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REC'D OCT 2 8 2005

October 26, 2005

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Via Federal Express

Office of Premarket Approval (HFS-200),
Center for Food Safety and Applied Nutrition,
Food and Drug Administration,
200 C St. SW., Washington, DC 20204

Re: GRAS Notification for Phospholipase A_2 derived from *Aspergillus niger*

Dear Sir or Madam:

As counsel for DSM Food Specialties, we are submitting under cover of this letter three copies of DSM Food Specialties' (DSM) GRAS notification of phospholipase A_2 from a genetically modified strain of *Aspergillus niger (A. niger).* DSM Food Specialties has determined through scientific procedures that phospholipase A_2 is generally recognized as safe for use in the food industry as a processing aid in the formation of lyso-phospholipids.

This use of phospholipase A2 derived from *A.niger* is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act (the act) because the notifier has determined that such use is generally recognized as safe (GRAS).

If for any reason the agency has any questions, or requires any additional information to aid its review of DSM's conclusion, please contact me at your earliest convenience.

Sincerely,

Gary L. Yingling

GLY:gly

cc: Dr. Linda Tarantino (w/o encl.)
DSM Food Specialties

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DC-763856 v1

DSM

REC'D OCT 2 8 2005

GRAS notification of phospholipase A_2 from a genetically modified strain of *Aspergillus niger*

Regulatory Affairs
P.O. Box 1
2600 MA Delft
THE NETHERLANDS

GRAS NOTIFICATION FOR PHOSPHOLIPASE A_2 FROM A GENETICALLY MODIFIED STRAIN OF *ASPERGILLUS NIGER*

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

DSM Food Specialties ("DSM") manufactures phospholipase A_2, which is produced by submerged fermentation of a selected, pure culture of *Aspergillus niger*. DSM produces two forms of the preparation; a spray-dried and granulated UF (ultra-filtration) concentrate that is standardized with granulated flour, and a column-purified liquid that is standardized with water. The trade names for the spray-dried product will be Bakezyme and Cakezyme (depending on the application) and the liquid product will be sold under the trade name Maxapal A2.

These phospholipase A_2 preparations are for use in the food industry as a processing aid in the formation of *lyso*-phospholipids, surface-active agents with emulsifying properties. The spray dried and granulated preparation and the liquid preparation are identical in enzyme identity, CAS number, production microorganism, and intended use in promotion of emulsification. Levels of use differ depending on the specific application (e.g., bread, fine bakery ware, egg-yolk-based dressings and sauces). The manufacturing process for both is identical with the exception that the liquid preparation is subject to further purification through column chromatography.

The spray-dried and granulated phospholipase A_2 preparation is to be used in the preparation of bread and fine bakery wares. The enzyme can achieve the same effects as chemical emulsifiers commonly used in the preparation of these foodstuffs, by imparting mixing tolerance, machineability, enhanced air retaining capacity and superior taste and texture. The liquid phospholipase A_2 preparation is intended for use as an egg-yolk treatment in the preparation of egg-yolk based sauces and dressings. The enzyme gives superior emulsifying properties and heat stability in these foodstuffs in comparison to unmodified phospholipids.

Phospholipase A_2 is a heat-stable, calcium-dependent carboxylic ester hydrolase, which catalyses the hydrolysis of 3-*sn*-phospholipids exclusively at the 2-position, giving rise to the formation of 1-acyl-3-*sn*-*lyso*-phospholipids and free fatty acids.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, DSM has determined that its phospholipase A_2 enzyme from GMO *A. niger* is a GRAS substance for the intended food applications 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, DSM's 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, *A. niger*, has a long history of safe use and is discussed in Section 2. FDA has previously affirmed as GRAS lipase, lactase, glucose oxidase, carbohydrase, catalase, glucose oxidase, pectinase, protease, and pectin lyase enzyme preparations from *A. niger*.

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, for phospholipase A_2. In Section 3 data showing phospholipase A_2 to be substantially equivalent to naturally occurring phospholipase A_2 are presented. The safety studies outlined in Section 7 indicate that *A. niger*

and phospholipase A_2 show no evidence of pathogenic or toxic substances. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 4, while Section 5 reviews the strictly hygienic composition, specifications as well as the self-limiting levels of use for phospholipase A_2. Finally, Section 6 provides information on the mode of action, applications, use levels and enzyme residues in final food product for phospholipase A_2.

1.1 Name and Address of Notifier

NOTIFIER

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The Netherlands

MANUFACTURER

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PERSON RESPONSIBLE FOR THE DOSSIER

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1.2 Common or Usual Name of Substance

DSM's phospholipase A_2 enzyme preparation from GMO *A. niger* is produced by submerged fermentation of a selected, pure culture of *A. niger*. The common or usual name of the substance is "phospholipase A_2." It is produced and sold in two forms: a spray-dried and granulated UF concentrate that is standardized with granulated flour, and a column-purified liquid that is standardized with water. The spray-dried product will be sold under the brand names Bakezyme and Cakezyme (depending on the application) and the liquid product will be sold under the brand name Maxapal A_2.

1.3 Applicable Conditions of Use

The spray dried form of phospholipase A_2 will be used for production of bread and egg-yolk-based fine bakery wares. If used for bread, it can be either added to flour at the mill or via the bread improver at the bakery. If used for fine bakery wares, it can be added either directly as one of the ingredients, or via the baking mix.

The liquid form of phospholipase A_2 will be used for the preparation of egg-yolk-based dressings and sauces. The enzyme is added to the liquid egg-yolk in order to hydrolyze the naturally occurring phospholipids. After hydrolysis, the egg-yolk is used to prepare the dressings and sauces.

1.3.1 Substances Used In

Phospholipase A_2 is to be used in foods containing natural phospholipids. Due to the action of phospholipase A_2 these phospholipids are hydrolyzed, resulting in the formation of *lyso*-phospholipids. *Lyso*-phospholipids are surface-active agents with emulsifying properties and are able to mimic the effects of chemical emulsifiers. After hydrolysis, phospholipase A_2 remains in the food as inactive protein. The use of phospholipase A_2 can thus be regarded as a processing aid because it has no function in the finished foodstuff.

1.3.2 Levels of Use

<u>Bread</u>: The average dosage of the enzyme depends on the flour type and quality, the addition of other ingredients like emulsifiers, e.g. lecithin, the time length of and temperatures during the bread-making process, and the type of bakery product produced. In general, phospholipase A_2 will be added in an amount between 2500 and 10000 EYU/kg flour. The maximum amount, which may be added to retain a beneficial effect, would be about 15000 EYU/kg of flour.

Egg-yolk based products (fine bakery wares, dressings and sauces): In general, phospholipase A_2 will be added in an amount between 10000 and 20000 EYU/kg egg-yolk. The maximum amount, which may be added to retain a beneficial effect, would be about 24000 EYU/kg egg-yolk.

1.3.3 Purposes

Phospholipase A_2 is intended to create *lyso*-phospholipids during the preparation of:

- bread (such as tin bread, buns and rolls, French sticks or batards, variety breads like multi grain types of bread, raisin bread, biscuits, crackers),
- egg-yolk based fine bakery wares (high-ratio cake, pound cake, Swiss rolls, snack cakes),
- egg-yolk based sauces, and dressings and other products.

1.3.4 Consumer Population

Because phospholipase A_2 is an enzyme protein naturally occurring in both animals and plants, DSM expects it will be digested as would any other protein occurring in food. It 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), and the phospholipids that form the substrates for phospholipase A_2 are natural constituents of various foods. The *lyso*-phospholipid reaction products of DSM's phospholipase A_2 are also formed in the human body from the action of pancreatic phospholipase A_2 on dietary phospholipids (Rossiter, 1968; Johnson and McDermott, 1974).

Phospholipase A_2 has long been recognized as a normal constituent of wheat flour (Nolte et al., 1974). The phospholipids that are the substrate of phospholipase A_2 and the *lyso*-lecithin formed as the end product of hydrolysis of lecithin by phospholipase A_2 are both also normal constituents of wheat flour (Eliasson and Larsson, 1993; Hargin and Morrison, 1980; Morrison et al., 1975; Clayton and Morrison, 1972). Indeed, *lyso*-lecithin formed by the action of phospholipase A_2 on lecithin was affirmed as GRAS in 1996 (21 C.F.R. 184.1063).

Lyso-lecithin is already naturally present in egg-yolk (Encyclopaedia of Food Science, Food Technology and Nutrition, 1993). Moreover, phospholipase A_2 from pig pancreas is used for the hydrolysis of egg-yolk and has a history of more than 25 years of common use in food as defined by 21 C.F.R. §170.3(f) (Dutilh and Groger, 1981).

As is shown in Section 6.4 of this dossier, the amount of denatured enzyme in the final food is expected to be about 1.7 – 9.1 mg/kg (= 0.00017 – 0.00091%) for bread and 0.9 – 1.8 mg/kg (=0.00009 – 0.00018%) for fine bakery ware. The amount of inactive enzyme in dressings and sauces is expected to be about 0.3 – 1.3 mg/kg (=0.00003 – 0.00013%).

Since phospholipase A_2 is present in food products at such low levels as an inactive protein, and because it is a naturally occurring substance in cells and tissues commonly ingested by humans, it is clear that the consumer population will be unaffected by the presence of phospholipase A_2 in food.

1.4 Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, DSM has determined, through scientific procedures, that its phospholipase A_2 enzyme preparation from GMO *A. niger* is GRAS for use as an enzyme for the hydrolysis of phospholipids in bread dough, fine bakery ware and egg-yolk-based sauces and dressings, in levels not to exceed good manufacturing practices.

1.5 Availability of Information for FDA Review

The data and information that are the basis for DSM's GRAS determination are available for the FDA's review and copies will be sent to FDA upon request. Requests for copies and arrangements for review of materials cited herein may be directed to:

Gary L. Yingling, Esq.
Kirkpatrick & Lockhart, Nicholson Graham LLP
1800 Massachusetts Ave., NW
Washington, DC 20036

2. PRODUCTION MICROORGANISM

2.1 Donor, Recipient Organism and Production Strain

Donor:

The mRNA coding for phospholipase A_2 was derived from pig pancreas tissue and used as a template to produce cDNA *in vitro*.

Recipient organism

The recipient organism used in the construction of the phospholipase A_2 production strain is a glucoamylase (also called amyloglucosidase) and protease negative *Aspergillus niger* strain designated ISO-502 and stored in the DSM Culture Collection as DS 30829. The strain ISO-502 was declared as suitable host strain for the construction of genetically modified organisms belonging to Group I safe micro-organisms by the Dutch authorities.

The strain ISO-502 is derived from the fully characterized DSM *Aspergillus niger* strain GAM-53 (DS 03043) by genetic modification. The strain GAM-53, was derived by several classical mutagenesis steps from *Aspergillus niger* strain NRRL 3122, a strain which was purchased from the Culture Collection Unit of the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, USA.

The fully characterized strain *Aspergillus niger* GAM-53 was isolated by DSM (then: Gist-brocades) in 1982 and selected for its enhanced production of the endogenous enzyme glucoamylase. Since that time, strains of the GAM-lineage have been used at DSM for the large-scale production of glucoamylase, an enzyme that is utilized worldwide in the starch processing industry.

The strain GAM-53 was taxonomically identified as *Aspergillus niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS). This is an independent, internationally recognized laboratory.

The strain GAM-53 contains 7 loci (i.e. the promoter and coding sequences) for the glucoamylase gene. These 7 loci were removed, creating so-called 'plug-sites' (also called Δ*gla*A loci) into which expression units containing various genes can be integrated ('plugged'). The 7 'plug-sites' were each provided with unique restriction sites (also called 'DNA-flags'), marking the location of the 'plug-sites' on the genome. In addition, the gene coding for protease was inactivated.

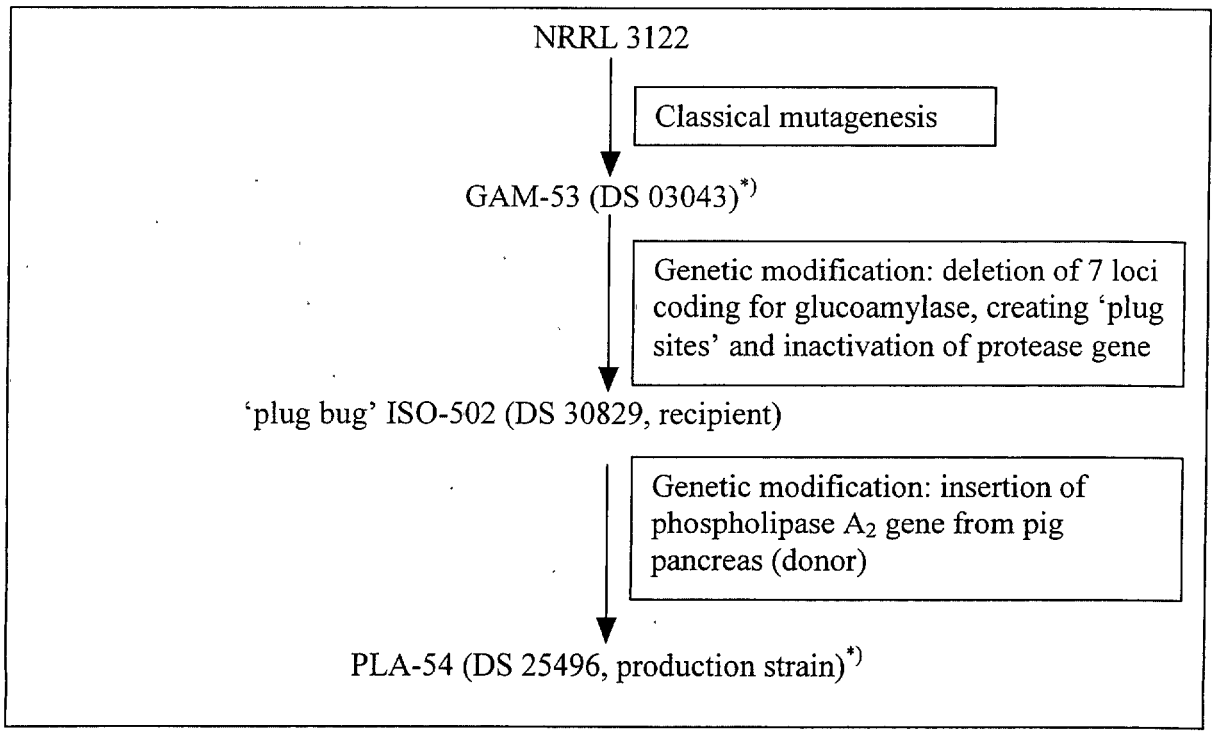
The resulting 'plug bug', designated ISO-502, was classified as a selfcloned GMO by the Dutch competent authorities. It is used as a basic strain for the genetic construction of various enzyme production strains. Apart from phospholipase A_2 (donor: pig pancreas), the recipient ISO-502 was also used for the construction of production strains for arabinofuranosidase (donor: *A. niger*), phytase (donor: *A. niger*), pectin methyl esterase (donor: *A. niger*), glucoamylase (donor: *A. niger*) and amylase (donor: *A. niger*). Additional strains are being made.

The exact technique describing the construction of the 'plug bug' ISO-502 and its advantages are described in literature (Selten et al, 1995 and van Dijck et al, 2003, included as Annex 2.1.1).

Production strain

The phospholipase A_2 production strain was obtained by further genetic modification of the *A. niger* strain ISO-502. The genetic modification techniques used are described in Section 2.2 of this dossier. The production strain was designated PLA-54 and stored in the DSM Culture Collection as DS 35496.

Below, a schematic presentation of the genealogy of the production strain is given:



Strain PLA-54 was taxonomically identified as *Aspergillus niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS).

As is shown in Section 2.4, the production strain complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) micro-organisms. It also meets the criteria for a safe production micro-organism as described by Pariza and Johnson (2001) and other expert groups (Berkowitz and Maryanski, 1989; EU guidelines of the Scientific Committee for Food, 1991; OECD, 1993; Jonas et al., 1996; Battershill, 1993).

2.2 Genetic modification

For the construction of the phospholipase A_2 production strain, two plasmids were used: one to derive the expression cassette, containing the phospholipase A_2 gene, and the other to derive the cassette, containing a selectable marker.

Phospholipase A_2 expression plasmid

The phospholipase A_2 expression plasmid contains strictly defined *A. niger* chromosomal elements (parts of the *A. niger* glucoamylase locus, the glucoamylase promoter and part of the glucoamylase *glaA* gene), the porcine phospholipase A_2 gene, and DNA from a well-characterized *Escherichia coli* vector pTZ18R.

The different elements of the plasmid are:

- 2.1 kb DNA from the flanking *gla*A terminator sequence from the parental *A. niger* strain GAM-53.
- 1.5 kb DNA from the *gla*A promoter from the parental *A. niger* strain GAM-53.
- 1.5 kb DNA from the 5' part of the GAM-53 *glaA* gene, coding for the 498 N-terminal amino acids of the glucoamylase, linked with a synthetic so-called "KEX2 proteolytic site" to the 0.5 kb DNA from the porcine phospholipase A_2 gene (de Geus et al., 1987). The use of a fusion protein comprising "truncated" glucoamylase for efficient production of heterologous proteins has been described Jeenes et al. (1993).
- 2.3 kb DNA from the 3'-flanking *gla*A terminator sequence from the parental *A. niger* strain GAM-53.
- DNA sequences from the *E. coli* plasmid pTZ18R. These sequences are removed prior to transformation of the phospholipase A_2 expression cassette into the host.

The plasmid map is shown in Annex 2.2.1. The nucleotide sequence of the expression cassette is shown in Annex 2.2.2.

Selectable marker plasmid

The selectable marker plasmid contains the same defined parts of the *A. niger* glucoamylase locus as the expression plasmid, the promoter sequence of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*A) gene from the *Aspergillus niger* related fungus *Aspergillus nidulans,* the *A. nidulans amdS* (acetamidase) selectable marker gene and DNA from a well-characterized *Escherichia coli* vector pTZ18R.

The different elements of the plasmid are:

- 2.1 kb DNA from the flanking *gla*A terminator sequence from the parental *A. niger* strain GAM-53.
- 0.9 kb DNA from the *gpd*A promoter from *A. nidulans*.
- 2.2. kb DNA from the *amdS* gene from *A. nidulans*

- 2.3 kb DNA from the 3'-flanking *gla*A terminator sequence from the parental *A. niger* strain GAM-53.
- DNA sequences from the *E. coli* plasmid pTZ18R. These sequences are removed prior to transformation of the amdS selection cassette into the host.

