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GRAS NOTIFICATION FOR PHOSPHOLIPASE A2 FROM A GENETICALLY MODIFIED STRAIN OF *TRICHODERMA REESEI*

AB ENZYMES GmbH

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1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36 (see 62 Fed. Reg. 18,938 (April 17, 1997)), AB Enzymes GmbH ("AB Enzymes") has determined that its Phospholipase A2 enzyme preparation from *Trichoderma reesei* (*T.reesei)* strain RF8793 expressing the gene encoding phospholipase A2 from *Aspergillus nishimurae* (*A. nishimurae)* (formerly *Aspergillus fumigates* (*A. fumigates*)), is a GRAS substance for the intended food applications based on scientific procedures and is therefore exempt from the requirement for premarket approval. Information on the enzyme and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme, its applicable conditions for use, AB Enzymes' basis for its GRAS determination and the availability of supporting information and reference materials for FDA's review can be found here in Section 1.

The production organism, *T. reesei*, has a long history of safe use; this is discussed in Section 2. The FDA has previously affirmed as GRAS several enzyme preparations from *T. reesei* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *T. reesei* strains.

Section 2 also describes the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from known harmful sequences.

In Section 3, data are presented that show the substantial equivalence of the phospholipase A2 to naturally occurring phospholipase A2. The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 4. Section 5 reviews the hygienic measurements, composition and specifications as well as the self-limiting levels of use for phospholipase A2. Section 6 provides information on the mode of action, applications, use levels of phospholipase A2 and enzyme

residues in final food products. The safety studies outlined in Section 7 indicate that phospholipase preparations from *T. reesei* show no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

1.1. Name and Address of Notifier

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[1] ROAL is a Joint Venture between Associated British Foods (UK) and Altia OY (Finland). Manufacturing and some research and development activities are performed for AB Enzymes by ROAL OY in Finland. ROAL coordinates its R&D activities independently while taking into account the market requirements reported by their sole distributor AB Enzymes GmbH.

1.2. Common or Usual Name of Substance

Phospholipase A2 enzyme preparation from *Trichoderma reesei* strain RF8793 expressing the gene encoding phospholipase A2 from *A. nishimurae* (formerly *A. fumigatus*).

The food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process.

1.3. Applicable Conditions of Use

pH value: Optimum: 3.5 – 5.5 Range: 3.0 – 6.0

Temperature: Optimum: 50 - 55°C Range: 50 – 58°C

1.3.1. Food Products Used in

Crude vegetable oil extracted from e.g. soya, rape seeds, sunflower seeds, or rice bran. Phospholipase A2 can also be used in the modification of lecithin.

1.3.2. Levels of Use

An appropriate enzyme preparation dosage is usually 120 to 3000 units, and the preferred dose is 750 to 1500 units per kilos of oil.

1.3.3. Purposes

Crude vegetable oil extracted from soya, rape seeds, and sunflower or rice bran has to be refined to remove phospholipids (referred to as gums), colours, free fatty acid, odouring and flavouring substances, S-compounds and metal ions. These impurities have a negative influence on downstream processing, taste and storage stability and therefore have to be reduced in the edible oil. A large part of the gums can be removed with water; however the remaining non-water soluble gums can either be removed by a caustic process or through an enzymatic process using phospholipases.

Phospholipase A2 degrades phospholipids into lysophospholipds during the degumming process. The formed lysophosholipids can easily be separated with the water phase from the oil due to its emulsifying properties. Constant phosphorus contents < 10 ppm can be achieved after centrifugation.

1.3.4. Consumer Population

Phospholipase A2 was the first phospholipase to be recognised. This enzyme is ubiquitous in nature and occurs naturally in animal and plant cells. It has been isolated from a number of food sources (including wheat flour) and is a natural constituent of the digestive pancreatic juice of mammals, including humans (*de Haas et al. (1968); Rossiter (1968); Johnson and McDermott (1974)*). Phospholipase A2 is a component of many animal and plant derived foods.

Similar Phospholipase A2 preparation, from *Aspergillus niger* and from *S. violaceumber* have already been the subject of a GRAS notification (respectively GRN 000183 - 2005, and GRN 000145-2004), and phospholipase A1s from different organisms are approved world-wide (*Appendix # 1*). So far, consumption of these phospholipases has not led to any adverse events or allergic reactions. Since the phospholipase A2 produced in genetically engineered *T. reesei* strain is a protein composed of natural amino acids, it will be digested in the human gastrointestinal tract just as any other food protein/enzyme.

Phospholipase A2 catalyses the hydrolysis of an ester bond (sn-2 ester bond) between a fatty acid and glycerol in phospholipids. Both, the substrate (phospholipids) and the products of this enzymatic reaction (lysophospholipids and free fatty acids) play important roles in a number of metabolic processes in all organisms, from bacteria to mammals. As a result, lysophospholipids and free fatty acids are quite abundant in the human diet. The natural occurrence of phospholipids in crude soybean oil is 2%, of which 90% is removed during the degumming process to improve color and enhance oxidative stability (*Nzai and Proctor (1998)*). Hence, there is no basis to believe that conversion of phospholipids to lysophospholipids and free fatty acids will have a significant effect, if any, on processed foods or on the human body.

As is shown in Section 6.4 of this dossier, the enzyme is expected to be denatured during the oil degumming process. Furthermore, based on the information given in section 6.3 of this dossier, the maximum amount of (denatured) enzyme in refined oils is expected to be about 11 mg TOS/kg oil (based on 500 PLU/kg oil). It is important to note that the refined oils are mainly used in the food industry as an ingredient, lowering even less the final amount of (denatured) enzyme in final foods. Since phospholipase A2 may be present in food products at only very low levels as an inactive protein, and because it is a naturally occurring substance in cells and tissues commonly ingested by humans without any harm, it is assumed that the consumer population will be unaffected by the presence of denatured phospholipase A2 in foodstuffs when used as processing aids.

