regulatory Toxicology and Pharmacology	Volume 33, pgs 173-186
000045 - 000048	Barbesgaard, Peder; Heldt-Hansen, Hans Peter; Diderichsen, Berge	On the safety of Aspergillus oryzae: a review	1992	Applied Microbiology and Biotechnology	Volume 36, pgs 569-572

NA- Not applicable