The plasmid map is shown in Annex 2.2.3.

Transformation and selection of the final production strain

The phosholipase A_2 expression cassettes and the selection marker cassettes, both completely devoid of any *E. coli* DNA sequences, are integrated into the genome of the recipient organism ISO-502 by co-transformation following standard methodology. Due to the homology in the 3'- and 3''-*gla*A parts of the two expression units, they are targeted to one of the seven Δ*gla*A loci.

Transformants are selected on their ability to utilize acetamide as sole carbon source. By further analysis transformants are selected that have multiple copies of the phospholipase A_2 expression cassette and one or more copies of the selection marker cassette integrated into one of the Δ*gla*A loci of the recipient strain. The selection of these transformants was done by PCR analyses, applying PLA2 and *gla*A specific primers.

By counter-selection on fluoro-acetamide containing plates, a natural variant of a transformant was selected in which the *amd*S selection marker was deleted as a result of a natural internal recombination event. The absence of the *amd*S marker was confirmed by Southern analysis. The resulting organism is thus not only totally free of *E. coli* DNA, but also of the *amd*S selection marker.

Starting with such a natural variant it is possible to multiply the region comprising the expression unit(s) and the "DNA-flag" into the other Δ*gla*A loci by so-called "gene conversion" (Selten et al., 1998), a natural spontaneous recombination event which does not involve mutagenic treatment. Strains that have an increase in the copy number of the "DNA flag" marking the filled Δ*gla*A locus and a consequent loss of the other "DNA-flags", can easily be identified by DNA gel electrophoresis.

From the available recombinants a strain was chosen that contained sufficient gene copies to allow for commercial attractive expression levels of the phospholipase A_2 enzyme: this strain was designated PLA-54.

The nucleotide sequence of the final phospholipase A_2 expression unit as present in the PLA-54 production organism has been determined.

The expression unit is translated into a glucoamylase-pro-phospholipase A_2 fusion protein. During secretion of this protein by the microbial cell, the endogenous *A. niger* kexine protease separates the pro-phospholipase A_2 from the glucoamylase part at the KEX2 proteolytic site. Also, the pro-part of the phospholipase A_2 is split off, resulting in the mature, active enzyme. The truncated secreted glucoamylase still has full enzymatic activity.

2.3 Stability of the Transformed Genetic Sequence

The strains belonging to the *A. niger* GAM-lineage - from which the host ISO-502 and the recombinant phospholipase A_2 production strain is derived - are genetically stable strains. The whole GAM-lineage is stored for more than 20 years at the DSM laboratory. New cultures are frequently derived from stock material and tested after many generations on morphological-, growth-, production- and product characteristics. These characteristics remain stable except that after plating out a low frequency of morphologic dissimilar colonies are found. This, however, is a normal phenomenon observed for the parental as well as the highly selected industrial strains. The stability of the ISO-strain and the phospholipase A_2 production strain does not differ from the parental GAM-strains.

Since the phospholipase A_2 expression unit is integrated into the genome and since the expression unit does not contain an *E. coli* origin for replication (all *E. coli* sequences have been removed from the plasmid prior to transformation, see Section 2.2), it is not possible that the expression unit will be transferred from the *A. niger* production organism to another, non-related, organism.

2.4 Good Industrial Large Scale Practice (GILSP)

The phospholipase A_2 production organism complies with all criteria for a genetically modified GILSP organism.

The host organism is non-pathogenic, does not produce adventitious agents and has an extended history of safe industrial use (see Section 7.1). The ancestor of the host, GAM 53 (see Section 2.1) has been shown to have a limited survival outside the optimal conditions of the industrial fermentor (see Annex 2.4.1). From the genetic modification performed, there are no reasons 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. In fact, besides the phospholipase gene, the insert only contains host-own sequences. No antibiotic resistance markers or other heterologous markers are present in the strain.

Therefore, the phospholipase A_2 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 Practise (GILSP), as endorsed by the Organisation of Economic Cooperation and Development (OECD). The production organism has been approved both by the Dutch and French competent authorities for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment. In the facilities of DSM Food Specialties for the large-scale production of food and feed enzyme products only fermentations are carried out not exceeding the GILSP level of physical containment.

2.5 Absence of transferable rDNA Sequences in the Enzyme Preparation

Tests have been performed that show that the phospholipase A_2 preparation does not contain any transferable rDNA.

2.6 Absence of Production Organism in the Product

DSM's phospholipase A_2 preparations include the absence of the production organism as an established requirement of the enzyme's manufacturing process, in accordance with the recommendations for safety evaluation by the International Food Biotechnology Committee (IFBC, 1990). All traces of the production organism are removed during the manufacturing process (see Section 4.4), ensuring that the dry and liquid enzyme preparations are free from the production organism *A. niger.*

2.7 Absence of Antibiotic Resistance Gene

As noted above, no antibiotic resistance markers or other heterologous markers are present in the strain. The enzyme preparations are tested to ensure the absence of antibiotic activity in accordance with the recommendation from the Joint Expert Committee of Food Additives of the FAO/WHO ("JECFA"). As is shown in Section 4.7 of this dossier, quality control testing of the finished phospholipase A_2 preparations ensures the enzymes do not contain antibiotic activity.

2.8 Absence of Toxins

Although absence of mycotoxins was mentioned in the specification requirements for fungal enzymes as laid down by the Food Chemicals Codex ("FCC") and JECFA in the past, this requirement has recently been deleted.

Instead, the FCC (5th edition) mentions the following: "*Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.*"

In the General Specifications for enzyme preparations laid down by JECFA in 2001, the following is said: "*The evaluation of enzyme preparations from fungal sources for toxigenicity shall include a determination that they do not contain toxicologically significant amounts of mycotoxins that are known to be synthesised by strains of the production organism's species related to the production micro-organism.*"

Instead of analysing the final enzyme preparations for mycotoxins, DSM therefore decided it more appropriate to test whether the phospholipase A_2 producing micro-organism possesses the intrinsic capacity to produce mycotoxins. This was tested under fermentation conditions, as well as conditions that induce toxin production. The test showed that the production strain does not produce any known toxins under these conditions. Test results are discussed in more detail in Section 7 of this dossier.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

- Systematic name : Phosphatidylcholine 2-acylhydrolase
- Common name : Phospholipase A_2
- Other names : Lecithinase A; Phosphatidase; Phosphatidolipase
- Enzyme Commission No. : 3.1.1.4
- CAS number : 9001-84-7

Phospholipase A_2 belongs to the class of carboxylic ester hydrolases.

3.2 Amino Acid Sequence

As the DNA coding for phospholipase A_2 is derived from the pig pancreas, the amino acid sequence of the enzyme expressed by *Aspergillus niger* is exactly the same. The amino acid sequence of the pig pancreas enzyme has been published in literature (Verheij et al., 1981).

The porcine phospholipase A_2 has a primary sequence of 123 amino acids and a calculated molecular weight of 13980 Da. (de Haas et al, 1968). The porcine pancreatic phospholipase A_2 is not glycosylated (Nieuwenhuizen et al., 1973).

3.3 Sequence Comparison to Other Phospholipase A_2 Enzymes

Comparison of the phospholipase A_2 expressed by *Aspergillus niger* to porcine phospholipase A_2 shows that both enzymes are identical. Using electrospray mass spectrometry, the *Aspergillus niger* phospholipase A_2 showed to have a molecular weight of 13982 Da, which is in good agreement with the theoretical mass of 13980 Da. The last 6 amino acids at the N-terminus of the protein show the same sequence for both the *Aspergillus niger* and the porcine phospholipase A_2, namely Ala_1-Leu_2-Trp_3-Gln_4-Phe_5-Arg_6. This sequence is in full agreement with the mature form of phospholipase A_2 described in literature (Verheij et al., 1981).

3.4 Enzymatic Activity

Principal Enzyme Activity

Phospholipase A_2 catalyses the hydrolysis of 3-*sn*-phospholipids exclusively at the 2-position, giving rise to the formation of 1-acyl-3-*sn*-*lyso*-phospholipids and free fatty acids. Although phospholipase A_2 is able to hydrolyse these phospholipids in their monomeric form, the enzyme is much more active when these substrates are organised in the form of micelles or lipid membranes. The presence of calcium ions is essential for the enzymatic activity of phospholipase A_2.

The US Food Chemical Codex (FCC, 5th edition) lists a method to measure the activity of phospholipase A_2 (see Annex 3.4.1). This method is called the egg-yolk test, as it uses egg-yolk as a substrate. A disadvantage of egg-yolk is that its composition - due to the fact that it is a natural product - is not constant. As a result, activity measurements vary depending on the egg-yolk used. Therefore, the activity measurement has to be repeated on various egg-yolks to get a more or less accurate (mean) value.

To circumvent the above problem, DSM used the FCC method merely to prepare a calibrated and validated phospholipase A_2 standard solution. The known activity of this standard (set at 100%) is then used to calculate the activity of unknown samples by means of a so-called relative method of analysis (see Annex 3.4.2). This method is based on a synthetic substrate (1,2-dithiodioctanoyl phosphatidylcholine), which has a more constant composition, and thus results in higher accuracy of the activity determination, than egg-yolk.

The activity of phospholipase A_2 for the FCC method is expressed in so-called Egg Yolk Units (EYU), whereas the activity of the DSM method is expressed in so-called Chromogenic Phospholipase Units (CPU). As one EYU is equal to one CPU, the abbreviation EYU is used in this dossier. One EYU of phospholipase A_2 activity is defined as the amount of enzyme producing 1 micromole of free fatty acid per minute under the conditions described for the egg-yolk test. The specific activity of *A. niger* phospholipase A_2 is 1000 EYU/mg. This specific activity is comparable with the specific activity found for porcine pancreatic phospholipase A_2 in the original egg-yolk test (Nieuwenhuizen et al, 1974).

The biochemical properties of phospholipase A_2 from porcine pancreas have been investigated extensively (see also Annex 3.4.3). The enzyme has shown to exhibit activity over a pH range between 5 and 9.5. Two pH optima are found, around pH 6.0 and pH 9.0. The temperature optimum for phospholipase A_2 activity is found in between 50 - 60 °C. The enzyme is inactivated (denatured) at temperatures above 80 °C.

Subsidiary enzymatic activities

Like any other living organism, the phospholipase A_2 production organism produces many other enzymes needed for the break down of nutrients and build up of cell material. Although phospholipase A_2 is being produced in excess, the enzyme preparation will also contain minor, non-standardized amounts of these other enzymes, such as glucoamylase, amylase and protease. These do not support the technological function of the enzyme preparation in the application.

For certain applications (e.g. egg-yolk-based sauces and dressings), the enzyme preparation is purified using column chromatography to remove the residual activities of glucoamylase and amylase.

4. MANUFACTURING PROCESS

4.1 Overview

Phospholipase A_2 from DSM is produced by a controlled submerged fermentation of a selected, pure culture of *Aspergillus niger* (see Section 2.1). The production process includes the fermentation process, recovery (downstream processing) and formulation of the product. An overview of the different steps involved is given in Annex 4.1.1.

4.2 Raw Materials

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. This is confirmed by the toxicological studies performed (see Section 7.4 of this dossier). The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

4.2.1 Raw materials for the fermentation process

The raw materials used in the fermentation process are listed below. The list includes the raw materials used for the pre-culture fermentation, the seed fermentation and the main fermentation.

- Dextrose, starch
- Spray dried corn steep liquor, corn steep solids
- Inorganic salts (ammonium sulphate, monosodium phosphate, monopotassium phosphate, manganese sulphate, zinc chloride, copper sulphate, cobalt chloride, ferrous sulphate, calcium chloride, magnesium sulphate)
- Citric acid
- Antifoam (CAS 78041-14-2)[1]
- Water

The fermentation medium used has been developed for optimum production of phospholipase A_2.

4.2.2 Raw materials for the recovery process

The raw materials used in the recovery process are listed below. The list includes the raw materials used for purification and formulation.

- Sodium benzoate
- Filter aid (perlite, dicalite BF)
- Chromatography resin (SP-Sepharose FF)
- Granulating agents (maltodextrin, salts (NaCl, MgSO4))
- Standardizing agent (flour)
- Water

[1] This antifoam is included on the ETA list as an addendum.

4.3 Fermentation Process

The fermentation process consists of three steps: pre-culture fermentation, seed fermentation and main fermentation. The whole process is performed in accordance with Good Food Manufacturing Practice (see Section 5.2).

Pre-culture fermentation

Conserved mycelium of a pure culture of *Aspergillus niger* is aseptically transferred to a sterile fermentation medium. The pre-culture is grown for 48 hours at 34°C, after which it is used as inoculum for the seed fermentation.

Seed fermentation

Before the pre-culture is aseptically transferred to the seed fermentor, the fermentor containing the fermentation medium is sterilised. Growth of the micro-organism takes place during 48 hours at a constant temperature of 34°C, a fixed pH and dissolved oxygen concentration. At the end of the fermentation, the complete content of the fermentor is aseptically transferred as inoculum to the main fermentation.

Main fermentation

Biosynthesis of phospholipase A_2 occurs during the main fermentation. To produce the enzyme of interest, a submerged, aerobic fed batch fermentation process is employed, using a stirred tank fermentor. The fermentor is equipped with devices for pH, temperature, oxygen and antifoam control, a top-mounted mechanical agitator and a bottom air sparger.

Before transfer of the inoculum, the fermentor and the fermentation medium are sterilized. Directly after inoculation till a few hours before the end of fermentation (duration approximately one week), the fermentor is continuously fed with aseptically introduced sterilised fermentation medium. The medium flow rate is used to keep the residual sugar concentration in the broth between 15 and 25 g/kg, in order to prevent carbon limitation.

Excessive foaming is prevented by the controlled addition of antifoam. During the fermentation the medium pH, the temperature and the dissolved oxygen tension are controlled

Growth of the production organism and increase of enzyme production are regularly monitored in the main fermentation by analysis of aseptically collected samples. After the fermentation has been stopped downstream processing will start.

4.4 Recovery Process

The fermentation is stopped by addition of sodium benzoate under conditions that effectively kill off the production organism.

After decreasing the temperature of the broth to approximately 15°C, the cell material is separated from the phospholipase A_2 by means of a simple membrane filtration process. Subsequently, the remaining particles are removed with a polish filtration and a germ reduction

filtration, and then concentrated by ultrafiltration (UF) by a factor 15-30. After Ultrafiltration and pH adjustment, the UF concentrate is polish-filtrated, followed by another germ reduction filtration.

4.5 Purification Process

For certain food applications, the UF concentrate is further purified by column chromatography. For this, a SP-Sepharose FF resin is used. The safety evaluation for the use of this column material has been performed.

4.6 Formulation and Standardization Process

In case of the column-purified product, the eluate (purified product) is diluted with water to a 1% solution and the pH adjusted. As a preservative, sodium benzoate is added to the final product. The product is standardised with water to an enzyme concentration of 10000 EYU/ml. This liquid product is used for certain applications – dressings and sauces.

For other applications - depending on the demands of the market - the UF concentrate is dried and granulated as such or granulated on maltodextrins or salts (e.g. NaCl, $MgSO_4$), resulting in a product with an enzyme activity ranging from 10.000 to 25.000 EYU/g and a particle size between 63 and 225 micrometer. Subsequently, the product is standardised with granulated flour to various enzyme concentrations.

4.7 Quality Control of Finished Product

In accordance with the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives (JECFA) of the FAO/WHO in 2001 and the FCC (5th edition), the final phospholipase A_2 preparations (dry and liquid) from *Aspergillus niger* meet the following specifications:

ITEM	NORM
Lead	< 5 mg/kg
Antimicrobial activity	Absent by test
Coliforms	< 30/g
Salmonella	0/25g
Escherichia coli	0/25g
Anaerobe sulphite reducing bacteria	< 30/g
Staphylococcus aureus	0/g
Listeria monocytes	0/25g
Yeasts	<10/g
Moulds	<10/g
Total viable count	$< 5*10^4$/g

The additional characteristics specified by DSM for the different formulations are:

Liquid preparations:

ITEM	NORM
Appearance	Pale yellow liquid
Phospholipase activity	10000 - 11500 EYU/ml
Glucoamylase activity	< 10 AGI/ml
Amylase activity	< 0.05 FAU/ml

Dry preparations:

ITEM	NORM
Appearance	Off white granulates
Phospholipase activity	10000 – 25000 EYU/g*
Glucoamylase activity	< 75000 AGI/g
Dry matter	> 92%

* activity depending on the formulation

5. COMPOSITION AND SPECIFICATIONS

5.1 Formulation

The common starting material for all formulations is the UF concentrate. Typically, its composition falls within the following ranges:

Item	Value	Unit
Enzyme activity	2200-3000	EYU/g
Water	78-85	%
Ash	1.2-1.4	%
Proteins (N x 6.25)	7.0-10	%
Sugars	4.5-8	%
Lipids	< 0.1	%

Apart from the enzyme complex, the phospholipase A_2 preparations will also contain some substances derived from the micro-organism and the fermentation medium. These harmless contaminations consist of polypeptides, proteins, carbohydrates and salts.

The Total Organic Solids ("TOS") of the phospholipase A_2 preparation were calculated from 3 different batches of the UF concentrate:

Calculation of the TOS					
Batch number	Water (%)	Ash (%)	TOS (%)	Activity (EYU/g)	EYU/g TOS
PLA 9901 (tox batch)	85.3	1.2	13.5	2230	16519
RER 101	81.4	0.71	17.9	4620	25810
RER 402	78.1	1.2	20.7	6940	33527
MEAN	81,6	1.04	17.4	4579	25285

In the case of the dry enzyme preparations, the UF concentrate is spray dried and formulated with the following carriers and ingredients (see also Section 4.6):

- Maltodextrins or salts (e.g. NaCl, $MgSO_4$)
- Granulated wheat flour

The TOS values of the final standardized dry enzyme preparations can be easily calculated on basis of the above values, taking the dilution factor into account. For instance, a standardized dry enzyme preparation containing 2500 EYU/g will have a TOS value of about 100 mg/g enzyme preparation.

000023

In the case of the liquid enzyme preparation, the UF concentrate is purified by column chromatography and subsequently formulated with the following carriers and additives (see also Section 4.6):

- Water
- Sodium benzoate

Because of the purification step, the column purified enzyme preparation has a higher activity per gram TOS than that of the UF concentrate. Analysis of the column purified preparation has shown that the activity per gram TOS is at least 800000, thus more than a factor 30 higher.

5.2 General Production Controls and Specifications

Commercial demands require a strictly controlled fermentation process.

The enzyme fermentation factory at Seclin, France, which has fermentation experience of over 60 years, has acquired the ISO 9001-2000 certification.