1.4. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, AB Enzymes GmbH has determined, through scientific procedures, that its Phospholipase A2 enzyme preparation from *T. reesei* expressing the gene encoding phospholipase A2 from *A. nishimurae/A. fumigauts* is GRAS for use as an enzyme for the degradation of vegetable phospholipids in oil degumming.

1.5. Availability of Information for FDA Review

A notification dossier providing a summary of the information that supports this GRAS determination is enclosed herein. The dossier includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration (FDA) for review and copying at reasonable times at a specific address set out in the notice or will be sent to FDA upon request. Please direct all inquiries regarding this GRAS determination to:

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2. PRODUCTION MICROORGANISM

2.1. Donor, Recipient Organism and Production Strain

Donor:

The phospholipase A2 (*pla2*) gene was isolated from the *Aspergillus fumigatus* strain RH3949. The donor strain RH3949 was first identified as *Aspergillus fumigatus* (*Appendix #2*) and more recently as *Aspergillus nishimurae* (*Appendix #3*). As the name *Aspergillus fumigatus* has been used in our publications, both names *Aspergillus fumigatus* and *Aspergillus nishimurae* are used interchangeably in this dossier for the donor organism.

Recipient Organism:

The recipient organism used in the construction of phospholipase A2 production strain is a *T. reesei* host strain. This strain, designated RF7720 was used in the construction of the phospholipase A2 production strain RF8793 which is the subject of this dossier. *T. reesei* RF7720 is a classical low protease mutant derived from the well characterized QM6a strain (Rut series) (*Nevalainen et al. 1994*).

T. reesei is an aerobic filamentous fungus (an ascomycete). It grows in mycelium form but starts to sporulate when cultivation conditions do not favor growth (e.g. due to lack of nutrients). *T. reesei* is a mesophilic organism which means that it prefers to grow at moderate temperatures. The cultures are typically fast growing at about 30° C (above 20°C and below 37°C). *T. reesei* prefers acidic to neutral pH (about 3.5 to 6) for growth. The colonies are at first transparent or white on agar media such as potato dextrose agar (PDA). The conidia are typically forming within one week of growth on agar in compact or loose tufts in shades of green. Sporulation is induced by daylight. Yellow pigment may be secreted into the agar by the growing fungal colonies, especially on PDA.

The classification of RF7720 (also referred to as RH32439) as *T. reesei* has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland (*Appendix #4*). Furthermore, the *T. reesei* host strain RF7720 is genetically stable as determined by Southern blot analysis. To study the genetic

stability of the Trichoderma reesei RF7720, the strain was cultured on Potatoe Dextrose Agar (Difco) for 4 days at 300C. The spores (first generation) were isolated and used as the inoculum for the next generation of spores (second generation). The procedure was repeated ten times. Southern blot analysis using the *T. reesei* cbhI-promoter as a probe showed in all samples two hybridizing bands of 9.0 and 1.6 kb. The hybridization patterns of the 10 generations were identical. The result indicates that the *Trichoderma reesei* RF7720 is genetically stable.

The taxonomic classification of the *T. reesei* is: Hypocreaceae, Hypocreales, Hypocreomycetidae, Sordariomycetes, Pezizomycotina, Ascomycota, Fungi, according to Index Fungorum database

AB Enzymes GmbH has been using *T. reesei* as an enzyme producer for many years without any safety problems. A GRAS notice was filed for pectin lyase enzyme preparation produced with *T. reesei* containing a gene from *Aspergillus niger* and FDA had no question and designated it as GRAS Notice No. GRN 000032 (*Appendix #5*). *T. reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production (e.g. cellulases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries). Currently, various *Trichoderma* enzymes are also used in the brewing process (β-glucanases), as macerating enzymes in fruit juice production (pectinases, cellulases, hemicellulases), as a feed additive to livestock (xylanases, endoglucanases) and for pet food processing. *T. reesei* - wild type or genetically modified - is widely accepted as safe production organism for a broad range of food enzymes.

Based on the available data, it is concluded that the organism *T. reesei* is non-pathogenic and non-toxigenic and is safe to use as the production organism for phospholipase A2.

Production Strain:

The production organism is strain T. *reesei* RF8793, which has been genetically modified by insertion of the *pla2* gene from the *Aspergillus fumigates/nishimurae strain* RH3949 (*Appendix #2*). The *T. reesei* strain RF8793 was constructed by transformation of the strain *T. reesei* RF7720 with a purified DNA fragment isolated from the plasmid pAB500-PL3949.

2.4. Good Industrial Large Scale Practice (GILSP)

The phospholipase A2 production strain RF8793 complies with all criteria for a genetically modified GILSP organism.

T. reesei is listed as a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules since. Class 1 Containment Agents are microorganisms with the lowest safety concern, such as baker's yeast.

As a result, *T. reesei* can be used under the lowest containment level at large scale, GILSP, as defined by OECD (*OECD, 1992*).

The host organism is non-pathogenic, does not produce adventitious agents under the fermentation conditions employed and has an extended history of safe industrial use (see Section 7.1). Indeed, the host *T. reesei* RF7720 was developed by conventional mutagenesis from the wild type strain QM6a strain (Rut series) that has been isolated from soil only at low altitudes and within a narrow belt around the equator (*Kubicek et al. (2008)*). The mycoparasitism-specific genes have been shown to be lost in *T. reesei* (*Kubicek et al. (2011)*).

Overall, industrial microorganisms modified to produce high levels of enzymes, in fermentation conditions (e.g. no competitive microorganisms, optimal nutrients and aeration that are not present in the natural environment) are not expected to have any competitive advantage against other microorganisms in nature, which themselves are well-adapted in their natural environment. The fitness of the industrial strains to survive is very likely reduced by their high performance characteristic: most of the energy is needed for the production of proteins in high amounts.