Technical measures

The batches of **primary seed material** are prepared, preserved and stored in such a way that contamination and degeneration is avoided and genetic stability is secured. The vials are clearly labelled and strict aseptic techniques are applied during the recovery of the culture.

Only sterilised **raw materials** are used to prepare the nutrient medium for the fermentation.

The **fermentor** is a contained system. Only sterilised air is used in the fermentation. Membrane valves, air filters and seals are regularly checked, cleaned and replaced if necessary. Prior to inoculation, the fermentor is cleaned, rinsed and sterilized. The sterilised nutrient medium and the complete biomass broth are transferred aseptically to the main fermentor. The methods used effectively prevent microbial contamination during fermentation.

The preparation of sterile media and the cleaning of the equipment are laid down in Quality Assurance documents and strictly followed.

Microbial contamination is prevented during **downstream processing** by several germ reduction filtrations. The filters are thoroughly cleaned for each production run.

Control measures

After preparation of a new batch of **primary seed material**, samples are checked for identity, viability and microbial purity. If these parameters are correct, the strain is tested for production capacity. Only if the productivity and the product quality meet the required standards, the new batch of primary seed material will be accepted for further production runs. Each time a vial from such a certified batch of primary seed material is used for production, the viability, purity and identity of the strain is checked.

The **raw materials** used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. This is confirmed by the toxicological studies performed (see Section 7.4 of this dossier). The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

At regular intervals during the **seed fermentation** manual samples are taken aseptically for analysis of pH, and microbiological quality in the laboratory.

During the **main fermentation** the dissolved oxygen content, pH, temperature, viscosity, enzyme activity and microbial quality are monitored. If microbial controls show that contamination has occurred, the fermentation will be discontinued.

Also during **downstream processing** samples are being taken and checked for the level of microbial contamination. If these checks show that contamination has occurred, the downstream processing will be discontinued.

The finished product is subjected to extensive controls and complies with JECFA and FCC specifications: see Section 4.7: Quality Control of Finished Product.

6. APPLICATION

6.1 Mode of Action

Phospholipase A_2 hydrolyzes natural phospholipids present in foodstuffs, resulting in the formation of *lyso*-phospholipids. *Lyso*-phospholipids are surface-active agents with emulsifying properties and are able to mimic the effects of chemical emulsifiers. After hydrolysis, phospholipase A_2 remains in the food as inactive protein. The use of phospholipase A_2 can thus be regarded as a processing aid, having no function anymore in the finished foodstuff.

6.2 Application

6.2.1 Bread

Bakeries all over the world use chemical emulsifiers in bakery dough products in order to strengthen the dough. Examples of such emulsifiers are DATEM (diacetyl tartaric acid ester of mono/di-glycerides), SSL (sodium stearoyl lactylate) and CSL (calcium stearoyl lactylate). Their E-numbers are 472e, 481 and 482, respectively.

Dough strengthening includes many functional qualities imparted to the dough and to the bread produced from it: improvement of dough mixing tolerance and machineability as well as enhancement of gas retaining capacity resulting into increased loaf volume, crispy crust, improved crumb texture and improved crumb softness.

Phospholipase A_2 can be used to replace the above-mentioned chemical emulsifiers. During mixing and subsequent fermentation of the dough, phospholipase A_2 hydrolyses the phospholipids naturally present in the wheat flour. This results in the formation of *lyso*-

phospholipids. *Lyso*-phospholipids are surface-active agents with emulsifying properties and are able to mimic the effects of the above-mentioned chemical emulsifiers.

However, the amount of available phospholipids in wheat flour is too low (about 0.3 %) to obtain a sufficient amount of *lyso*-phospholipids after addition of phospholipase A_2. Therefore, by adding (soy) lecithin (0.5 % w/w) the concentration of phospholipids in the wheat flour can be increased. Soy lecithin (E322) is commonly used for bread making and contains about 30% of phospholipids. After hydrolysis of the lecithin by phospholipase A_2, an amount of *lyso*-phospholipids (*lyso*-lecithin) is formed which is sufficient for an optimum effect in bread making.

In Annex 6.2.1, the results of both laboratory and commercial scale bakery trials are given. They confirm the benefits of using phospholipase A_2 in the absence and presence of soy lecithin.

6.2.2 Egg-yolk-based fine bakery wares

Bakeries all over the world use chemical emulsifiers in the production of bakery batter products. Examples of such emulsifiers are mono/diglycerides of fatty acids and polyglycerol esters of fatty acids. Their E-numbers are 471 and 475, respectively.

The efficiency of air incorporation and retention in batter-derived products like sponge and pound cakes depend on the film-forming capacity and the speed by which the air bubbles escape from the batter. Emulsifier/stabilisers are applied to provide the necessary aeration and gas bubble stability during the baking process until the cake structure is set. Emulsifiers also help to achieve a uniform distribution of bubble sizes in the batter. The bubble structure and distribution depends on the type of emulsifier used.

Phospholipase A_2 can be used to replace at least some of the chemical emulsifiers mentioned above. During mixing of the ingredients, phospholipase A_2 hydrolyses the phospholipids naturally present in the eggs that are part of the batter recipe. This reaction results in the formation of *lyso*-phospholipids. *Lyso*-phospholipids are surface-active agents with emulsifying properties and are able to mimic the effects of the mentioned chemical emulsifiers.

In Annex 6.2.2 results of laboratory scale bakery tests are given.

6.2.3 Egg-yolk-based sauces and dressings

Egg-yolk is used in mayonnaise, sauces and dressings because of its emulsifying properties. These emulsifying properties are due to the naturally occurring phospholipids in egg-yolk. Egg-yolk contains about 9% phospholipids, 70% of which is lecithin and 3% *lyso*-lecithin. The emulsifying properties of *lyso*-phospholipids like *lyso*-lecithin are much higher than the ones of phospholipids.

Conversion of phospholipids into *lyso*-phospholipids by phospholipase A_2 thus gives the egg-yolk superior emulsifying properties.

The conversion of phospholipids into *lyso*-phospholipids also increases the heat-stability of the egg-yolk. Egg-yolk treated with phospholipase A_2 is heat-stable up to 70-80°C, whereas untreated egg-yolk is stable only up to 60°C.

In addition, the treatment with phospholipase A_2 results in egg-yolk with a 30-35% higher viscosity than the untreated egg-yolk.

Due to these improved characteristics, phospholipase A_2-treated egg-yolk can be used in more applications than untreated egg-yolk, including substitution of chemical emulsifiers. Moreover, the heat-stable properties of phospholipase A_2-treated egg-yolk allow warm pasteurization, resulting in increased microbial safety and a longer shelf-life.

DSM conducted laboratory tests on the efficiency of phospholipase A_2 in the hydrolysis of egg-yolk phospholipids. DSM then compared the firmness and heat stability of mayonnaise made with hydrolyzed egg-yolk to mayonnaise produced with untreated egg-yolk. Test results demonstrated the positive effect of phospholipase A_2-treated egg-yolk in the production of mayonnaise. These tests are described in Annex 6.2.3.

6.3 Use Levels

Enzyme preparations are generally used in quantum satis ("Q.S."). The average dosage of the enzyme depends on the flour type and quality, the addition of other ingredients such as emulsifiers like lecithin, time length of the bread making process and temperature during that process, and the type of product being made. Section 1.3.2 describes the levels of use expected to result in beneficial effect.

6.4 Enzyme Residues in the Final Food

6.4.1 Baked goods (bread and fine bakery ware):

The action of phospholipase A_2 takes place in the dough during mixing, proofing, and in the early stage of baking. As mentioned in Section 3.4, the enzyme is inactivated (denatured) at temperatures above 80°C. Because the temperature inside the dough during baking reaches levels higher than 80°C no enzyme activity is expected to remain in the finished product.

To verify this assumption an experiment was conducted using the laboratory procedure described in Annex 6.4.1. Phospholipase A_2 was added in the fairly high amount of 10.000 EYU/kg flour. Following baking the enzyme was extracted from the breadcrumb and its activity was determined. Under the conditions used no phospholipase A_2 activity could be detected.

Based on the information given in Section 1.3.2 and the specific activity of the enzyme of 1000 EYU/mg (see Section 3.4), the following calculation can be made:

Final food	Enzyme use level in food ingredient	Amount of ingredient in final food	Residual amount of (denatured) enzyme in final food (in EYU)	Residual amount of (denatured) enzyme in final food (in mg)
Bread	2500-10000 EYU/kg flour	670-910 g flour/kg bread	1675-9100 EYU/kg bread	1.675 – 9.1 mg/kg bread
Fine bakery wares	10000-20000 EYU/kg egg-yolk	87.5 g egg-yolk/kg fine bakery ware	875-1750 EYU/kg fine bakery ware	0.875 – 1.75 mg/kg fine bakery ware

6.4.2 Egg-yolk-based dressings and sauces:

Phospholipase A_2 hydrolyses the phospholipids naturally present in egg-yolk. After hydrolysis, the substrate for the enzyme (i.e. the phospholipids) is depleted and the enzyme remains inactive in the egg-yolk like any other protein.

Based on the information given in Section 1.3.2 and the specific activity of the enzyme, the following calculation can be made:

Final food	Enzyme use level in food ingredient	Amount of ingredient in final food	Residual amount of (inactive) enzyme in final food	Residual amount of (inactive) enzyme in final food
Dressings and sauces	10000-20000 EYU/kg egg-yolk	33-65 g egg-yolk/kg dressing	330-1300 EYU/kg dressing	0.33 – 1.3 mg/kg dressing

6.4.3 Main Reaction Products and Possible Reaction Products Considered Normal Dietary Constituents

Phospholipase A_2 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). The substrates for phospholipase A_2, phospholipids, are naturally constituents of various foods. The reaction products, *lyso*-phospholipids, form in the human body from the action of pancreatic phospholipase A_2 on dietary phospholipids (Rossiter, 1968; Johnson and McDermott, 1974).

Enzyme modified lecithin, i.e. *lyso*-lecithin formed by the action of phospholipase A2 on lecithin, was affirmed as GRAS in 1996 (21 C.F.R. 184.1063).

Baked goods

Phospholipase A_2 is a normal constituent of wheat flour (Nolte et al., 1974). Also naturally constituents of wheat flour are the substrate of phospholipase A_2, phospholipids and the end product of the hydrolysis of lecithin by phospholipase A_2, *lyso*-lecithin (Eliasson and Larsson, 1993; Hargin and Morrison, 1980; Morrison et al., 1975; Clayton and Morrison, 1972).

Based on the above it can be concluded that the addition of extra phospholipase A_2 with lecithin therefore does not lead to new or unintended reaction products in foods produced with wheat flour.

Dressings and sauces

The reaction product *Lyso*-lecithin is already naturally present in egg-yolk (Encyclopaedia of Food Science, Food Technology and Nutrition, 1993). Moreover, phospholipase A_2 from pig pancreas has been used for the hydrolysis of egg-yolk already for more than 25 years (Dutilh and Groger, 1981).

Based on the above it can be concluded that the addition of phospholipase A_2 from *Aspergillus niger* does not lead to new or unintended reaction products in egg-yolk-based food products.

6.4.4 Possible Effects on Nutrients

Based on the information given above, there are no reasons to expect any possible effects on the nutrients in baked goods, sauces or dressings.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the IFBC, food or food ingredients are safe to consume if they have been produced, according to current Good Manufacturing Practices, from a nontoxigenic and nonpathogenic organism (IFBC, 1990). A nontoxigenic organism is defined 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" (Pariza and Foster, 1983).

A niger is not a human pathogen and it is not toxigenic. It is known to naturally occur in foods. The fungus is commonly present in products like rice, seeds, nuts, olives and dried fruits.

For several decades, *A. niger* has been safely used in the commercial production of organic acids and various food enzymes, such as glucose oxidase, pectinase, alpha-amylase and glucoamylase. Industrial production of citric acid by *A. niger* has taken place since 1919 (Schuster et al., 2002, attached as Annex 7.1.1).

This long experience of industrial use has resulted in a good knowledge of the characteristics of *A. niger* and understanding of the metabolic reactions.

The long industrial use and wide distribution of *A. niger* in nature has never led to any pathogenic symptoms. The nonpathogenic nature of *A. niger* has been confirmed by several experimental studies (see Annex 7.1.1). *A. niger* is therefore generally accepted as a nonpathogenic organism.

Even though products from *A. niger* have been used in food for many decades, there has never been any evidence that the industrial strains used are able to produce toxins. The non-toxicogenicity has been confirmed by toxicological tests, as well as batch testing of the various end products for toxins.

The toxicological studies performed on various enzyme preparations from *A. niger* provided the basis for a safety evaluation by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO in 1987 (see Annex 7.1.2). Although not justified by the results of the toxicological studies, JECFA first allocated a numerical Acceptable Daily Intake (ADI) to enzyme preparations of *A. niger*, based on the concern that some strains may produce unknown toxins. Two expert reports submitted to JECFA in 1988 concluded that the production of toxins was highly unlikely (see Annex 7.1.3). The long history of use as an enzyme source, the numerous toxicological studies and the two expert reports caused JECFA to review its decision in 1990 and change the ADI for enzyme preparations derived from *A. niger* into "not specified." See Annex 7.1.4 to this notification for JECFA's 1990 review.

In addition to the positive evaluation of JECFA, countries which regulate the use of enzymes, such as the USA, France, Denmark, Australia and Canada, have accepted the use of enzymes from *A. niger* in a number of food applications.

Strains belonging to the *Aspergillus niger* GAM-lineage as well as the host strain ISO-502 from DSM were declared suitable host strains for the construction of genetically modified organisms belonging to Group I safe micro-organisms by the Dutch authorities.

The *A. niger* GAM-53 strain, which is being used as the parental strain of the host organism, has already been used as host for the selection of genetically modified production strains, by the process of random integration, for the production of the enzymes phytase and xylanase. These strains are used by DSM on industrial scale since 1991 and 1996, respectively.

The recombinant phospholipase A_2 production strain PLA54 (DS35496) has been classified by the Dutch authorities as well as the French Genetic Committee as a Group I safe micro-organism. Consequently, the strain was approved for large-scale production of phospholipase A_2 in the DSM factory in Seclin, France.

Based on the genetic modification performed (see Section 2.2), there are no reasons to assume that the recombinant production strain should be less safe than the original GAM-53 (DS 03043) strain. In fact, it has been shown that the DSM GAM/ISO lineage of *A. niger* strains are safe hosts for the over-expression of enzymes to the extent that for new enzymes the safety is already covered by the safety studies performed on other enzyme production strains derived from this lineage and consequently new safety studies are superfluous (van Dijck et al, 2003, see Annex 2.1.1).

At the end of the fermentation, the recombinant production organism is effectively killed off (see Section 4.4).

Specific tests have been performed to confirm that the recombinant phospholipase A_2 production strain is not able to produce any toxins under fermentation conditions, nor under conditions that

are known to induce toxin production in general. The results of these tests showed that the production strain does not produce any known toxins under these conditions.

7.2 Safety of the Phospholipase A_2 Enzyme

As noted above, enzymes produced by *Aspergillus niger* have already been used for food production for several decades. In the USA, various enzymes from *Aspergillus niger* have been notified as "GRAS" (Generally Recognised as Safe), such as carbohydrases (amylase and glycoamylase), proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132) and lipase (GRN 000111).

A similar phospholipase A_2 preparation, from *S. violaceoruber,* has already been the subject of a GRAS notification, in 2004 (GRN 000145).

The Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO has evaluated several enzymes from Aspergillus niger for their safety (WHO Food Additives Series 6 (1975) and 22 (1988)). All these enzymes have received an ADI "not specified" by JECFA.

Since it is generally known that commercial enzyme preparations of *Aspergillus niger* are not toxic and since phospholipase A_2 is a natural constituent of pancreatic juice and food (see Section 6.4), it is not expected that phospholipase A_2 would have any toxic properties.

The enzyme preparation phospholipase A_2 derived from *Aspergillus niger* strain PLA-54, over-expressing the phospholipase A_2 gene from porcine pancreas, was evaluated according the Pariza & Johnson Decision Tree. The decision tree is based on the safety evaluation methodology published by Pariza and Foster in a 1983 article, which was extended by the IFBC into the decision tree format and published in 1991. In 2001, Pariza and Johnson published an update. DSM's decision tree analysis, based on the most recent update of the decision tree, is described in Annex 7.2.

To confirm the assumption that phospholipase A_2 would not have any toxic properties and to further establish the toxicological safety of the use of phospholipase from *Aspergillus niger* in food, the following additional studies were performed on the UF concentrate:

- Acute oral toxicity 2x,
- Subacute (14-day) oral toxicity study,
- Subchronic (90-day) oral toxicity study,
- Ames test,
- Chromosomal aberration, *in vitro,*
- Micronucleus mice, *in vivo.*

No adverse effects or mutagenic activity were discovered in the studies, which are described in further detail at Section 7.4.1.

7.2.1 Allergenicity

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are

food allergens. Below we describe briefly why ingestion of enzymes used as food processing aids is unlikely to elicit an allergic response after consumption.

Enzymes are proteins with highly specialized catalytic functions. They are produced by all living organisms and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are natural protein molecules that act as very efficient catalysts of biochemical reactions.

The unique role of enzymes in food processing is as a catalyst. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a food. The use levels are based on the activity of the enzyme, not the amount of the enzyme product. Under these circumstances, the enzyme used is classified as an incidental additive, and where it has no functional effect on the finished food and is present in an insignificant amount, it need not be included in the ingredient label.

Because exposure to enzymes used as processing aids in food is very low, even if they were potentially allergenic by the oral route, the likelihood of allergic sensitization of consumers to these proteins is virtually nil. The absence of food allergenicity has been confirmed by an extensive literature search and survey of producers' files, in which no cases have been found of people that have been sensitized or that reacted allergic by ingestion of food prepared with various enzymes (see Annex 7.2.1). Even people who ingest high daily doses of enzymes as digestive aids are not reported to have gastrointestinal allergy to enzymes even after many years of daily intake.

7.2.2 Leading Enzyme Publications on the Safety of Phospholipase A_2 Enzymes or Enzymes That are Closely Related

The safety of the production organism is the point of focus as to the safety of the enzyme which will be used in food processing. In this case, the production organism *A. niger*, has been demonstrated to be nontoxigenic and nonpathogenic 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 nonpathogenic organism was very unlikely to produce a disease under ordinary circumstances. In their publication, *A. niger* is included in the authors' listing of the organisms being used in the industry.

The FDA has also accepted the Enzyme Technical Association's GRAS Notifications stating that carbohydrase, pectinase, protease, glucose oxidase and catalase (GRAS No. 000089), lipase (GRAS No. 000111) and lactase (GRAS No. 000132) enzyme preparations from *A. niger* are generally recognized as safe. *A. niger* is listed as a production organism for enzymes (Pariza and Johnson, 2001) and has a long history of safe use (see Annex 7.1.1).

As is clear from the information provided in this notification, there have been genetic modifications to the *A. niger* used by DSM, 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.

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 IFBC 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.

Phospholipase A_2 was included in the safety evaluation by Pariza and Johnson (2001), and the result of a 90-day oral toxicity study was included in a publication by van Dijck et al. in 2003 (see Annex 2.1.1). Egg-yolk hydrolyzed with phospholipase A_2 has been found to have no effect on the physiological parameters of rats in short term feeding trials (Dutilh and Groger, 1981).

In addition, *lyso*-lecithin formed by phospholipase A_2 was affirmed as GRAS in 1996 by FDA (21 C.F.R. § 184.1063).

7.2.3 Substantial Equivalence

Several expert groups, as well as FDA and FDA scientists, have discussed the concept of substantial equivalence relative to food safety assessment. Essentially, all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA has applied this concept in the determination that several enzyme preparations are safe for use in food. In particular, FDA has considered differences in glycosylation between enzyme proteins. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to chemical modifications, or site-direct mutagenesis, would not raise safety concerns.

As is described in Section 3.3 of this dossier, the phospholipase A_2 enzyme produced by GMO *A. niger* is substantially equivalent to the phospholipase A_2 enzyme derived from pig pancreas, an enzyme which already has a history of safe use.

7.3 Safety of the Manufacturing Process

Phospholipase A_2 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 A_2 preparation is produced in accordance with current good manufacturing practices, 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 using DSM's phospholipase A_2 preparation.

7.4.1. Safety Studies in Summary

All safety studies were performed according to international accepted guidelines (OECD) and are in compliance with the principles of Good Laboratory Practice (FDA/OECD).

To check for possible irritating effects on the gastrointestinal tract due to bioactivity of the phospholipase A_2 preparation when used in oral gavage studies, both an EDTA inactivated tox-batch (batch PLA9901-inactivated; 70 EYU/g) and a phospholipase A_2 enriched tox-batch (batch PLA9901-enriched; 9750 EYU/g) were prepared and tested at a single high oral dose in the rat. Since no adverse effects were observed it was decided to perform the necessary toxicity studies with the native, active preparation (batch PLA9901; 2230 EYU/g, TOS 13.5%).

Levels used in the 14- and 90-day oral gavage studies were chosen to provide a sufficient margin of safety towards expected exposure (see Section 7.5) and to reach the toxicologically relevant limit dose of 1000 mg/kg above which testing is only necessary when expected exposure warrants this.

1. Acute oral toxicity

(a). Acute oral toxicity with enriched tox-batch

The tox-batch (PLA9901, enriched) was administered by oral gavage to three Wistar rats of each sex at 23400 mg/kg body weight (bw). Animals were subjected to daily observations and weekly determination of body weight. Macroscopic examination was performed after terminal sacrifice (day 15).

Results

No mortality occurred
Lethargy was noted in all males on day 1 and red staining of the neck was noted in one female on days 1,2 and 10
The mean body weight gain shown by the animals over the study period was considered to be normal
No abnormalities were found at macroscopic post mortem examination of the animals.

(b). Acute oral toxicity with inactivated (denatured) tox-batch

The tox-batch (PLA9901, inactivated) was administered by oral gavage to three Wistar rats of each sex at 21200 mg/kg bw. Animals were subjected to daily observations and weekly determination of body weight. Macroscopic examination was performed after terminal sacrifice (day 15).

Results

No mortality occurred

000034

Lethargy was noted in all animals on day 1.
The body weight gain shown by the animals over the study period was considered to be normal.
No abnormalities were found at macroscopic post mortem examination of the animals.

2. 14-days oral toxicity

The subacute oral toxicity was examined in a 14-day study with four groups of five male and five female Wistar rats, which received the tox-batch (PLA9901), by oral gavage, at dosages of 500, 2000 and 10000 mg/kg bw/day. A similar constituted control group received water. Clinical signs, body weights, food consumption, organ weights, haematology and clinical biochemistry parameters and macroscopy were studied.

Results

From the results present a No Observed Adverse Effect level (NOAEL) of 10000 mg/kg/day was concluded. Dose levels for the 90-day oral toxicity study in Wistar rats were selected to be 0, 500, 2000 and 10000 mg/kg bw/day.

3. 90-days oral toxicity

The sub-chronic oral toxicity of the tox-batch (batch PLA9901) was examined in a 90-day study with groups of 10 male and 10 female SPF-bred CD rats. The rats received the tox-batch daily by gavage at dose levels of 500, 2000 and 10000 mg/kg/day. A similar constituted control group received the vehicle (water) alone. In addition to the 14-day study, ophthalmoscopy, histopathology and functional performance were examined.

Results

From the results a No Observed Effect level (NOEL) of 2000 mg/kg/day was concluded. The No Observed Adverse Effect Level (NOAEL), based on the absence of any functional disturbance and morphological changes in the treated animals, was concluded to be 10000 mg/kg/day.

4. Mutagenicity tests

(a). AMES test

The tox-batch (batch PLA9901) was examined for its mutagenic potency in four histidine-requiring *Salmonella typhimurium* mutant strains: TA 98, TA 100, TA 1535 and TA 1537 and one tryptophan-requiring *Escherichia coli* mutant strain WP2uvrA in two independent experiments.

Tester bacteria were exposed to five concentrations ranging from 100 to 5000 µg dry matter/plate in the absence and presence of rat liver-derived metabolic activation system (S9-mix). Negative and positive controls were run simultaneously with the test.

The tox-batch did not induce a dose-related increase in the number of revertant colonies in each of the five tester strains both in the absence and presence of S9-metabolic activation. This result was confirmed in an independently repeated experiment. The positive control substances gave the expected strong increase in the number of revertants.

Based on the results of this study it is concluded that the tox-batch is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay.

(b). Chromosomal aberration test, *in vitro*

The tox-batch (batch PLA 9901) was examined for its effect on the induction of chromosomal aberrations in cultured human peripheral lymphocytes in the presence and absence of a metabolic activation system (Acroclor-1254 induced rat liver S9-mix).

(i). Experiment 1
In both the absence and presence of S9-mix the tox-batch was incubated with the cells during 3 hours in concentrations up to 5000 µg/ml with a 24 hours fixation time. No statistically or biologically significant increase in the number of cells with chromosome aberrations was observed.

(ii). Experiment 2
In the absence of S9-mix, at the continuous treatment of 24 hours, the tox-batch did not induce a statistically or biologically significant increase in the number of cells with chromosome aberrations at concentrations tested as high as 3300 µg/ml. However, at the continuous treatment time of 48 hours, the tox-batch induced a statistically significant dose-related increase in the number of cells with chromosome aberrations at toxic concentrations of 4200 and 5000 µg/ml.

In the presence of S9-mix, concentrations up to 5000 µg/ml were incubated for 3 hours with a fixation time of 48 hours. 1000 µg/ml induced a statistically significant increase in the number of cells with chromosome aberrations when gaps were included. Since no statistically significant increase was found when gaps were excluded, the increase was observed at the lowest concentration of 1000 µg/ml only, and, moreover, the number of cells with chromosome aberrations was within the historical control data range, the increase is not considered biological relevant.

To obtain more information about the clastogenic results found in experiment 2 an additional experiment (experiment 3) was performed in the absence of S9-mix, with a 48-hour continuous treatment time with the inactivated form of the tox-batch up to 1300 µg/ml.

(iii). Experiment 3
The inactivated tox-batch induced a statistically significant dose-related increase in the number of cells with chromosome aberrations at all concentrations tested.

Since there is a dose-related statistically significant increase in the number of cells with chromosome aberrations following treatment with the tox-batch and with the inactivated tox-batch in the absence of a metabolic activation system at the continuous treatment of 48 hours, the tox-batch and inactivated tox-batch should be considered as clastogenic under the experimental conditions of this *in vitro* test.

It must be noted that EDTA was used for the inactivation of tox-batch. Some published articles showed that EDTA has the ability to induce chromosome aberrations, this might have affected the clastogenic result observed in the third experiment.

(c). Mouse micronucleus test

The tox-batch (batch PLA 9901) was examined for its genotoxic effect on erythrocytes in bone marrow. Six groups each compromising 5 males and 5 females, received a single oral intubation. Two groups were dosed with 2000 mg/kg body weight, two groups were dosed with 1000 mg/kg body weight and two groups were dosed with 500 mg/kg body weight. Another group treated with a single oral intubation of cyclophosphamide (CP) at 50 mg/kg body weight served as positive control.

Cyclophosphamide induced a statistically significant increase in the number of micronucleated polychromatic erythrocytes in both sexes.

No increase in the frequency of micronucleated polychromatic erythrocytes was observed in the polychromatic erythrocytes of the bone marrow of animals treated with the tox-batch.

It is concluded that the tox-batch is not mutagenic in the micronucleus test under experimental conditions of the test.

7.5 Estimates of Human Consumption and Safety Margin

On the basis of the information given in Section 6.4, the following estimation of the human consumption can be made:

Final food	**Residual amount of (inactive) enzyme in final food (EYU/kg)**	**90th percentile intake level (g food/ per person/day[1])**	**Estimated daily intake of (inactive) enzyme (EYU/kg body weight/day)[2]**
Bread	1675-9100	172	4.8-26.1
Fine bakery wares	875-1750	88	1.3-2.6
Dressings and sauces	330-1300	36	0.2-0.8
Total			**6.3-29.5**

1) Intake levels based on Wilkinson Enns et al (1997). 90th percentile is 2 times the intake level.
2) Calculated for a person of 60 kg.

The 90-day oral toxicity study (see Section 7.4) showed a No Observed Adverse Effect Level (NOAEL) of 10000 mg enzyme preparation/kg body weight/day, which corresponds to 22300 EYU/kg body weight/day. Thus the Margin of Safety lies between 22300/29.5=756 and 22300/6.3=3540.

7.6 Results and Conclusion

Results of the toxicity and mutagenicity tests described in Section 7.4.1 demonstrate the safety of DSM's phospholipase A_2 preparation, 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 *A. niger* and phospholipase A_2 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 DSMs' conclusion that phospholipase A_2 preparation from *A. niger* is GRAS for the intended conditions of use.

8. LIST OF APPENDICES

2.1.1	Dijck, P.W.M. van, Selten, G.C.M., Hempenius, R.A., On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains, Regulat. Toxicol. Pharmacol. 38:27-35 (2003)
2.2.1	Figure of the phospholipase A_2 expression plasmid, the vector pGBTOPPLA-1
2.2.2	Nucleotide sequence of the expression unit
2.2.3	Map of the selectable marker plasmid
2.4.1	Survival studies of host organism GAM 53 outside the fermentor
3.4.1	Method of analysis: FCC method
3.4.2	Method of analysis: DSM method ALS00622.UK1
3.4.3	Physical-chemical characteristics of phospholipase A_2
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6.2.1	Results of laboratory and commercial scale bakery experiments
6.2.2	Results of laboratory scale experiments on the production of fine bakery wares
6.2.3	Results of laboratory scale experiments on the production of mayonnaise
6.4.1	Proof of inactivation of phospholipase A_2 during baking
7.1.1	Schuster, E., Dunn-Coleman, N., Frisvad, J.C., Dijck, P.W.M van, *On the Safety of Aspergillus niger – a Review*, Appl. Micobiol. Biotechnol. 59:426-435 (2002).
7.1.2	JECFA safety evaluation of *A. niger* as a source of enzymes to be used in food, 1987
7.1.3	Expert reports submitted to JECFA by Professor J.W. Bennett and Dr. M.O. Moss on the probability of mycotoxins being present in industrial enzyme preparations obtained from fungi (September 1988)
7.1.4	JECFA reconsideration of safety of *A. niger* as a source for enzymes to be used in food, 1990
7.2	Pariza & Johnson Decision Tree Analysis of Phospholipase A2
7.2.1	Report from the Amfep Working Group on Consumer Allergy: risk from enzyme residues in food (August 1998)

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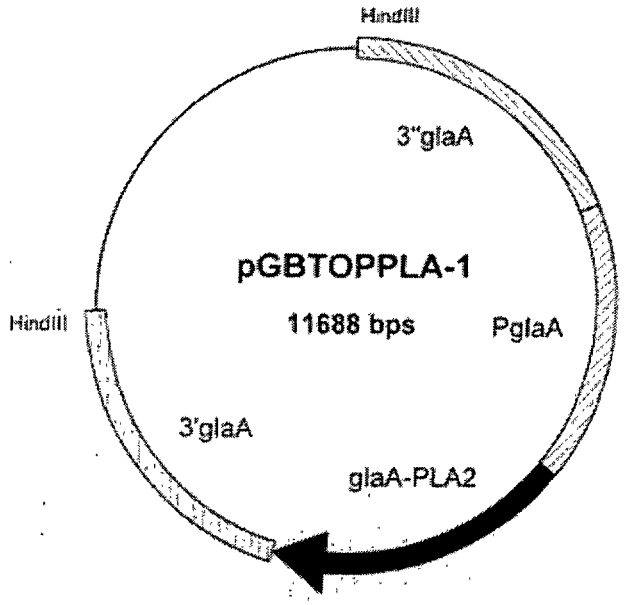
Annex 2.1.1

Pages 000043 - 000051 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Annex 2.2.1

Annex 2.2.1
Figure of the vector pGBTOPPLA-1



Annex 2.2.2

Annex 2.2.2

Nucleotide sequence of the expression unit in the recombinant production strain

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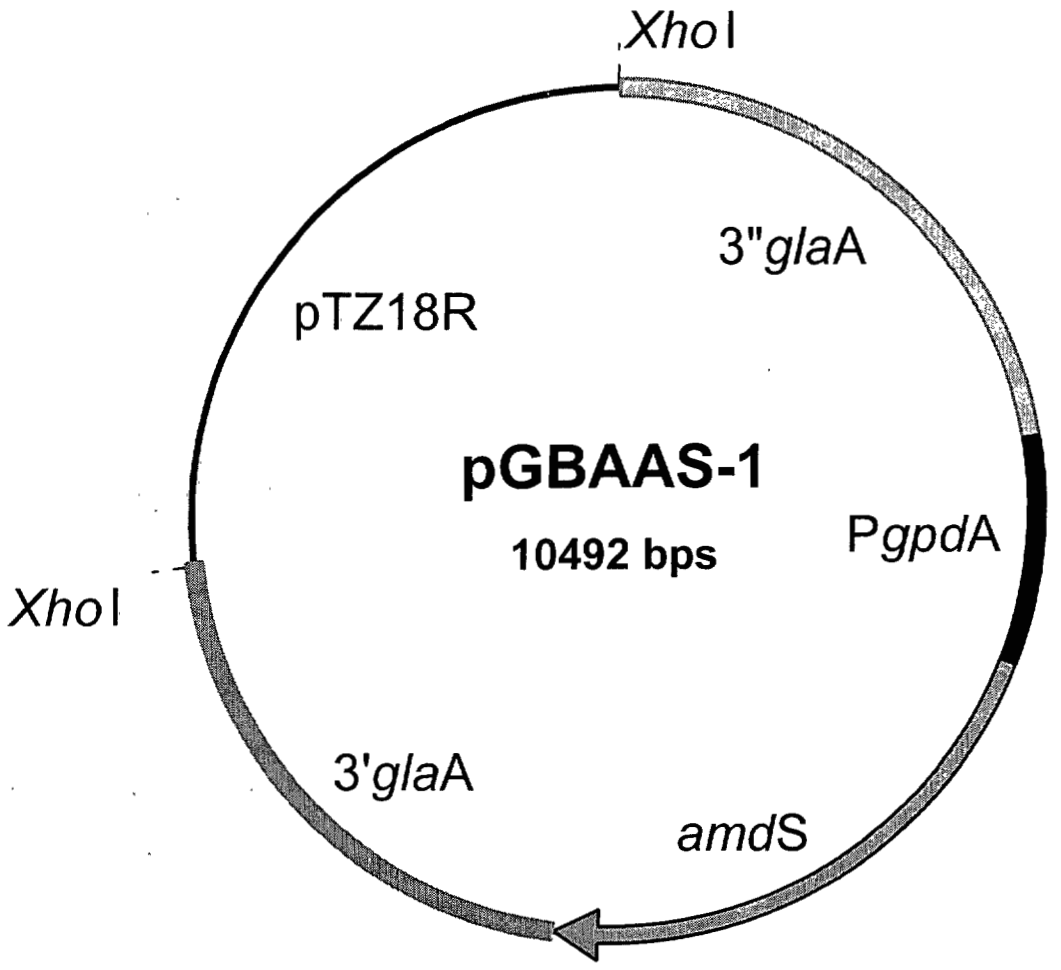


The pGBAAS-1 *amdS* selection vector

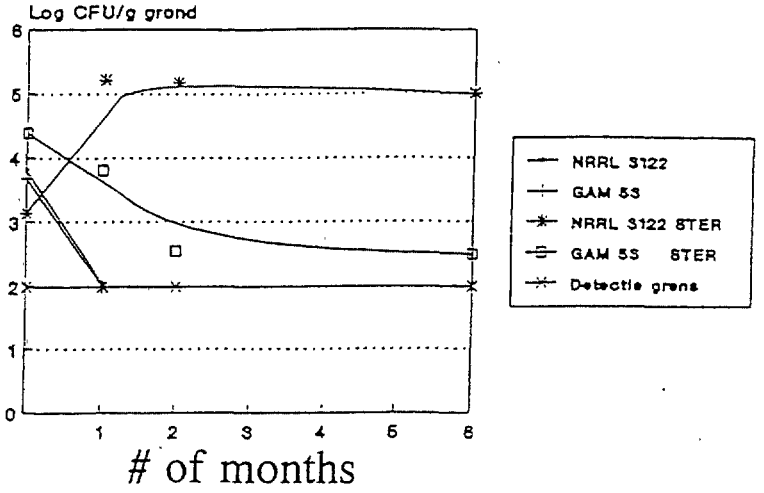
Annex 2.4.1

Surviving studies of *Aspergillus niger* strains in soil, surface water, and waste water.