From the genetic modification performed, there is no reason to believe that the survival of the genetically modified production organism would be different when compared to its ancestor. The DNA insert is fully characterized and is free from known harmful sequences. No antibiotic resistance markers or other heterologous markers are present in the strain. We consider thus that the colonization capacity

of *T. reesei* RF8793 in the environment is rather low because of its adaptation to fermentation conditions.

Therefore, the *T. reesei* production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. This is the concept of Good Industrial Large Scale Practice (GILSP), as endorsed by the Organization of Economic Cooperation and Development (OECD). The production organism has been approved by the Finnish competent authorities for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment.

2.5. Absence of the Production Organism in the Product

The down-stream process following the fermentation includes unit operations to separate the production strain. The procedures are executed by trained staff according to documented standard operating procedures complying with the requirements of the quality system.

The phospholipase A2 enzyme produced from *T. reesei* strain RF8793 is recovered from the fermentation broth by a widely used process that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch.

2.6. Absence of Transferable rDNA Sequences in the Enzyme Preparation

As described above the expression cassette is well characterized and does not contain any undefined or harmful sequences (see section 2.2). The absence of the pUC plasmid containing the Ap selectable marker in the fungal genome of RF8793 was characterized by Southern blot analysis. No hybridizing band was detected in all samples of host strain and production strain indicating the absence of the pUC-plasmid in the fungal genome. Furthermore, the colonization capacity of the strain in the environment is rather low due to its adaptation to fermentation conditions. Furthermore, samples of the phospholipase A2 enzyme preparation were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. Two primer sets were first tested using pAB500-PL3943 plasmid DNA and *T. reesei* RF8793 genomic DNA as controls. The results indicated that there is no

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Phospholipase A2 activity is represented as PLU, or units of phospholipase, and PLU g^{-1} is the definition for phospholipase activity. 1 Unit of phospholipase corresponds to that amount of enzyme, which releases 1 µmol of fatty acids per minute under the described conditions.

4. MANUFACTURING PROCESS

4.1. Overview

Like all food enzymes, the phospholipase A2 enzyme described in this dossier is manufactured in accordance with current Good Manufacturing Practices for Food (cGMPs) and the principals of Hazard Analysis of Critical Control Points (HACCP). Compliance to Food Hygiene Regulation is regularly controlled by relevant food inspection services in Finland.

The phospholipase A2 described herein is produced by controlled fed-batch submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. Finally, the measures taken to comply with cGMPs and HACCP are provided. A manufacturing flow-chart is given in *Appendix 8*.

It should be noted that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in a vast majority of cases, the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

4.2. Fermentation

The production of food enzymes from microbial sources follows the process involving fermentation as described below. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. The main fermentation steps are:

- Inoculum
- Seed fermentation

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4.3. Recovery

The purpose of the recovery process is:

- to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein,
- to concentrate the desired enzyme protein and to improve the ratio enzyme activity/Total Organic Substance (TOS).

During fermentation, the enzyme protein is excreted by the producing microorganism into the fermentation medium. During recovery, the enzyme-containing fermentation medium is separated from the biomass.

This Section first describes the materials used during recovery (downstream processing), followed by a description of the different recovery process steps:

- Pre-treatment
- Primary solid/ liquid separation
- Concentration
- Polish and germ filtration

The nature, number and sequence of the different types of unit operations described below may vary, depending on the specific enzyme production plant.

4.3.1. Materials

Materials used, if necessary, during recovery of the food enzyme include:

- Flocculants
- Filter aids
- pH adjustment agents

Potable water can also be used in addition to the above mentioned materials during recovery.

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The clear filtrate is concentrated with ultrafiltration. Ultrafiltration is a membrane filtration process applied to increase the ratio of high molecular weight molecules (such as the enzyme protein) versus the low molecular weight molecules, thus increasing the enzyme activity/TOS ratio.

4.3.5. Polish and germ filtration

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied at various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

4.4. Formulation and Packaging

Following formulation, the final product is defined as a 'food enzyme preparation.' Food enzymes can be sold as dry or liquid preparations, depending on the final application where the enzyme is intended to be used. Typically, food enzyme preparations sold to the oil degumming industry are sold in liquid form. For all kinds of food enzyme preparations, the food enzyme is standardized and preserved with food ingredients or food additives which are approved in the USA according to ruling legal provisions.

Phospholipase A2 enzyme preparation from *T. reesei* RF8793 is typically sold as a liquid food enzyme preparation and is thus standardised with food grade ingredients and adjusted to the desired activity. The preparation is in certain cases stabilized with preservatives.

Phospholipase A2 enzyme preparation from *T. reesei* RF8793 is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations, and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations. Labels conform to relevant legislation.

4.5. Quality Control of Finished Product

The final enzyme product complies with the recommended specifications of the FAO/WHO's Joint Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

Property	Requirement	Testing rate
Total viable counts	< 50000 g^{-1}	every lot
Yeasts and fungi	each < 1000 g^{-1}	every lot
E. coli	not present in 25 g	Semifinals, spot sampling in finals every 10th lot
Salmonella	not present in 25 g	Semifinals, spot sampling in finals every 10th lot
Coliform counts	< 30 g^{-1}	every lot
Arsenic	< 3 ppm	Spot samples from raw materials and semifinals
Lead	< 2 ppm	Spot samples from raw materials and semifinals
Heavy metals	< 30 ppm	Spot samples from raw materials and semifinals
Antibacterial Activity	not detectable	at least 1 lot/y

In addition, the following specifications were set:

Property	Requirement	
Phospholipase Activity (kPLU g^{-1})	min.	10
pH	3.6 – 4.0	
Appearance	Light brown liquid	
Density	1.1-1.2 g/mL	

5. COMPOSITION AND SPECIFICATIONS

5.1. Formulation

The composition of the final enzyme product is as follows:

Composition of Final Product		
Constituent	**%**	**% spec. Protein**
Phospholipase A2	6.7	0.3
Glycerol	30.0	
Sorbitol	30.0	
Water	33.3	

5.2. General Production Controls and Specifications

In order to comply with cGMPs and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account and controlled during production as described below:

Identity and purity of the producing microorganism:

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore it is essential that the identity and purity of the microorganism is controlled.

Production of the required enzyme protein is based on a well-defined Master (MCB) and Working Cell Bank (WCB). A Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The MCB is prepared from a selected strain. The WCB is derived by sub-culturing of one or more ampoules

of the MCB. A WCB is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

Microbiological hygiene:

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Microbial contamination would immediately result in decreased growth of the production organism, and consequently, in a low yield of the desired enzyme protein, resulting in a rejected product.

Measures utilized by ROAL OY to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

- Hygienic design of equipment:
    - all equipment is designed, constructed and used to prevent contamination by foreign micro-organisms
- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the production area and equipment: all fermentors, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place), where hot caustic soda and nitric acid are used as cleaning agents. After cleaning, the vessels are inspected manually; all valves and connections not in use for the fermentation are sealed by steam at more than 120°C; critical parts of down-stream equipment are sanitized with disinfectants approved for food industry
- Sterilization of all fermentation media:
    - all the media are sterilized with steam injection in fermentors or media tanks (at 121°C for at least 20 min at pH 4.3 – 4.8.).
- Use of sterile air for aeration of the fermentors:
    - Air and ammonia water are sterilized with filtration (by passing a sterile filter).
- Hygienic processing:

- Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor
- Maintaining a positive pressure in the fermentor

- Germ filtration

In parallel, hygienic conditions in production are furthermore ensured by:

- Training of staff:
  - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- pest control
- Inspection and release by independent quality organization according to version-controlled specifications
- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production take place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

Chemical contaminants:

It is also important that the raw materials used during fermentation are of suitable quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

In addition to these control measures in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high quality product (cGMPs). The whole

process is controlled with a computer control system (Metso DNA) which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

Absence of significant microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring of fermentation parameters may include:

- pH
- Temperature
- Dissolved oxygen content
- CO2

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):

This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

6. APPLICATION

6.1. Mode of Action

Like any other enzyme, phospholipase A2 acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

The degumming process to remove phospholipids is the most important step in the refining process. Phospholipase A2 can be used for the enzymatic hydrolysis of phospholipids. The formed lysophosholipids can easily be separated with the water phase from the oil due to its emulsifying properties. Constant phosphorus contents < 10 ppm can be achieved after centrifugation. These properties are very useful process for enzymatic degumming of vegetable oils.

6.2. Application

Vegetable oil extracted from soy, rape seed, sunflower, cotton, corn or rice bran contains impurities. They consist of phospholipids, colours, free fatty acid, odouring and flavouring substances, S-compounds and metal ions. If these impurities are not removed before the oil is exposed to the high temperatures used in deodorization quality problems can well emerge. Taste and stability of the oil would be affected as well.

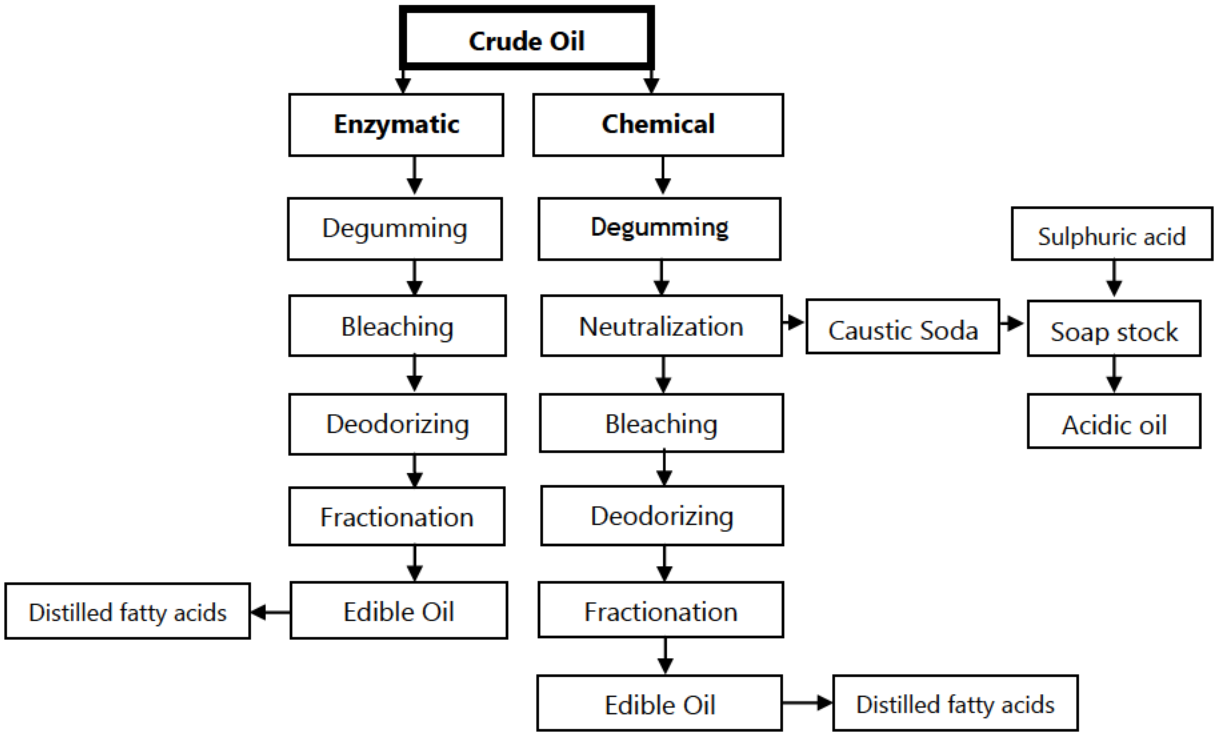
Compared with conventional chemical refining of edible oils, physical methods are becoming more technological advantageous. The oil degumming process is a type of physical refining being used as conventional processes (chemical refining) cannot guarantee the achievement of low gum content required for physical refining. In the chemical refining process, gums work as emulsifiers and are responsible for the major part of the oil losses. The yield loss, the apparatus requirements and energy expenditures are also great in conventional degumming (*Yang et al., 2005*). Chemical refining of oil uses about 10-15% of water to total crude oil and sulphuric acid for neutralization of fatty acids, the resultant waste water (soap stock) is highly acidic. It is during the separation of the soap stock that