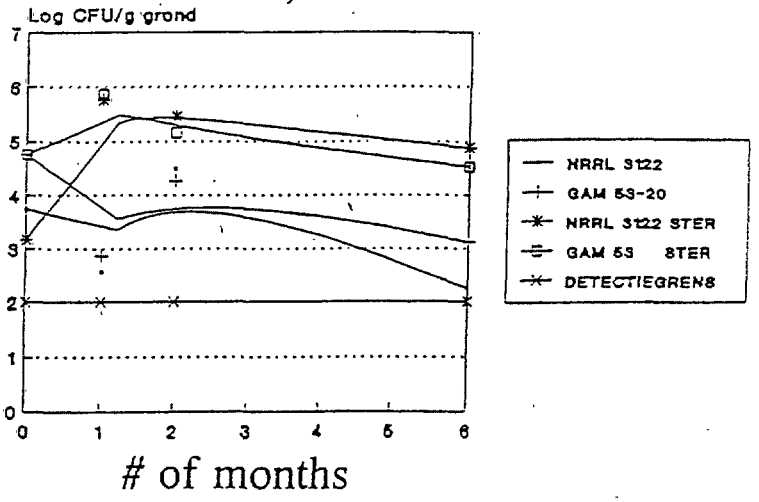
Aspergillus niger was inoculated in three different environments, namely: soil, surface water and sewage (waste water of Gist-brocades production plant in Delft (The Netherlands)).
As positive control, that is to say to judge whether the environment contained sufficient substrates for the organisms to survive in the absence of competitive (micro)organisms, and that there were no toxic components present, additional inoculations were performed in sterile environments. Sampling was performed during 6 months.
Experimental results are shown in this annex (see figures 1,2 and 3, in which the data are presented for each environment at 8°C as well as 25°C.
In independent incubations the wild-type *Aspergillus niger* (NRRL 3122) and an industrial strain (GAM-53) were compared in sterile and non-sterile environments.
In all sterile environments at 25°C inoculated with *A. niger* spores, having a titre between 10^4-10^5, colony forming units/gram sample were found. At 8°C the wild-type strain was surviving well in soil as well as in surface water, whereas in waste water it was decreased beneath the detection level.
In the surface water environment the industrial strain decreased rapidly (within 2 months) below the detection level, whereas in soil instabilisation occurred when inoculated with a low (10^2/g soil) titre.

In natural environments and in the presence of competitive micro-organisms surviving *Aspergillus niger* strains were found after 6 month only in soil samples at 25°C, inoculated with at least 10^3 spores/g. In both aqueous environments at 8°C and 25°C and in soil of 8°C a fast decline of surviving strains was observed: within three months and often even faster titres decreased below the detection level.
During optimal conditions (when there is no competition) *Aspergillus niger* survives well in different environments, and the industrial strain usually less compared to the wild-type. Under natural condition *Aspergillus niger* survives only in soil at 25°C (after 6 month titre is decreased only 50 times). In the other environments under different conditions both tested *Aspergillus* strains were not detectable any more within 1 to 3 months, showing that the industrial strain *A. niger* GAM-53 is biological restricted with respect to growth and environmental surviving.

Incubation of *A. niger* mycelium in soil, 8^0C

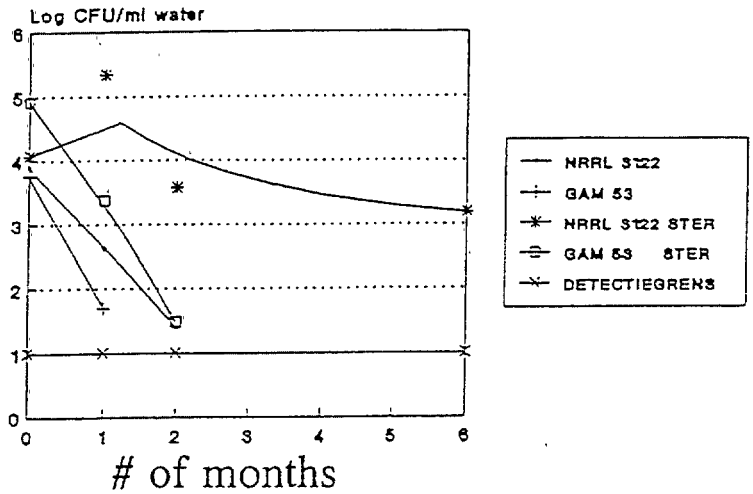


Incubation of *A. niger* mycelium in soil, 25^0C

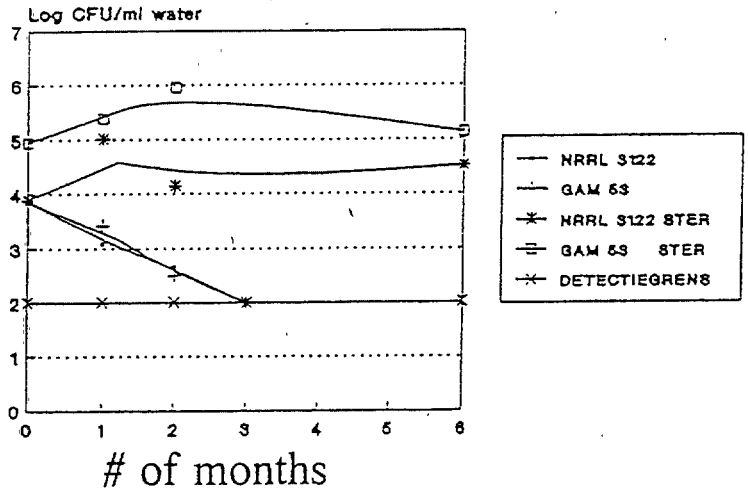


Surviving of *Aspergillus niger* I

Incubation of *A. niger* mycelium in surface water, 8^0C

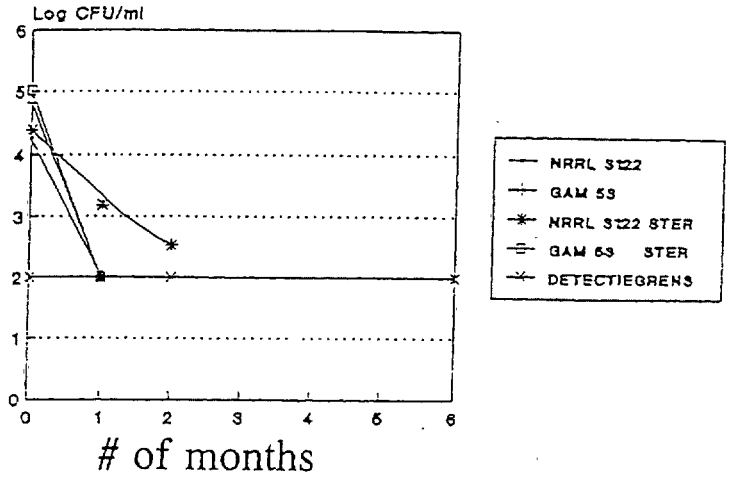


Incubation of *A. niger* mycelium in surface water, 25^0C

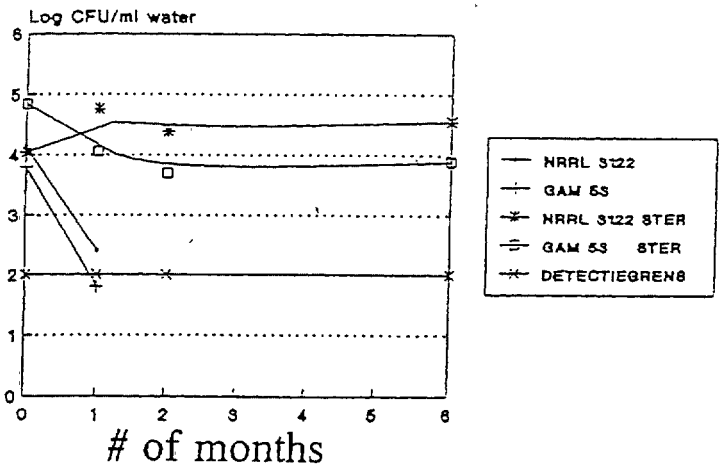


Surviving of *Aspergillus niger* II

Incubation of *A. niger* mycelium in waste water, 8^0C



Incubation of *A. niger* mycelium in waste water, 25^0C



Surviving of *Aspergillus niger* III

Annex 3.4.1

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Annex 3.4.2

Annex 3.4.2
Method of analysis: DSM method ALS61895

DSM

METHOD OF ANALYSIS		No. ALS00622.UK1
Department: DSM-R/ALS	Title: Determination of the Phospholipase A2 activity using the pH-Stat method.	
Valid from:	Product: Fermentation and down stream processing samples	
Author: T. Kemper Date:		Validated method YES
Approved by Expert: W. Bijleveld Date:	Approved by Management: L.A.M. Jansen Date:	Approved by GLP-coordinator: W.J. van Zoest Date:

1 INTRODUCTION

The activity of phospholipase A2 is determined titrating the fatty acid that has been released in unit time with a sodium hydroxide solution using a pH-Stat set-up.
The determination is carried out at pH 8.0 and a temperature of 40 °C.
The activity is expressed in EYU, where one unit is defined as the amount of enzyme that releases 1 μmole of acid per minute from lecithin at pH 8 0 and 40 °C.
The substrate consists of egg yolk, water, Ca^{2+} and sodium deoxycholate.
The CV (coefficient of variation) of a single determination is 5 %.

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2. APPARATUS AND CONDITIONS

2.1 Apparatus

1. A Radiometer pH-stat unit, consisting of:
   - pH meter PHM82 STANDARD pH METER
   - titrator TTT80 TITRATOR
   - burette ABU80 AUTOBURET
   - TTA80 TITRATION ASSEMBLY with double-walled reaction vessel
   - Recorder REA 160 TITRIGRAPH MODULE with REA270 PHSTAT UNIT
   - pH electrode REF 201
   - PHG 201
2. Water bath with thermostat
3. Magnetic stirrer IKA Combimag RCO
4. Stopwatch

Or equivalent equipment or requisites.

2.2 Conditions

Temperature is 40°C and pH is 8.0.

3 MATERIALS

3.1 Chemicals

Please note that the manufacturer states the expiry date on the jar.

1. DOC:	Sodium deoxycholate (Merck, article no. 6504), Storage temperature: Room temperature, Place of storage: Chemicals cupboard.
2. $CaCl_2.2H_2O$:	Calcium chloride with 2 molecules water of crystallisation. (Merck article no. 1.02382) Storage temperature: Room temperature, Place of storage: chemicals cupboard.
3. NaOH:	Sodium hydroxide solution 1 mole /L (1 N). (Merck, Article no.1.09137.1000). Storage temperature: Room temperature, Place of storage: chemicals cupboard.
4. TRIS:	2-amino-2-(hydroxymethyl)- 1,3 propanediol. (Gibco, UltraPure, Article no. 15504-012). Storage temperature: Room temperature, Place of storage: chemicals cupboard.
5. NaCl:	Merck no. 106404. Keep at room temperature
6. Eggs.	Battery cage eggs from the supermarket, but not older than two weeks. Storage temperature: 2 to 8 °C, Place of storage: refrigerator
7. Buffer pH 7.0:	Phosphate buffer pH 7.00. (Merck, article no. 1.09439).
8. Buffer pH 9.0:	Boric acid buffer pH 9.00 (Merck, article no. 1.09461).
9. HCl	Fuming hydrochloricacid 37% p.a. (Merck1.00317)
10. Triton X-100:	Triton X-100 (Merck, article no 1.1229)

Or equivalent chemicals.

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3.2 References and standards

Phospholipase A2 standard — Purified phospholipase A2.

3 3 Reagents

1. Demineralized water: Distilled water that has been purified with a Millipore UF water purification filter.
2. DOC solution: Sodium deoxycholate. Dissolve 4.5 g Na-DOC in 500 ml demineralized water water. Keep at the refrigerator. Can be kept for 6 months.
3. $CaCl_2$ solution: Dissolve 16.5 g $CaCl_2.2H_2O$ in 500 ml of demineralized water. This solution can be kept in the refrigerator for 1 year.
4. NaOH (50 mM): Pipet 5 ml 1 M NaOH into a 100 ml volumetric flask, using a pipette. Fill the flask with Milli-Q water and homogenise Prepare freshly on each day that the analysis is performed.

4.HCL (4 M) Add 118 ml hydrocloricacid 37% to 250 ml demineralized water. This solution can be kept in the refrigerator for 1 year.

5. TRIS buffer: Dissolve successively in 2 L demineralized water: 48.4 g Tris, 11.6 g NaCl, 4.4 g $CaCl_2$. $2H_2O$ and 4 g Triton X-100. Adjust to pH 8.3 using 4 M hydrochloric acid. Keep at 4 °C. Can be kept for 1 year.

8. Substrate: Break the eggs, rinse the yolks with demineralized water and place the yolks in a suitable measuring cylinder (the volume depends on the final volume of the substrate. One egg is used to make 251 ml of substrate). Add demineralized water up to the required volume (up to 100 ml for one egg, 200 ml for 2 eggs etc.). Add 6 ml of calcium chloride for each egg, 120 ml of demineralized water and 25 ml of NaDOC solution. Stir for 10 minutes with the magnetic stirrer. Adjust at room temperature to pH 8.3, using sodium hydroxide. Prepare freshly on each day that the analysis is performed.

4 PROCEDURE

4.1 Preparation

Set the water bath to 40 °C. Calibrate the electrode at 40 °C with the pH 7 buffer, turning the "BUFFER" control on the pH meter until the scale reads 6.95, followed by turning the "SENS" control, using the pH 9 buffer, until the scale reads 8.85. Flush the burette with 50 ml of NaOH solution.

Set the titrator to:

-ENDPOINT	pH 8 0
- Titration	UP-scale
- PROPORTIONAL BAND	0.1
- DELAY SEC	pH STAT

Set burette to:

- switch	AUTO
-VOLUME	1/10
- SPEED	30

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Set recorder to:

- SPAN	14
- PEN	SERVO
- CHART SPEED	1 cm/min
- Set to remote	

4.2 Pre-treatment of control sample
Dilute the control sample to 20 EYU/ml with Tris buffer. The activity of the control sample is determined at the beginning and at the end of a sample series by adding 150 µl of the dilution to the substrate.

4.3 Pre-treatment of reference samples
Not applicable

4.4 Pre-treatment of samples

4.4.1 Dilution of samples
Dilute the sample with TRIS buffer pH 8.0 to an estimated activity of approx. 10 EYU/ml, using the dilution apparatus. The sample is diluted when the unit of activity has to be in EYU/ml.

4.5.2 Weighing of samples
Depending on the estimated activity, weigh 1, 2 or 3 g of sample into a 10, 25 or 50 ml volumetric flask. Extract for 30 minutes with Tris buffer. The sample is weighed when the unit of activity has to be in EYU/g.

4.6 Preparation for measurement
Using a pipette, transfer 30 ml of substrate to a beaker and place it in the water bath at 40 °C., so that the substrate will be pre-heated. After some time, pour the substrate in the reaction vessel and wait until the temperature has reached 39 °C.

4.7 Determination

4.7.1 Determination of the blank
As soon as the substrate has reached a temperature of 39 °C, start the pH stat by pushing the <start> button of the titrator and placing the recorder in the 'down position'. During the period of stabilisation, the substrate will reach a temperature of 40 °C. The titration is carried out for 15 minutes at a titration rate (speed) of 10. The blank value can be calculated from the trace obtained. This blank determination is carried out at the beginning, of a series.

4.7.2 Determination of samples/standards
The linear measuring range lies between 1-3 EYU/ 30 ml of substrate. Depending on the activity of the diluted sample, 50 to 500 µl of the dilution can be added to the substrate. Clean the pipette with ethanol after the addition in order to remove traces of Triton. When there are traces of Triton left on the pipette, the tip of the pipette will not fit properly on the pipette.

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Take care to set the 'speed' to 30. As soon as the temperature of the substrate has reached 39 °C, start the pH stat by pushing the <start> button of the titrator and placing the recorder in the 'down position'. After a stabilisation period of approx. 3 minutes, add the required volume of sample/ standard. Allow the titration to stabilise for approx. 2 minutes and then follow the titration for approx. 5 minutes.
The time of the procedure can be increased (e.g. when stabilisation takes longer), but it cannot be decreased.

It is of utmost importance to obtain a linear consumption of at least 3 minutes, in order to be able to properly draw a tangent!

After approx. 3 minutes, determine the consumption of sodium hydroxide for approx. 20 seconds (stopwatch) and calculate the sodium hydroxide consumption per minute. If the consumption is too low, extra sample can be added If consumption is too high, the analysis is interrupted and re-started with a smaller volume
The minimum consumption is 0.02 ml/min, 1 EYU/30 ml of substrate.
The maximum consumption is 0.06 ml/min, 3 EYU/30 ml of substrate.

5 CALCULATIONS

5.1 Calculation procedure

Determining the slope is carried out using a tangent line This tangent is drawn on the curve at a point approx. 3 minutes after the addition of the sample in a manner that allows the tangential plane with the linear portion of the trace to be at its maximum. When the tangent has been drawn, two points are found where the tangent coincides with the intersection of the lines of the recorder paper. Between these points, the T and V squares are counted. In the direction of "time", each square represents one minute and in the direction perpendicular to it, one square represents 25 µl.

$$U/ml = \{(V_m/T_m \times 0.025) - (V_b/T_b \times 0.25)\} \times (100 \times 50 \times F_v)/Vol$$

T= number of squares in the direction of time
T_m for the sample
T_b for the blank
V = the number of squares in the direction perpendicular to it.
V_m for the sample
V_b for the blank
F_v = the factor of dilution with TRIS buffer
Vol = number of µl added to the reaction vessel
50 = the concentration (in mM) of the NaOH solution.

6 SYSTEM SUITABILITY TEST

The control at the beginning of de series has to be equal to the squaled value of the control ±10% before samples may be analysed.

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7 STATISTICAL INFORMATION

The method has been validated for begin- and end of fermentation samples and ultrafiltrates. The reproducibility is less then 5%. The calibration line is linear from 1 to 3 EYU/ml.