results in high losses of oil. Furthermore, the soap stock needs to be treated before disposal; this involves the use of harsh chemicals with a potentially negative impact on the environment.

Enzymatic Oil Degumming:

The enzymatic method of oil degumming (physical degumming) is a process whereby enzymes such as phospholipase A2 converts nonhydratable phosphatides (phospholipids) into hydratable forms (lysophospholipids). An advantage of using phospholipase in the degumming process is that they can effectively remove most of the phosphatides from crude oil thereby reducing the consumption of excess caustic acid over the calculated amount during the subsequent refining operation and minimizing refining loss (*Mukherjee et al., 2013*). Low and constant gum contents, typically below 10 ppm, can be achieved after centrifugation.

The Oil Refining Process: Enzymatic VS Chemical Refining



6.3. Use Levels

Dosage shall be determined by means of tests in real processing conditions since many factors can influence the performance of the enzyme including the raw materials variations, temperature, pH, reaction time, and equipment etc.

An appropriate phospholipase A2 enzyme dosage is usually between 120 and 3,000 PLU per kilos of oil treated and preferably around 500 PLU per kilos of oil. This corresponds to 11 mg TOS/kg of oil.[2]

6.4. Enzyme Residues in the Final Food

6.4.1. Residues of Inactive Enzyme in Various Applications

In general, enzymes perform their technological function during food processing. Like the endogenous enzymes present in food, they do not perform any technological function in the final food. The reasons why the enzymes do not typically exert enzymatic activity in the final food could be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing, lack of water activity, inadequate pH, etc. In some cases (e.g. after alcohol distillation, products that result from processing of starch, and oil degumming), the enzymes may no longer be present in the final food.

In the production of high-quality edible oils, where the phospholipase A2 is mainly intended to be used, the refining process comprises the steps of degumming, bleaching and deodorization. After hydrolysis of glycerophospholipids by phospholipase A2, the lysophospholipid fractions dissolve in aqueous phase and the fatty acids produced remain in the oil phase. Phospholipase A2 remains in water phase while

[2] Enzyme concentrate (tox batch) is 91% TOS with an activity of 41200 PLU/g (equivalent to 45 PLU/mg TOS).
Enzyme preparation has a minimum activity of 10 000 PLU/g, i.e. 222 mg TOS/g.
Max use level of enzyme preparation is 50 g of enzyme preparation (with 10 000 PLU/g) / ton oil (equivalent to 500 PLU/kg oil) resulting in 11 mg TOS/kg oil.

catalysing the hydrolysis of oil.

The next step following the degumming step is a bleaching step typically with bleaching earth, a strong absorbent which would remove proteins in case they are not removed by the previous centrifugation step.

Finally, a distilling step is carried out in order to remove free fatty acids: this step is performed at a temperature above 190°C, inactivating all potentially remaining protein.

Therefore, it can be assured based on the current evidence and physical refining process results in the removal or inactivation of the food enzyme product during processing, with no resultant enzyme activity present in the final food product.

6.4.2. Possible Effects on Nutrients

As the catalytic activity of the enzyme preparation is very specific, i.e. hydrolysis of the sn-2 ester bond between a fatty acid and glycerol in phospholipids, it is not to be expected that the enzyme preparation will have any effect on other constituents or nutrients in food.

7. SAFETY EVALUATION

7.1. Safety of the Production Strain

The insertion of the expression cassette into the genome of the recipient strain *T. reesei* RF7720, results in the recombinant *T. reesei* strain RF8793. The production strain only differs from its recipient strain by its production of phospholipase due to expression of the pla2 gene from *A. fumigatus*.

T. reesei is an industrially important filamentous fungus and has been used as producer of different hydrolases such as xylanase and cellulase for food, animal feed, and pulp and paper industries. It is also used as host for production of heterologous proteins in the same areas. Like many other organisms

with a long safe history of industrial use, *T. reesei* strains have been and are being used by many commercial companies in the construction of production strains by genetic engineering.

Trichoderma are metabolically versatile aerobic mesophilic imperfect fungi and are common in soil in all climate zones (*Nevalainen et al. (*1994*)*). According to *Kuhls et al*. (1996), *T. reesei* is a clonal, asexual derivative of the ascomycete *Hypocrea jecorina* and can be identified by PCR-fingerprinting assay and sequence analyses of the nuclear ribosomal DNA region containing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rRNA gene (*Kuhls et al. (*1996*)*).

T. reesei is classified as a Biosafety Level 1 (BSL-1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Public Health Service guidelines. BSL-1 microorganisms are not known to cause disease in healthy adult humans. *T. reesei* is regarded as non-pathogenic and non-toxigenic. The safety of this organism as an enzyme producer has been reviewed by *Nevalainen et al. (*1994*), Blumenthal (*2004*),* and *Olempska-Beer (*2006*)*.

The transformed expression cassette, fully characterized and free from potential hazards, is stably integrated into the fungal genome (see section 2.3) and is no more susceptible to any further natural mutations than any other genes in the fungal genome. Also, the transformation does not increase the natural mutation frequency. If there were any mutations in the genes affecting the relevant characteristics of the fungus, this would likely be noticed in the growth characteristics in the fermentation and/or in the product obtained, and no such changes have been observed. The possibility of mutations is further decreased by inoculating the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C. There is no indication that this genetic modification will have a negative effect on the safety properties. Therefore, it can be concluded that the *T. reesei* strain RF8793 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food and feed processing.

7.2. Safety of the Phospholipase A2 Enzyme

This enzyme hydrolyzes the phospholipids to form fatty acid and lysophospholipid. It has an optimum temperature for activity of 50°C, a broad pH optimum between 3- 5 and a very low activity of lipase. These properties are very useful for enzymatic degumming of vegetable oils.

The phospholipase A2 enzyme preparation produced by *T. reesei* RF8793 also contains other *T. reesei* enzymes such as endogenous cellulase, xylanase and protease at very low levels. These enzymes are not used to hydrolyze the phospholipids and therefore do not contribute to the required effect in the application.

Phospholipase A2 PL3949 described in this application was isolated from an environmental isolate strain *A. nishimurae* RH3949.

A. nishimurae is a ubiquitous filamentous fungus which plays an important role under natural conditions in the aerobic decomposition of organic materials. *A. nishimurae* secretes multiple extracellular phospholipases, including PLA, PLB, PLC and PLD.

A. nishimurae RH3949 is an environmental isolate. *Birch et al*. (2004) suggested, "Phospholipid acyl hydrolase activity for the environmental isolates may be more important for growth in the environmental than it is for clinical isolates growing in the body". According to *Rementeria et al. (*2005*)*, "there is no unique essential virulence factor for development of this fungus in the patient and its virulence appears to be under polygenetic control". While the phospholipase have been considered as virulence factors in *C. albicans* or *C. neoformans*, "in clinical isolates of *A. fumigatus* the production of phospholipases is lower than environmental isolates, making unlikely, if not excluding, their involvement in the virulence of the fungus" (*Abad et al. (*2010)).

Furthermore, a wide range of food enzymes, including phospholipases, have been on the market for decades and have been approved on the market for use in food on the basis of safety documentation. Phospholipases are used to make emulsifier-like products such as lysolecithin and monoglyceride.

Phospholipase is used to improve the yield in cheese production. In bread making phospholipases are used to improve bread volume and dough stability. For degumming of vegetable oils Phospholipase A2s are used in the refining process that influences the stability of refined oils (*De Maria et al.* (2007)).

The use of phospholipase A2 as a food processing aid has been approved in Australia and New Zealand isolated from *S. violaceoruber* (*Appendix #10*), and isolated from porcine pancreas expressed in *Aspergillus niger* (*Appendix #11*). Canada has approved the use of phospholipases as a processing aid expressed in *Steptomyces violaceoruber* (used in modified lecithin, unstandardized egg products), *Aspergillus oryzae* (used in cheddar cheese), *and Aspergillus niger* (used in bread, unstandardized bakery products, unstandardized egg whole and yolk, and modified lecithin). Phospholipase has also been approved as a processing aid expressed in *Aspergillus oryzae* (GRN 142), *Streptomyces violaceoruber* (GRN 145), *Aspergillus niger* (GRN 183), and *Pichia pastoris* (GRN 204).

Finally phospholipases are natural constituents of foods: phospholipase is a normal constituent of wheat flour (*Verlotta et al.* (2013)), and is one of the digestive enzymes present in the pancreatic juice of mammals, including humans (*de Haas et al. (*1968); *Rossiter*, (1968); *Johnson and McDermott*, (1974)).

Based on these points and the long history of safe use of *T. reesei* strains in the production of food enzymes it is concluded that phospholipase A2 from production strain *T. reesei* RF8793 is safe for use as a food processing aid.

7.2.1. Allergenicity

Virtually all food allergens are proteins, although only a small percentage of proteins are allergens. Any food containing protein has the potential to cause allergic reactions, however a few food groups are known to cause allergies more frequently than others. These major allergenic food groups are: milk, eggs, fish, crustacea (shrimp, lobster, and crab), soybeans, peanuts, tree nuts and wheat. Allergens from these food groups account for more than 90% of food allergic reactions. The prevalence of allergic sensitivities to specific foods varies between countries, depending on the frequency with which the

foods are consumed and the age at which it is introduction into the diet. Although no general characteristics can be defined that make a protein an allergen, size and structure, glycosylation, solubility, resistance to heat and sensitivity to enzymatic and acidic degradation are believed to play a role. Most food allergens, perhaps especially those that do cause systemic effects, are resistant to digestion, proteolysis, and other forms of hydrolysis.

To evaluate the potential allergenicity of phospholipase A2 enzyme from *T. reesei* RF8793, the sequence comparison with known allergenic proteins was done using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 12, February 7, 2012), which contains the amino acid sequences of known and putative allergenic proteins. The analyses were performed using the full-length alignment program, by searching for 80 amino acid alignment program to find identities greater than 35% and by searching for a stretch of eight amino acids program.

The resulting alignment of the full-length phospholipase A2 protein sequence to any allergenic proteins in Allergen Database showed no matches greater than 34% identity (*Appendix #12*). According to *Aalberse R.C.* (2000) "cross-reactivity is rare below 50%. In most situations, cross-reactivity requires more than 70% identity".

In addition, the phospholipase A2 protein sequence showed no similarity to the known allergens by searching for 80 amino acid alignments to find identities greater than 35% or by searching for a stretch of eight amino acids (*Appendix #12*).

Therefore, it is concluded the Phospholipase A2 enzyme from *T. reesei* expressing the gene encoding phospholipase A2 from *A. nishimurae/A. fumigauts* is considered non-allergenic.