8 REMARKS

None

9 REFERENCES

Validation Report No. 00622.UK1. The validation of the phospholipase A2 egg yolk method.
Optimisation PLA2 egg yolk assay ARS00622.NL2, ALS reference: 2000-086

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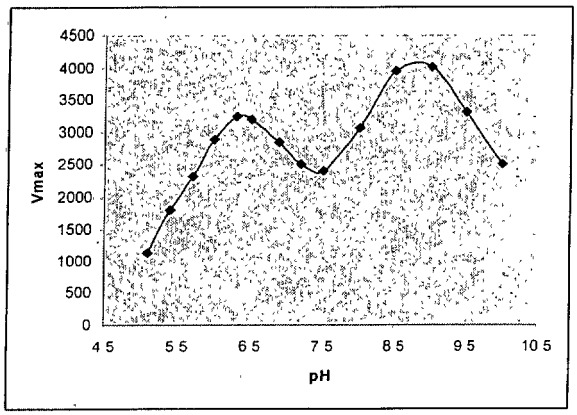
Annex 3.4.3

Annex 3.4.3
Physical-chemical characteristics of phospholipase A_2

Ca^{2+}-ions are an essential cofactor for phospholipase A_2. One Ca^{2+}-ion is strongly bound to the active site and is essential for enzymatic activity. At alkaline pH, porcine pancreatic phospholipase A_2 is known to bind two Ca^{2+}-ions[1]. When saturating conditions for both Ca^{2+}-binding sites are applied, the pH-profile for porcine phospholipase A_2 activity is as shown in Fig. 1[2].

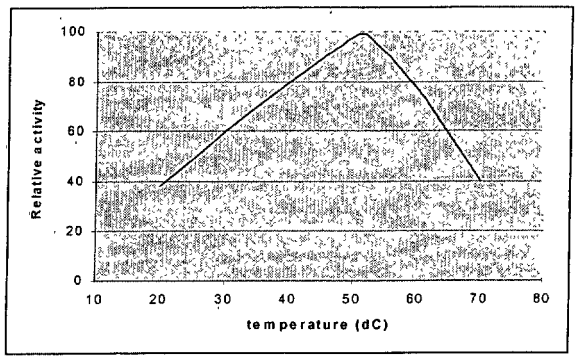
Fig. 1 pH dependence of porcine pancreas phospholipase A2.



In some publications[3,4] only the alkaline optimum is reported. In those cases kinetic analyses were only done at one Ca^{2+}- concentration. In wheat flour an average of about 5 mmol Ca^{2+}-ions/kg flour is present[5], sufficient to saturate the first Ca^{2+}-binding site at pH 5.5 under dough conditions. The temperature profile for the activity of phospholipase A_2 is shown in Fig. 2[4].

Fig. 2 Temperature dependence of phospholipase A2

The optimum temperature for phospholipase A_2 is around 50 °C. Because of the seven



disulphide bridges present in the enzyme molecule, phospholipase A_2 is a rather stable enzyme. Stability of phospholipase A_2 at different pH values after 2 hrs of storage in buffer at different temperatures is shown in Fig. 3[4].

---

[1] van Dam-Mieras, M.C.E., Slotboom, A.J., Pieterson, W.A., de Haas, G.H. (1975) Biochemistry 14, 5387-5394
[2] Dutilh, C.E.(1976) Ph.D Thesis, State University Utrecht (Holland)
[3] Dutilh, C.E., van Doren, P.J., Verheul, F.E.A.M., de Haas, G.H. (1975) Eur. J. Biochem. 53, 91 - 97
[4] Novo Nordisk Technical product sheet Lecitase
[5] Pyler, E.J. (1988) in Baking Science & Technology, 3rd ed. (Sosland Publ. Company, Kansas City, U.S.A., ed. E.J. Pyler), Volume I, 342

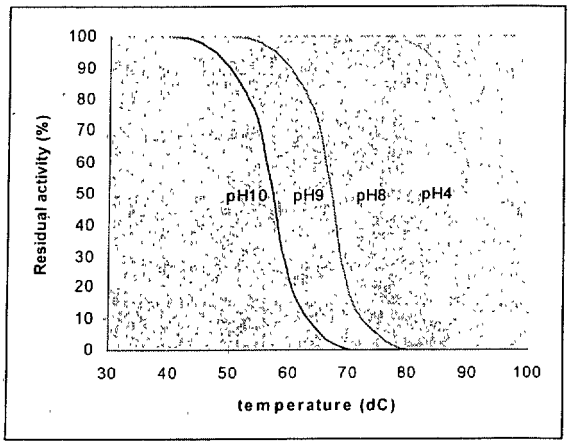
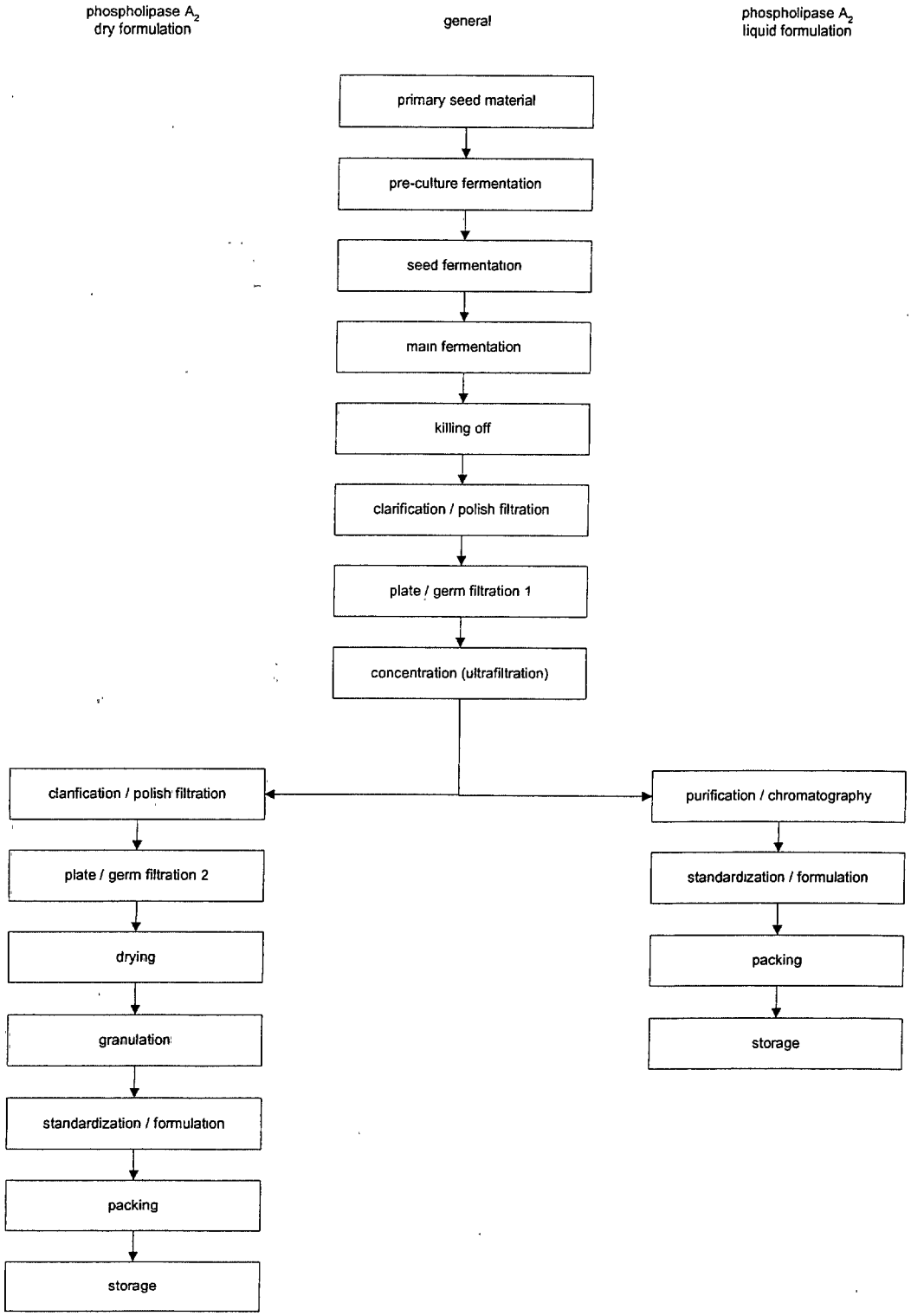


Fig. 3 Stability of phospholipase A2 at different pH values after 2 hrs storage in buffer at different temperatures.

From this graph it is clear that phospholipase A_2 is not stable at alkaline pH and more stable at lower pH. At dough-pH the enzyme will be inactivated at temperatures above 80 °C.

Annex 4.1.1

Annex 4.1.1
Flow diagram of manufacturing process



Annex 6.2.1

Annex 6.2.1

Results of laboratory and commercial scale baking trials using phospholipase A_2

A. Laboratory scale baking trials

Preparation of pup loaves in a direct bread-making process was done by mixing 200 g of wheat flour, 1.4 g Fermipan® instant yeast, 3.2 g salt, 0.8 g calcium chloride ($CaCl_2.2H_2O$), 25 ppm ascorbic acid, 10 ppm Fermizyme® P_{200} (fungal alpha-amylase), 60 ppm Fermizyme® H_{2000} (fungal hemicellulase), 56 % water, and varying amounts of phospholipase A_2 with and without added oil-containing soy lecithin in a pin mixer for 6 minutes and 15 seconds. The dough temperature is 27 ^{0}C. Machineability of the dough is analysed by hand by a qualified baker. Directly after mixing the dough is divided into two 150 g pieces, which are rounded and proofed for 45 minutes in a proofing cabinet at 31 ^{0}C and 85 % RH.

After this period the doughs are rounded again and given an intermediate proof of 25 minutes at 31 ^{0}C and 85 % RH. Afterwards the doughs are analysed again by hand to check machineability, moulded, shaped, panned and given a final proof of 70 minutes in a proofing cabinet at 31 ^{0}C and 85 % RH. Afterwards the fully proofed doughs are baked in an electric oven at 225 ^{0}C for 20 minutes. After cooling down to room temperature the volumes of the loaves are determined by use of the rapeseed displacement method. After 24 hrs of storage at room temperature the crumb structure quality is analysed by a qualified baker.

Results on loaf volume increase are summarised in Fig. 1. The influence of phospholipase A_2 on other dough and bread characteristics are summarised in Table I.

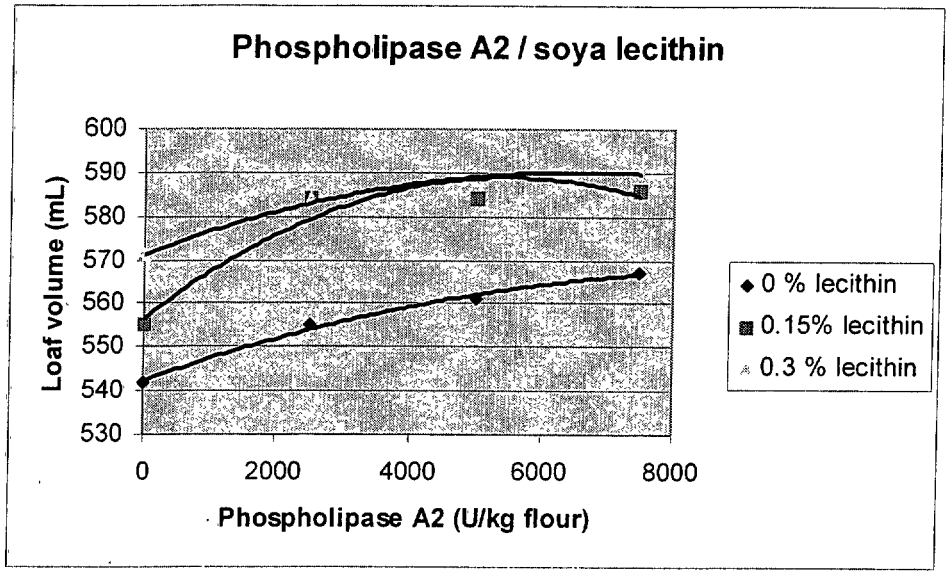

Phospholipase A2 / soya lecithin
Loaf volume (mL)
600
590
580
570
560
550
540
530
0
2000
4000
6000
8000
Phospholipase A2 (U/kg flour)
0 % lecithin
0.15% lecithin
0.3 % lecithin

Fig. 1

Table I

Composition	*Dough machineability after*		*Break & shred*	*Crumb structure*	*Loaf volume*
	mixing	*moulding*			(ml)
Reference	good	good	6	7	542
+ 2500 U/kg PLA_2	good	good	6	7	555
+ 5000 U/kg PLA_2	little slack	good	6	7	561
+ 7500 U/kg PLA_2	slack	little slack	6	6.5	567
+ 0.15% soy lecithin (lec.)	good	good	6.5	7	555
+ 0.15% lec. + 2500 U/kg PLA_2	little stiff	good	7	7.5	584
+ 0.15% lec. + 5000 U/kg PLA_2	little stiff	good	7.5	7.5	584
+ 0.15% lec. + 7500 U/kg PLA_2	little stiff	little stiff	7	7	586
+ 0.3% soy lecithin	good	good	7	7	571
+ 0.3% lec. + 2500 U/kg PLA_2	little stiff	good	7.5	8	584
+ 0.3% lec. + 5000 U/kg PLA_2	little stiff	good	8	7.5	588
+ 0.3% lec. + 7500 U/kg PLA_2	little stiff	little stiff	7	8	590
+ 0.3% DATEM	little stiff	little stiff	8	7.5	593

From the results summarised in Fig. 1 and Table I it may be concluded that the combination of soy lecithin plus phospholipase A_2 improves bread quality in all its characteristics. The obtained bread quality at the optimum dosage of ingredients (0.3 % soy lecithin + 5000 U of phospholipase A_2/kg of wheat flour) is fully comparable to the bread quality obtained when 0.3 % DATEM is applied.

B. Commercial scale: French batard

Preparation of French batard type of bread in a direct process was done by mixing 2 kg of wheat flour, 2.25 % salt, 3.5 % compressed yeast, 60 ppm Fermizyme® H1000 (fungal alpha-amylase and hemicellulase), 0.2 % oil-containing soy lecithin in the absence and presence of various levels of phospholipase A_2 or 0.15 % DATEM (Panodan 90, Grindsted), and 62 % water in a spiral mixer (3 min. in 1st speed, 13 min.

in 2nd speed). The dough temperature is 24 ^{0}C. Machineability of the dough is analysed by hand by a qualified baker.

The dough is given a bulk proof of 30 min. at ambient temperature (~21 ^{0}C). Afterwards the dough is divided into 350 g pieces, which are moulded and given a final proof of 120 min. at 25 ^{0}C and 85 % RH. The doughs are baked in an electric oven at 250 ^{0}C for 25 min.. After cooling down to room temperature the volumes of the loaves are determined by use of the rape seed displacement method.

Results are gathered in Table II. From these results it may be concluded that lyso-phospholipids, obtained by the hydrolytic action of phospholipase A_2 on wheat and exogenous lipids, greatly improve the baking potential of wheat flour up to yielding bread volumes at least equivalent to those obtained by adding a chemical emulsifier in the form of DATEM.

Table II

Composition	***Phospholipase A_2 (U/kg flour)***	***Dough machineability after mixing***	***Dough tolerance after fermentation***	***Loaf volume (ml)***
Ref.+ 0.2 % soy lecithin	1000	good	satisfactory	1850
Ref.+ 0.2 % soy lecithin	1500	good	satisfactory	1917
Ref.+ 0.2 % soy lecithin	2000	good	satisfactory	1975
Ref.+ 0.2 % soy lecithin	2500	good	satisfactory	2060
Ref.+ 0.15 % DATEM	0	good	satisfactory	2025

Annex 6.2.2

[redacted]

Annex 6.2.2
Results of laboratory scale experiments on the production of egg-based fine bakery wares with phospholipase A2

A. Production of pound cake

Pound cake (basic recipe) was prepared by adding 500 g margarine into the mixer bowl (Hobart) and whisk it into a homogeneous mass. Afterwards, a premix of 334 g wheat flour (Kluut), 143 g wheat starch (Puramyl SP), 500 g sugar, 10 g baking salts, 7 g salt, 6 g sorbic acid, 1.6 g citric acid (blanc), and either 1.2% Admul 5306 (Quest International) or 100 EYU phospholipase A_2 and 500 g whole egg were added. The whole of ingredients was mixed: 1 minute in speed 1, and 1.5 minutes in speed 2.

Directly after mixing the specific weight was determined. Afterwards, 4 x 375 g batter was placed in cake forms and baked for 50 minutes at 180 °C. The resulting characteristics for the batter and the finished product are shown in Table 1. In Table 2 sensory observations during a shelf-life period of 5 weeks are given.

Table 1 Batter density and cake charactistics

Batter	blanc	Admul 5306	Phospholipase A_2
Density batter (g/ml)	0.79	0.87	0.83
Cake			
External view	good	good	good
Internal structure	irregular, coarse	regular	regular, woolly

Table 2 Organoleptic analyses

	blanc			Admul 5306			Phospholipase A_2		
Storage (wks)	1	3	5	1	3	5	1	3	5
Softness									
Compressibility	-	-	--	0	0	0	0	+	++
Sensory analysis									
Coherence	-	--	--	0	0	0	0	+	+
Humidity	--	--	--	0	0	0	0	0	+
Bite	-	--	--	0	0	0	++	++	++
Sweetness	0	+	+	0	0	0	0	0	0

From the results shown in Table 1 it may be concluded that the effect of phospholipase A_2 on the batter density is very similar to the effect of emulsifier Admul 5306. The produced cakes are similar in external characteristics. The texture of cakes prepared with phospholipase A_2 is as regular but more woolly when compared to cakes prepared with Admul.

Sensory analysis of the various products during a shelf-life period of 5 weeks showed the importance of the presence of Admul as emulsifier to improve coherence and humidity of the baked product. Phospholipase A_2 showed to be able to replace the emulsifier system in this application: improved coherence and softness was seen.

B. Production of muffins

Muffins were prepared by mixing 500 g wheat flour (Reigerl), 141 g whole egg powder, 4.7 g egg white powder, 35.2 g dextrose, 470 g sugar, 4.7 g salt, 16.4 g baking salts, 1.6 g citric acid, 3.5 g sorbic acid, 475 g oil, 475 g water (blanc), either 2.4 g Admul 5306 or 150 EYU phospholipase A_2 into the mixer bowl (Hobart) and whisk it into a homogeneous mass during 7 minutes in speed 1.

Directly after mixing the specific weight was determined. Afterwards, 10 x 65 g batter was placed in a muffin tray and baked for 23 minutes at 195 °C. The resulting characteristics for the batter and the finished product are shown in Table 3.

Table 3 Batter density and muffins characteristics

Batter	blanc	Admul 5306	Phospholipase A_2
Density batter (g/l)	1090	1080	1070
Batter surface	bumpy	smooth	little bit bumpy
Muffin			
External view	good	good	good
Internal structure	irregular, open	regular, open	regular, open

From these results it is clear that phospholipase A2 is able to replace the commercial emulsifier, only the surface of the batter is not as smooth as found in the case of Admul 5306.

In Table 4 sensory observations during a shelf-life period of 5 weeks are shown.

Table 4 Organoleptic analyses

	Blanc		Admul 5306		Phospholipase A_2	
Storage (wks)	1	5	1	5	1	5
Crumb softness	-	--	0	0	0	+
Sensory analysis						
Coherence	0	-	0	0	0	0
Humidity	0	--	0	0	0	+
Bite	-	--	0	0	+	0

When compared to the blanc, emulsifier addition improves crumb softness of the muffin, and results in good mouth feel. Replacing the emulsifier by phospholipase A_2 does not really change crumb softness and organoleptic characteristics of the muffin, but the bite is somewhat improved. After five weeks of storage, the crumb softness of muffins prepared by phospholipase A_2 is somewhat better when compared to muffins prepared by emulsifier.

From these results it may be concluded that the emulsifier Admul 5306 in muffin production can be replaced by phospholipase A2 .

Annex 4.2.3

ANNEX 6.2.3

Result of laboratory scale experiments of the use of phospholipase A2 in the production of mayonnaise

The time-dependent efficiency of phospholipase A2 from *Aspergillus niger* to hydrolyse the egg yolk phospholipids PC (phosphatidylcholine) and PE (phosphatidylethanolamine) into *lyso*-PC and *lyso*-PE was tested.

Subsequently, the textural properties (firmness and heat-stability) of mayonnaise produced with egg yolks hydrolysed to various degrees were compared to mayonnaise produced with untreated egg yolk.

A. Production of hydrolysed egg yolk

Egg yolk was hydrolysed with phospholipase A2 at a dosage of 890 EYU/100 gram egg yolk and a fixed temperature of 52 ^{0}C. The time of hydrolyses varied from 0.5 – 15 h. After hydrolysis, the percentage of PC, PE, *lyso*-PC and *lyso*-PE in egg yolk was analysed by means of Nuclear Magnetic Resonance (NMR).

The results are shown in Table 1:

Incubation time (hours)	PC → LPC (% in dry egg yolk)		PE → LPE (% in dry egg yolk)	
0.0	11.1	0.3	2.6	0.1
0.5	5.1	4.0	0.8	1.2
1.0	3.1	4.9	0.4	1.4
2.0	1.7	5.5	0.2	1.5
3.0	1.2	6.3	0.2	1.6
4.0	0.8	6.3	0.1	1.7
5.0	0.7	6.2	0.1	1.7
6.0	0.6	6.3	0.1	1.7
8.0	0.5	6.5	0	1.7
15.0	0.3	6.4	0	1.6

Table 1 Time dependent degree of hydrolysis of egg yolk PC and PE into *lyso*-PC (LPC) and *lyso*-PE (LPE) after incubation with phospholipase A2[1]

As can be seen in this Table, the maximal percentages of *lyso*-PC and *lyso*-PE were formed after 3 hours of hydrolysis. After that, the percentage of LPC did not increase any further, even though the amount of PC decreased. It might be possible that *lyso*-PC is hydrolysed further into components that are not detectable with the method used.

B. Production of mayonnaise with hydrolysed egg yolk

Mayonnaises were prepared with untreated egg yolk, as well as egg yolks hydrolysed with phospholipase A2 to various degrees, according to the recipe given in Table 2. Afterwards, firmness and heat-stability of the mayonnaises were measured.

000090

[1] Full (1005) hydrolysis of PC and PE would result in 7.1% LPC and 1.6% LPE, respectively.

ANNEX 6.2.3

Ingredient	%
Sun flower oil	70
Egg yolk	6.5
Sugar	2
Salt	1.5
Vinegar	2.5
Mustard	0.5
Potassium sorbate	0.1
Water	16.9

Table 2 Recipe for production of mayonnaise

The firmness of the mayonnaises produced with the various egg yolks was measured after storage of 1 – 2 days at 4 ^{0}C. The firmness was measured by Stable Micro System TA-XT2 analyser with a bulb / cylinder probe. The pressure in grams needed to penetrate a cylinder in the product (specific speed and penetration depth) is an indication for mayonnaise firmness.

The results of the firmness measurements are presented in Table 3.

Hydrolysis time (hours)	Firmness (grams)	LPC (% in dry egg yolk)	LPE (% in dry egg yolk)
0.0	11.7	0.3	0.1
0.5	20.2	4.0	1.2
1.0	21.2	4.9	1.4
2.0	22.4	5.5	1.5
3.0	24.9	6.3	1.6
4.0	25.1	6.3	1.7
5.0	25.9	6.2	1.7
6.0	26.7	6.3	1.7
8.0	26.7	6.5	1.7
15.0	26.0	6.4	1.6

Table 3. Firmness of mayonnaises prepared with egg yolk hydrolysed to various degrees and corresponding concentrations of *lyso*-PC (LPC) and *lyso*-PE (LPE)

As can be seen it this Table, mayonnaises prepared with hydrolysed egg yolks are significantly firmer than mayonnaise prepared with untreated egg yolk (0 hours incubation time). The Table also shows that the firmness of the mayonnaise is related to the percentage of *lyso*-PC and *lyso*-PE present in the egg yolk. Egg yolk that was hydrolysed longer than three hours did not result in further significant increase of the mayonnaise firmness. This is in agreement with the fact that the percentage of *lyso*-PC and *lyso*-PE in egg yolk reaches the maximum level after 3 hours of hydrolysis. It may thus be concluded that the increased firmness of mayonnaise can be ascribed to the superior emulsifying properties of *lyso*-PC and *lyso*-PE present in hydrolysed egg yolk prepared with phospholipase A2.

The heat-stability of the mayonnaises produced with the various egg yolks was measured by the droplet size of the mayonnaise before and after heating samples for 45 minutes in a water bath of 90 ^{0}C. Droplet size measurement was performed by microscope, equipped with special application for circle / diameter measurement. 50 – 60 droplets were chosen manually for further analyses and calculations.

Droplet size increase after heat treatment of mayonnaises indicates emulsion instability.

ANNEX 6.2.3

The results of the stability measurements are given in Table 4.

Egg yolk hydrolysis (hours)	Before heat treatment (µm)		After heat treatment (µm)	
		SD		SD
0	5.07	1.01	10.25	5.34
0.5	5.03	1.65	5.06	0.95
1	4.54	0.90	4.58	1.41
15	4.49	0.72	4.83	1.16

Table 4. Droplet size of mayonnaises prepared with hydrolysed egg yolk (SD: standard deviation)

As is shown in this Table, the droplet size of the different mayonnaises before heat treatment did not differ very much. The droplet size of the mayonnaise prepared with 1 hour hydrolysed egg yolk was slightly decreased, thus having a slightly higher stability. After heat treatment, the droplet size of mayonnaise prepared with untreated egg yolk was significantly increased when compared to the corresponding sample before heat treatment. Also, the variation in size (expressed as SD) was significantly increased. On the other hand, the droplet size of the heat-treated mayonnaises prepared with hydrolysed egg yolks was still the same as before heat treatment.

It may thus be concluded that the use of egg yolk hydrolysed with phospholipase A2 results in a mayonnaise with higher heat stability.

Annex 6.4.1

Annex 6.4.1

Proof of inactivation of phospholipase A_2 during baking

In 10 % (w/w) phospholipid solution plus non-limiting quantities of Ca^{2+} - ions porcine pancreatic phospholipase A_2 is stable at pH 4.0 up to ~80 °C. At higher pH's the stability of phospholipase A_2 is decreasing.

In wheat dough on an average the starting pH is around 5.2 and during the bread-making process decreasing to about 4.5. This particular situation might lead to a minor percentage of enzyme surviving the baking phase.

In order to conclude upon survival of phospholipase A_2 after baking some baking trials were done followed by analysis of the quantity of phospholipase A_2 activity still present in the crumb after baking.

1. Baking trials

Preparation of pup loaves in an intermediate time bread-making process was done by mixing 200 g wheat flour, 0.7 % (w/w) Fermipan® instant dried yeast, 2 % (w/w) salt, 56 % (w/w) water, 25 ppm ascorbic acid, 10 ppm Fermizyme P_{200} (fungal α-amylase), 60 ppm Fermizyme HS_{2000} (fungal hemicellulase), 0 or 0.3 % (w/w) soy lecithin, and 0, 5000 or 10000 U/kg of phospholipase A_2 in a pin mixer for 6 minutes and 15 seconds. The dough temperature was 27 °C. Directly after baking the dough is divided into 150 g dough pieces, and proofed for 45 minutes in a proofing cabinet at 31 °C and 85 % RH.

After this period the doughs are moulded, and given an intermediate proof of 25 minutes at 31 °C and 85 % RH. Afterwards the doughs are again moulded, shaped and panned and given a final proof time of 70 minutes at 31 °C and 85 % RH. At the end of this period the doughs are baked in an electric oven at 225 °C for 20 minutes. After cooling down to room temperature the volumes of the loaves are obtained by the rapeseed displacement method.

Afterwards the breads are packed in plastic and stored in the refrigerator at 4 °C up to the next day when sample preparation took place. The results are summarised in Table 1.

Table 1

PL A_2 (U/kg)	soy lecithin (%)	dough height < baking (mm)	dough height >baking (mm)	loaf volume (ml)
0	0	71	82	536
5.000	0.3	71	90	572
10.000	0	72	86	551
10.000	0.3	70	86	545

It has to be stated that the added level of 10.000 U/kg phospholipase A_2 is very high for application and in general not applied in bread making. See also Fig. 5.1.

It may also be concluded that phospholipase A_2 indeed was present in those doughs where it was planned to be present and active.

2. Sample preparation

The day after baking the breads were cut into slices from which the crusts were removed. From every bread sample 25 g crumb was taken.

To check the extraction efficiency to 25 g crumb of the reference bread a quantity of PLA_2 solution was added corresponding to 5.000 or 10.000 U/kg. The bread samples were dried overnight by lyophilisation. The dried crumbs samples were ground in a coffee mill.

3. Sample analysis

The phospholipase A_2 content of the samples was analysed in the so-called egg-yolk test. This is a standard test described by Nieuwenhuizen et al. [1]. The substrate used is egg-yolk lipoprotein. In this assay the consumption of alkali needed to keep the pH constant, when phospholipase A_2 liberates fatty acids in the medium, is recorded automatically. The hydrolysis is linear with time for at least three minutes after injection of up to 8 units of enzyme. The slope of the line representing the alkali consumption is proportional to the amount of enzyme injected into the medium. The assay is done at pH 8.0 and 40 °C.

One unit of phospholipase A_2 is defined as the amount of enzyme producing 1 micro-equivalent of free fatty acid per minute.

At first the dried crumb powders were added directly into the egg-yolk / detergent solution. However, in those cases a bad dosage / activity correlation was found. Afterwards the

sample powders were extracted with water previous to analysis. The results are shown in Table 2. The standard deviation in PLA_2 analyses is in the order of 10 %.

Table 2

Sample	*Soy lecithin (%)*	*PLA_2 added (U/g crumb)*	*PLA_2 found (U/g crumb)*	*Recovery (%)*
Reference	**0**	**0**	**0.12**	**-**
Ref. + PLA_2 added to dough	**0**	**6.5**	**0.10**	**0**
	0.3	**13.1**	**0.16**	**0.5**
	0	**13.1**	**0.16**	**0.5**
Ref. + PLA_2 added to crumb	**0.3**	**6.4**	**3.3**	**52**
	0	**6.4**	**3.4**	**53**
	0.3	**12.7**	**7.1**	**56**

From these results it is clear that presence of soy lecithin does not influence phospholipase A_2 survival during baking and/or analysis. It is also clear that only about 50 % of the phospholipase A_2 units added to the crumb after baking are analysed. Reason for this partial recovery will be the extraction procedure previous to analysis.

However, in bread crumb produced from doughs containing up to 10.000 U/kg not any residual activity is found back.

N.B. In another bread-making method using the same recipe including 0.3 % lecithin a quantity of 15.000 U/kg PLA_2 was introduced and also here no residual activity could be traced in the crumb after baking.

4. Conclusion

Phospholipase A_2 is fully inactivated during the baking process resulting into no residual activity of this enzyme in the bread after baking.

5. Literature

1. Nieuwenhuizen, W., Kunze, H., & G.H. de Haas (1974) Meth. Enzymol. 32B, 147

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Annex 7.1.1

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Annex 7.1.3

Annex 7.1.3

Expert reports of Prof. J.W. Bennett and Dr. M.O. Moss on the probability of mycotoxins being present in industrial enzyme preparations obtained from fungi (September 1988)

DATE: September 14, 1988

SUBJECT: Proposed Acceptable Daily Intake (ADI) Levels For Enzymes From Organisms Not Commonly Considered To Be Constituents of Food

FROM: J. W. Bennett, Ph.D.
Professor of Biology
Tulane University

I have read the report of the Joint FAO/WHO Expert Committee of Food Additives 31st meeting, Geneva, February 16-25, 1987 This report reiterates a conclusion reached at an earlier meeting of JECFA that an acceptable daily intake (ADI) should be established for certain enzyme preparations derived from microorganisms not normally used as food, or for enzyme preparations not removed from the food products to which they are added. This conclusion is based, in part, on the notion "that source organisms may produce toxins under certain conditions of growth" Neither the name of the putative toxins, nor the name of the organisms implicated as toxin producers, was given in the report. I would assume that the "offending" species are Aspergillus niger, Trichoderma harzianum, Trichoderma reesei, Penicillium funiculosum, and Aspergillus alliaceus since these are the producing organisms for enzymes for which the Joint FAO/WHO Expert Committee seeks to establish ADI's. Since none of these

species has been documented to produce mycotoxin in industrial applications, my comments below pertain more toward hypothetical situations, involving the introduction of new producing strains in the future, than to the species for which the ADI's are currently proposed. Based on the lack of documented evidence of toxin production in industrial settings, it is my opinion, that there is no reason to establish ADI's for the enzymes or species listed in the Table (ICS/87.13 Page 3 of the Summary and Conclusions of the Joint FAO/WHO Expert Committee on Food Additives entitled "Acceptable daily intakes, other toxicology information, and information on specifications" (Part A. Food additives, Enzyme preparations).

Before speaking to the questions raised by the report of the Joint FAO/WHO Expert Committee of Food Additives, it is important that certain terms be defined. Selected references, cited by author and date, are included in the text below. A bibliography is affixed at the end of the report.

Mycotoxins are fungal secondary metabolites that evoke a toxic response when introduced in low concentration to higher vertebrates, and other animals, by a natural route. Pharmacologically active fungal products such as antibiotics (which are toxic to bacteria and ethanol which is toxic to

animals but only in high concentration) are excluded from this definition (Bennett, 1987

Secondary metabolites are low molecular weight compounds of enormous chemical diversity and restricted taxonomic distribution that are normally synthesized after active growth has ceased Secondary metabolites are biosynthesized from small precursor molecules e.g., acetate, malonate, isoprene, amino acids) via a series of enzymatic conversions. Production of secondary metabolites is both species and strain specific Bennett & Ciegler, 1983).

Species are basic taxonomic units. Fungal species are named in accordance with the rules governed by the International Code of Botanical Nomenclature. The term "strain" derives from the International Code of Nomenclature of Bacteria. A strain constitutes the descendants of a single isolation in pure culture, sometimes showing marked differences in economic significance from other strains or isolations. Strain is analogous to "clone" in the International Code of Botanical Nomenclature (Jeffrey 1977; Bennett, 1985

The ability to produce a mycotoxin or other secondary metabolite is a characteristic of a species. Within the species different strains may vary in their biosynthetic potential: some strains may be high producers, some may be low producers, some

may be non-producers. The most common variant is the non-producer

Having defined the relevant terms, it is now possible to address certain issues raised by the report of the Joint FAO/WHO Expert Committee. The commentary below is organized as a series of questions and answers.

1. Do non-toxicogenic species of fungi develop strains that produce detectable levels of mycotoxins? Is the fact that mycotoxins are secondary metabolites relevant to this questions?

   No. Non-toxicogenic species of fungi do not become toxicogenic. However, the reverse is true. It is quite easy to isolate non-toxicogenic mutants and variants as clones "strains" from toxicogenic species

   The fact that mycotoxins are secondary metabolites is very relevant. Unlike enzymes, which are direct gene products synthesized directly from a structural gene via a series of RNA and amino acid intermediates, secondary metabolites are the result of numerous biosynthetic steps, each step enzymatically catalyzed. In most cases we do not know the exact number of steps in a biosynthetic pathway for a given secondary metabolite. Therefore, we do not know the number of genes required to encode for the enzymes of the pathway However, <u>all</u> secondary metabolites are biosynthesized by

multistep pathways with many genes and many enzymes involved.

2. Can conventional mutation (by mutagens or UV) or changes in medium or growth conditions cause a demonstrated non-toxin producer to begin producing toxins?

No. "You can't get something from nothing". Organisms which lack the structural genes for the enzymes of a mycotoxin pathway cannot be turned into toxin producers by simple mutation or changes in environmental parameters. In order for a non-toxicogenic species to become toxicogenic it would have to acquire the genes for an entire biosynthetic pathway.

A basic precept from genetics is analogous here: Deletions do not revert. Put another way, the absence of genetic material cannot mutate. Nor can it be expressed. Again note that the reverse is possible. Toxicogenic species may mutate to non-toxicogenic strains; and under certain growth conditions, toxicogenic strains may not express the genetic material for toxin production.

3. Since enzymes are primary metabolites which are ordinarily produced in the logarithmic phase of growth, what is the likelihood that mycotoxins, which are secondary metabolites, would be co-produced with the enzymes?

Usually there would be no co-production of secondary metabolites with the enzymes harvested during growth phase. Modern fermentation technology relies heavily on submerged cultures for growing production strains of fungi. Commercial enzymes are usually isolated from actively growing cultures. Because filamentous fungi grow in the form of thread-like hyphal cells, this early phase of growth, roughly analogous to logarithmic growth in single-celled organisms, has been given a special name: "trophophase" Similarly, in the jargon of fungal physiology, the period after active growth has ceased is called "idiophase". Idiophase is roughly analogous to lag phase or stationary phase for single-celled organisms. Most of the time, no secondary metabolites are produced during trophophase (Turner, 1971, pp. 18-20). Since this early growth phase is the phase during which most commercial enzymes are harvested, even in toxicogenic species it is possible to avoid accumulation of toxins by early harvesting of the fermentation cultures.

It is also relevant that the majority of mycotoxins are only sparingly soluble in water. Chemical separations of most mycotoxins use nonpolar solvents (Cole and Cox, 1981

Enzymes, on the other hand, are isolated with water and other polar solvents.