7.2.2. Leading Publications on the Safety of Phospholipase Enzymes or Enzymes that are Closely Related

The production organism *T. reesei* has been demonstrated to be non-toxigenic and non-pathogenic and any food ingredient (enzyme) from that organism will exhibit the same safety properties if manufactured under current Good Manufacturing Practices ("cGMPs"). *Pariza and Foster* (1983) noted that a non-pathogenic organism was very unlikely to produce a disease under ordinary circumstances. In their publication, *T. reesei* is included in the authors' listing of the organisms being used in the industry. The evaluation of the safety of the genetic modification should be examined based on the concepts outlined in the *Pariza and Foster* (1983) paper. Their basic concepts were further developed by the JFBC in 1990, the EU Scientific Committee for Food in 1991, the OECD in 1993, ILSI Europe Novel Food Task Force in 1996 and FAO/WHO in 1996. Basically, the components of these evaluations start with an identified host strain, descriptions of the plasmid used and the source and fraction of the material introduced, and an outline of the genetic construction of the production strain. This information is found in Section 2.

The FDA has also accepted the GRAS Notifications stating that pectinlyase (GRN 32), chymosine (GRN 230), transglucosidase (GRN 315), protease (GRN 333), glucoamylase (GRN 372) enzyme preparations from *T. reesei* are generally recognized as safe. *T. reesei* is listed as a production organism for enzymes (*Pariza and Johnson* (2001)) and has a long history of safe use (also see Section 7.1).

As is clear from the information provided in this notification, there have been genetic modifications to the *T.reesei* used by AB enzymes, but these genetic modifications are thoroughly well characterized and specific in that the DNA encoded does not express any harmful or toxic substance. The safety studies described in Section 7.4 of this dossier support the fact that the genetic modification did not result in any toxic effects.

7.2.3. Substantial Equivalence

The substantial equivalence is determined by comparing the structure of enzymes**,** the enzyme activities and intended uses, the production organism and production process. Lipases and phospholipases type

A are triacylglycerol hydrolyzing enzymes and belong to the members of carboxylic ester hydrolases (EC 3.1.1), which catalyze the hydrolysis of the carboxylic ester bonds of triglycerides or phosphoglyceride lipids to release carboxylic acids. Lipases and phospholipases are enzymes which have been used widely in food industry. Several lipases and phospholipases were notified as GRAS for use in baking application, in the production of cheese, in egg yolk industry and for enzymatic degumming of vegetable oils.

Some lipases also have phospholipase activity. *Thermomyces lanuginosus* lipase (accession no. AAC08588) has no phospholipase activity; however, it can be modified for example by changing several amino acids near the active site to have activity towards phospholipids. The new lipolytic enzyme contains the amino acid residues 1-284 originated from the N-terminal region of *T. lanuginosus* lipase with three amino acids substitutions ($G^{113}A$, $D^{118}W$ and $E^{121}K$) and the catalytic triad consisting of Ser^{167}, His^{280} and Asp^{223}. The C- terminal 7 residues of the *T. lanuginosus* lipase were substituted by 55 residues of the C-terminal of the *Fusarium oxysporum* lipase. This lipolytic enzyme with both lipase and phospholipase activities is a commercial enzyme preparation from Novozymes (Lecitase Ultra) used for enzymatic degumming of vegetable oils and is the subject of GRAS notification GRN No. 103 to which FDA has no question.

The Phospholipase A2 enzyme from *T. reesei* RF 8793 is 53% homologous to the *T. lanuginosus* lipase as well as to the enzyme core (modified *T. lanuginosus* lipase sequence) of the new lipolytic enzyme (Lecitase Ultra). Generally a sequence homology greater than 50% would indicate a similar structure. The additional sequence alignment shows the pentapeptide consensus Gly-X-Ser-X-Gly sequence and the catalytic residues Asp and His are well conserved in all enzymes.

The Phospholipase A2 enzyme is a commercial phospholipase product from AB Enzymes GmbH. The enzyme hydrolyzes phospholipids to release fatty acids and is active at pH between 3- 5. This property makes it very useful for enzymatic degumming of vegetable oil.

Phospholipase A2 is substantially equivalent to the lipolytic enzyme from *T. lanuginosus* (Lecitase Ultra) based on the sequence homology and structure similarity, the functionally enzymatic equivalence and intended uses and the safety of the production strain. It can be conclude that the Phospholipase A2 enzyme preparation from *T. reesei* RF8793 is safe.

7.3. Safety of the Manufacturing Process

Phospholipase A2 enzyme preparation from *T. reesei* RF8793 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 4, the phospholipase A2 preparation is produced in accordance with cGMPs using ingredients that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

7.4. Safety Studies

This section describes the studies performed to evaluate the safety of the phospholipase A2 preparation. All safety studies were performed according to internationally accepted guidelines (OECD or FDA) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

7.4.1. Summary of Safety Studies

The following studies were performed with the phospholipase A2 enzyme preparation from *T. reesei* RF8793:

- Ames Test (*Appendix #24)*
- Chromosome aberration test (*Appendix #25*)
- 90-day oral toxicity study in rats (*Appendix #26*)

These safety studies were conducted using the enzyme preparation concentrate, not with the diluted final product. All studies were conducted using the same production batch, Batch No. LF 10082 B3, with 91.0 % TOS. Dose calculations for the experiments were adjusted to account for TOS.

7.4.2. Results of the Safety Studies

7.4.2.1. Ames Test

This study was performed to investigate the potential of phospholipase A2 enzyme preparation from *T. reesei* RF8793 to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in two independent experiments both with and without S9 liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations calculated to the total organic substance (TOS):

Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 Lg/plate

Experiment II: 33; 100; 333; 1000; 2500; and 5000 Lg/plate

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with the phospholipase enzyme preparation at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). A minor but dose dependent increase in revertant colony numbers was observed following treatment with the phospholipase enzyme preparation in the absence of metabolic activation in strain TA 98 in experiment I and in strain TA 1537 in experiment II. In both strains the threshold of two (TA 98) and three times (TA 1537) the number of the corresponding solvent control was not reached. Therefore this minor increase is judged as biologically irrelevant. Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

These studies show that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frame shifts in the genome of the strains used. Therefore, the phospholipase A2 enzyme preparation from *T. reesei* RF8793 is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

7.4.2.2. Chromosomal Aberration Test

This test assesses the potential of the phospholipase A2 enzyme preparation from *T. reesei* RF8793, to induce structural chromosome aberrations in V79 cells of the Chinese hamster *in vitro* in two independent experiments.

Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without metabolic activation. In Experiment II the exposure period was 18 hours without S9 mix. The chromosomes were prepared 18 hours after start of treatment with the test item. The highest applied concentration 5495.0 µg/mL (5000.0 µg/mL adjusted to TOS) was chosen with respect to the current OECD Guideline 473. Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations

No relevant increase in polyploidy metaphases was found after treatment with the test item as compared to the frequencies of the control cultures. In conclusion, no biologically relevant increases of chromosomal aberrations were observed and the phospholipase A2 enzyme preparation from *T. reesei* RF8793 is classified as non-clastogenic.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, phospholipase A2 enzyme preparation from *T. reesei* RF8793 did not induce gene mutations by base pair changes or frame shifts in the genome of the strains used.

7.4.2.3. 90-Day Sub-Chronic Toxicity Study

In this sub-chronic toxicity study, the phospholipase A2 enzyme preparation from *T. reesei* RF8793 was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 100, 300 and 1000 mg/kg body weight/day for a period of 91/92 days. A control group was treated similarly with the vehicle, bidistilled water, only.

Oral administration of the phospholipase A2 enzyme preparation to Wistar rats at doses of 100, 300 and 1000 mg/kg/day, for 91/92 days resulted in no test item-related deaths of definitive toxicological relevance, no test item-related clinical signs at daily or weekly observations (weeks 1-12), no effects on the functional observational battery (week 13, including no effects upon grip strength or locomotor activity), no test item-related effects upon mean absolute or relative food consumption, no changes in mean absolute body weight or body weight gain, no ophthalmoscopic findings, no effects upon the hematology, clinical biochemistry or urinalysis parameters, no differences in organs weights and no macroscopic or microscopic findings of toxicological relevance.

Based on the results of this study, 1000 mg/kg body weight/day of the phospholipase A2 enzyme preparation from *T. reesei* RF8793 was established as the no-observed effect-level (NOEL) and as the no-observed-adverse-effect-level (NOAEL).

7.5. Estimates of Human Consumption and Safety Margin

7.5.1. Estimate Dietary Exposure

As described herein phospholipase A2 is used in the manufacturing of a wide variety of crude oils. The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (*Hansen* (1966); *Douglass et al.* (1997)). This method enables one to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Average consumption over the course of a lifetime/kg body weight/day	***Total solid food (kg)***	***Total non-milk beverages (l)***	***Processed food (=50% of total solid food) (kg)***	***Soft drinks (=25% of total beverages) (l)***
	0.025	0.1	**0.0125**	**0.025**

In Section 6.3., the recommended use levels of phospholipase A2 from *T. reesei* RF8793 are given, based on the raw materials used in the food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food (or beverage) is obtained per kg raw material and it is assumed that all the TOS will end up in the final product and the wide variety of food products based on edible oils that are available to consumers[3]. It is therefore assumed that refined oils are used in the manufacturing of all solid and liquid foods.

[3] A w de variety of products based on edible oils is available to the consuming public. Salad and cooking oils, salad dressings, mayonnaise, deep frying oils, margarines and spreads, chocolate fats, ice cream fats, bakery fats, confectionery filling and coating fats, vegetable fats for dairy products and fats for infant nutrit on are some of the widely available products that are based entirely on fats and oils or contain fat or oil as a principal ingredient. Many of these products also are sold in commercial quant ties to food processors, snack food manufacturers, bakeries and restaurants.

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food or food ingredient	Ratio RM/final food	Maximal level in final food (mg TOS/kg food)
Solid	Oil degumming	Crude vegetable oil	11	Refined oil used in solid foods	1	11
Liquid	Oil degumming	Crude vegetable oil	11	Refined oil used in liquid foods	1	11

The Total TMDI can be calculated on basis of the **maximal** values found in food multiplied by the average consumption of food /kg body weight/day. Consequently, the Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverages (mg TOS/kg bodyweight/day)	Total TMDI (mg TOS/kg body weight/day)
11x0.0125=0.1375	11 x 0.025=0.275	**0.4125**

It should be stressed that this Total TMDI is based on very conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL solid and liquid foods contain edible oils that have been processed with this enzyme

- It is assumed that ALL producers apply the highest use level per application;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food and beverages containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime;
- Assumptions regarding food (and beverage) intake of the general population are overestimates of the actual average levels (*Douglass et al.* (1997)).

7.5.2 Safety Margin

Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions were observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 1000 mg TOS/kg body weight/day.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). The Total TMDI of the food enzyme is 0.4125 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 2424 (e.g. 1000/0.4125)

As is explained above, the Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

8. Conclusion

Results of the toxicity and mutagenicity tests described in Section 7.4 demonstrate the safety of phospholipase A2 preparation from *T. reesei* RF8793, which showed no toxicity or mutagenicity across a variety of test conditions. The data resulting from these studies is consistent with the long history of safe use for *T. reesei* and phospholipase A2 in food processing, and in keeping with the conclusions found in a review of relevant literature. Based upon these factors, as well as upon the limited and well characterized genetic modifications allowing for safe production of the enzyme preparations, it is AB Enzymes' conclusion that phospholipase A2 enzyme preparation from *T. reesei* RF8793 expressing the gene encoding phospholipase A2 from *A. nishimurae/A. fumigauts* is GRAS for the intended conditions of use.

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