4. It is common practice for industry to test organisms for toxicogenicity and pathogenicity and products for non-specific toxicity before introducing them into commercial production and to test specifically for a toxin known to be associated with a given species. Is it appropriate for JECFA to impose testing for aflatoxin B1, ochratoxin A, sterig-matocystin, T-2 toxin and zearalenone in all fungal-derived enzyme preparations?

Once a producing species has been demonstrated as non-toxicogenic, it is a waste of time and money to test each lot of a commercial preparation for toxin production

If a species lacks the genetic material to biosynthesize a toxin, it will remain non-toxicogenic. Biosynthetic capacity is part of a species definition

A clumsy but colorful analogy could be drawn from the animal world. It would not make sense to test chickens and their eggs for milk production; nor would it be logical to assay cows and milk for the presence of feathers. Some vertebrates make milk; some make feathers. However, just because an organism is a vertebrate does not mean it will make either of these substances. Similarly, although some species of fungi make aflatoxin or T-2 toxin, it does not make sense to test <u>all</u> fungal preparations for aflatoxin and T-2 toxin

Specifically, there is no reason to test Aspergillus niger, Penicillium funiculosum, Trichoderma harzianum or T. reesei for aflatoxin B_1, sterigmatocystin, ochratoxin, T-2 toxin or zearalenone. Since some strains of Aspergillus alliaceus are known ochratoxin producers, enzyme preparations from this species might be tested for this one toxin. It would not be necessary to test A. alliaceus preparations for aflatoxin B_1, sterigmatocystin, T-2 toxin, or zearalenone.

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Sep. 1988

THE OCCURRENCE AND SIGNIFICANCE OF MYCOTOXINS

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THE OCCURENCE AND SIGNIFICANCE OF MYCOTOXINS

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1. Description of mycotoxins

A conservative estimate suggests that there are at least 100,000 species of fungi (Hawksworth, Sutton & Ainsworth, 1983) and many of these are able to produce one or more low molecular weight organic compounds known as secondary metabolites. These metabolites are a structurally diverse group of molecules (Turner & Aldridge, 1983) some of which have biological activity as antibiotics, phytotoxins and mycotoxins. The term mycotoxin is generally confined to those toxic metabolites produced by moulds growing on foods, animal feeds, or the raw materials and additives used in their manufacture.

The biological activity of mycotoxins is characterized by a toxic response when consumed by man or animals. Depending on the type of mycotoxin and animal species, even low concentrations of mycotoxins can create an acutely toxic, carcinogenic, oestrogenic or immuno-suppressive effect. A number of fungi producing macroscopic fruiting bodies (mushrooms and toadstools) also produce toxic metabolites and these are a hazard when such fruiting bodies are eaten. It is convenient to deal with these compounds separately and not include them as mycotixons.

2. Mycotoxins as natural contaminants in food

Of the several hundred known toxic mould metabolites (see Moreau, 1974; Wyllie & Morehouse, 1977; Watson, 1985) only about three dozen have been shown to occur as natural contaminants in food (Krogh, 1987). Table 1 lists the majority of these with the species of mould known to produce them.

A further selection of mycotoxins, such as the satratoxins, verrucarins, sporidesmins and slaframine, have been identified in animal feeds and fodders.

3. Ability of mycotoxin production depends on species as well as circumstances

Some mycotoxins are only produced by a limited number of strains of one or two species of fungi, whereas others may be produced by a large number of species. Thus the aflatoxins are only known to be produced by Aspergillus flavus and A. parasiticus, whereas ochratoxin is produced by several species of Aspergillus and Penicillium. It is not the case that species of mould traditionally used as constituents of food of producing mycotoxins.

Thus, Aspergillus oryzae, used extensively in the production of koji for the manufacture of a wide range of foods, is able to produce cyclopiazonic acid and β-nitro propionic acid, and Penicillium roquefortii, used in the manufacture of all the blue cheeses of the world, can produce PR-toxin, roquefortine and several other toxic metabolites. Because processes, and strain properties, are developed to optimise such qualities as biomass and industrial enzyme production (and are generally inversely related to those developed to optimize, or even permit, secondary metabolite formation), the production of koji and blue cheese is not associated with any known mycotoxin problem. In a sense, it is the process, rather than the organism, which is safe.

4. Species specific mycotoxins

Table 2 lists some of the secondary metabolites associated with species of mould used for the production of enzymes.
Only Aspergillus alliaceus is known to produce one of the mycotoxins (ochratoxin) included in those routinely tested for using the method of Patterson and Roberts (i.e. aflatoxin B_1, ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone).
The major justification for looking for these mycotoxins in products from species not associated with their production must presumably be concern for carry over from contaminated raw materials, or a failure to maintain a pure culture during the manufacturing process.

5. Effect of mutations on mycotoxin production

The biosynthetic pathways leading to the production of mycotoxins are frequently complex involving many steps (Steyn, 1980). The majority, if not all, of these steps will involve an enzyme which in turn will be coded for by a gene.
Thus many genes may be involved in the production of a particular mycotoxin. It is thus a common experience that the ability to produce a particular mycotoxin is readily lost during routine subculture of the producing strain. In fact, those who are trying to industrially produce secondary metabolites need to take special care to avoid this happening. It is also relatively easy to lose the capability of producing a mycotoxin by a deliberate programme of mutation.
Since the chance to obtain a mutation defect in one of the many genes involved in mycotoxin synthesis is much higher than that of a mutation repair of one or more specific defects, the situation in which a non-toxigenic strain becomes toxigenic is far less common. Only one author (Benkhammar et al. (1985) has reported obtaining cyclopiazonic acid producing mutants of Aspergillus oryzae by treating a non-toxigenic strain with a mutagenic N-nitroso-guanidine derivative.

6. Mycotoxin and enzyme production: likelihood of co-production

The growth and morphological and biochemical differentiation of filamentous fungi involve the sequential induction, formation and repression of many hundreds of enzymes, some of which are involved in the biosynthesis of mycotoxins.
However, the relatively small number of enzymes of industrial interest are usually associated with the earlier stages of vigorous growth and their production is directly growth related. This is in contrast to the production of mycotoxins most of which occurs during the later stages of development and their optimum production is often associated with some form of stress on growth processes.
In a limited study of strains of Aspergillus flavus and closely related species at the University of Surrey, it was found that an inverse correlation occurs between the ability of strains to produce aflatoxin and the ability to produce and secrete high levels of growth related catabolic enzymes such as amylases. Such observations are entirely compatible with the suggestion that Aspergillus oryzae and A. sojae are "domesticated" forms of A. flavus and A. parasiticus respectively (Wicklow, 1984).

7. Mycotoxins and enzyme purification: likelihood of co-isolation

The enzymes of particular interest in the food industry are globular proteins which are high molecular weight water soluble compounds in contrast to the low molecular weight secondary metabolites many of which are more soluble in organic solvents than in water.
If secondary metabolites, including mycotoxins, were present in the production liquors from which enzyme are obtained, it is highly probable that some stages in down stream processing, such as ultra filtration, will effect a partial removal.

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TABLE 1: Mycotoxins identified as natural contaminants in food associated commodities.

MYCOTOXIN	MAJOR PRODUCING SPECIES
AFLATOXINS	*Aspergillus flavus, A. parasiticus*
OCHRATOXIN	*Aspergillus ochraceus,*
	Penicillium viridicatum
CITRININ	*Penicillium citrinum*
PENICILLIC ACID	*Penicillium spp., Aspergillus spp.*
PATULIN	*Penicillium expansum,*
	Aspergillus clavatus
STERIGMATOCYSTIN	*Aspergillus versicolor*
MYCOPHENOLIC ACID	*Penicillium roquefortii*
PENITREM A	*Penicillium aurantiogriseum*
P R TOXIN	*Penicillium roquefortii*
VIOMELLEIN	*Aspergillus ochraceus*
	Penicillium viridicatum
CYTOCHALASIN E	*Aspergillus clavatus*
CITREOVIRIDIN	*Penicillium citreonigrum*
CYCLOPIAZONIC ACID	*Aspergillus flavus,*
	Penicillium aurantiogriseum
ROQUEFORTINE	*Penicillium roquefortii*
ISOFUMIGACLAVINE	*Penicillium roquefortii*
ZERALENONE	*Fusarium* spp
ZEARALENOL	*Fusarium* spp
TRICHOTHECENES	*Fusarium* spp
MONILIFORMIN	*Fusarium* spp
TENUAZONIC ACID	*Alternaria* spp
ALTERNARIOL	*Alternaria* spp
ALTENUENE	*Alternaria* spp
ERGOT ALKALOIDS	*Claviceps* spp

TABLE 2: Examples of secondary metabolites reported to be produced by moulds used for the manufacture of enzymes.

MOULD SPECIES	METABOLITES
Aspergillus alliaceus	OCHRATOXINS A and B*
Aspergillus niger	RUBROFUSARIN B NIGERONE AURASPERONE NEOECHINULIN NIGRAGILLIN ASPERRUBROL
Aspergillus oryzae	B-NITROPROPIONIC ACID* MALTORYZINE* CYCLOPIAZONIC ACID* KOJIC ACID ORYZACIDIN ASPERGILLOMARASMINS
Penicillium funiculosum	11-DEACETOXY WORTMANNIN FUNICULOSIN SPICULISPORIC ACID
Trichoderma harzianum	IOSNITRINIC ACID*

* recognised as mycotoxins

APPENDIX 1

Search Strategy Used

Set	Items	Description
S1	5328	MYCOTOXIN
S2	16258	AFLATOXIN
S3	195	DIHYDROXYFLAV?
S4	736	DIACETOXYSCIRPENOL
S5	2352	OCHRATOXIN
S6	238	LUTEOSKYRIN
S7	0	EPOXY(W)TRICOTHECENE
S8	1226	STERIGMATOCYSTIN
S9	172721	TOXIN? ?
S10	2352	T(2W)2(2W)TOXIN
S11	2094	ZEARALENONE
S12	36	TRICOTHECENE
S13	394	RUBRATOXIN
S14	1506	PATULIN
S15	22946	S1 OR S2 OR S3 OR S4 OR S5
S16	173438	S6 OR S7 OR S8 OR S9 OR S10
S17	3802	S11 OR S12 OR S13 OR S14
S18	183474	S15 OR S16 OR S17
S19	1276181	ENZYME? ?
S20	261508	MANUFACTUR?
S21	2117	S19 AND S20
S22	80623	DEEP
S23	885707	CULTURE? ?
S24	2056	S22 AND S23
S25	4156	S21 OR S24
S26	77	S18 AND S25

The effect of the above strategy is that a reference is printed out if it contains one or more of the toxin keywords (S1 - S14) AND either Enzyme Manufacture OR Manufacture of Enzymes OR Manufacturing Enzymes etc., OR Deep Culture OR Deep Cultures. This gives a fairly wide coverage without overproducing results which swamp out relevant references and waste time, money and effort.

APPENDIX 2 - FILE SEARCHED

Files searched	Host	Major Journals Covered
Biotechnology	Orbit	Derwent Biotechnology Abst.
Current Awareness in Biotechnological Sciences	Orbit	Current Advances in Biotechnology Current Advances in Microbiol. Current Advances in Molecular Biol. Current Advances in Cell + Dev. Biol. Current Advances in Toxicology and many more
Biosis Previews	Dialog	Biological Abstracts
EMBASE	Dialog	Abstracts & Citations from 4000 worldwide Biomedical Journals
International Pharmaceutical Abstracts	Dialog	500 Pharmaceutical, medical + related Journals
Life Sciences Collection	Dialog	Industrial + Applied Microbiology, Microbiological abstracts
Chemcial Exposure	Dialog	Databank
Martindale on line	Dialog	Databank
Medline	Dialog	Index Medicus (3000 International Journals)
Occupational Safety & Health	Dialog	400 Journals 70,000 monographs
Chemical Regulations & Guideline system	Dialog	US Federal Databank on controlled substances
Drug information full text	Dialog	
Agrochemicals Handbook	Dialog	
CA Search	Dialog	Chemical abstracts
Merck index on line	Dialog	Merck index

Annex 7.1.4

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Annex 7.2

Annex 7.2

SAFETY EVALUATION using the PARIZA & JOHNSON DECISION TREE of PLA2 (Phospholipase A2 from porcine pancreas) : an enzyme derived from *Aspergillus niger* strain PLA-54

Introduction

The "Decision Tree for evaluation of the relative safety of food ingredients derived from genetically modified organisms" was published in 1991. This publication from the International Food Biotechnology Council (IFBC) was an extension, based on an earlier publication by Pariza and Foster in 1983[1]. The 1991 IFBC Decision Tree was updated by Pariza and Johnson in 2001[2].

The enzyme preparation phospholipase A2 derived from *Aspergillus niger* strain PLA-54, over-expressing the phospholipase A2 gene from porcine pancreas was evaluated according the Pariza & Johnson Decision Tree. The result is described below.

Decision Tree

1. Is the production strain genetically modified?
   YES
   The strain *Aspergillus niger* PLA-54 is derived from host ISO-502, which is a genetically modified strain (GMO self-clone) derived from the DSM GAM lineage of *A. niger* strains.
   If yes, go to 2.

2. Is the production strain modified using rDNA techniques?
   YES (see question 1)
   If yes, go to 3.

3. Issues related to the introduced DNA are addressed in 3a-3e.

3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?
   YES
   If yes, go to 3c.
   Phospholipase A_2 from porcine or bovine pancreas has a history of safe use. The enzyme is generically listed as one of two enzymes used for production of enzyme-modified lecithin, which is affirmed as GRAS in 21CFR184.1063. Phospolipases from microbial sources can be found in the lists of GRAS notices of CFSAN/Office of Food Additive Safety (phospholipase A_2 from *S. violaceoruber* – GRN000145; phospholipase A1 from *F. venenatum* overexpressed in *A. oryzae* – GRN000142).

Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?
   YES
   If yes, go to 3c.

[1] Pariza M.W. and Foster E.M. J. Food Protection **46**. (1983), 453-468
[2] Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. **33** (2001) 173-186

This test article is made by a new generation of DSM *A. niger* production strains, for which the safety has been covered by safety studies on previous test articles from the same safe strain lineage3. However, since the product was developed before this paper was published, safety tests have been done and the NOAEL of the tox-batch was 10,000 mg/kg bw/day, corresponding to a Margin of Safety of at least 650 for all intended applications (90th percentile intake method). This is sufficiently high to ensure safety.

3c. Is the test article free of transferable antibiotic resistance gene DNA?
YES
If yes, go to 3e.

3d. Does (Do) the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animals?
NA

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?
NA
The final production strain is complete marker gene free and devoid of any uncharacterized heterologous DNA.

4. Is the introduced DNA randomly integrated into the chromosome?
NO
If no, go to 6.
The introduced DNA is targetedly integrated.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects, which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?
NA
Nevertheless, the strain has been analyzed with respect to its potential to produce secondary metabolites, including mycotoxins. The strain PLA-54 showed no potential to produce secondary metabolites or mycotoxins of importance in food. This was supported by analyses on broth and concentrated UF samples.
If yes, go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?
YES
Many strains of this safe strain lineage exist, for which safety data are available, that can be or have been tested through the Pariza & Johnson Decision Tree evaluation scheme.
If yes, the test article is ACCEPTED.

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3 Van Dijck, P.W.M. et al. On the Safety of a new generation of DSM aspergillus niger enzyme production strains. Reg. Toxicol. Pharmacol. 38:27-35 (2003).

Annex 7.2.1

WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

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Copenhagen, August 1998

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Summary

In recent years, claims have been made by the media and some consumer organisations that enzyme residues in bread and other foods can result in allergic responses in the consumers of that food.

AMFEP established an Expert Group to evaluate whether residual enzymes in foods are an allergy risk for consumers. The Expert Group was asked to investigate existing scientific data and to report the results of the findings.

The main questions were whether enzymes in, for example, bread can sensitise a consumer of the bread, and subsequently if the presence of the enzyme residue could induce symptoms of allergy.

A further question was if a person with existing allergy to common allergens could develop allergy symptoms upon eating foods containing residual enzymes by cross reaction. This is not uncommon in the case of food allergy.

The literature survey was made to search for general food allergy, epidemiology and to find cases of food related enzyme allergy. In addition a survey of enzyme producers' files was carried out to look for adverse reactions to food enzymes.

High daily doses of industrial enzymes in are prescribed for patients with insufficient function of the pancreas. The literature on adverse events was reviewed and telephone interviews were undertaken with authorities and university hospital departments to check if experience of enzyme related gastrointestinal allergy were observed but not published.

Studies of common food allergy indicate a relatively low prevalence of about 2% of populations in Europe and the United States. There is however, a significant discrepancy between the perception of being allergic to foods (15%) and those that can be verified as food allergy (2%).

Yet, there are no firm data of the doses required to sensitise a person via the gastrointestinal tract, but the doses required to induce sensitisation seem to be very high. Indeed, patients with insufficient enzyme production of the pancreas need to take industrial enzymes in doses 100.000 - I million times higher than the amounts found in food.

There are no published cases of people that have been sensitised by the ingestion of food with residual enzymes, and even people who ingest high daily doses of enzymes as digestive aids are not reported to have gastrointestinal allergy to enzymes, even after many years of daily intake.

There are a few case histories of people who had reactions to papain, extracted from the papaya fruit. Papain in powder form is used as a meat tenderiser in some countries. It is unclear if the sensitisation in these cases occurred by inhalation of the powder or by ingestion of the meat with the papain.

One case history described a person who reacted with hay-fever upon eating a lactase tablet. This case was incomplete in describing the possible source of sensitisation.

There are 2 cases of people with baker's asthma and allergy to α-amylase, and wheat flour who developed symptoms after the ingestion of bread. The symptoms were somewhat more pronounced after bread prepared with α-amylase than bread without. One case with occupational allergy to α-amylase reacted upon ingestion of a very high test-dose of pure α-amylase, but not at lower doses. Four other persons with occupational α-amylase allergy did not react at any dose.

The question of cross reactions between common moulds and enzymes produced in related moulds was described in a double blind placebo controlled food challenge study of asthma patients with allergy to Aspergillus fumigatus. This mould is closely related to *Aspergillus oryzae* and - *niger* which are used for the production of industrial α-amylase. None of the test persons could be challenged to elicited symptoms by eating bread prepared with enzymes.

The expert group concludes that there are no scientific indications that the small amounts of enzymes in bread and other foods can sensitise or induce allergy reactions in consumers.

Employees with respiratory occupational enzyme allergy should be informed that in rare cases, symptoms may be induced by ingestion of food with residual enzymes. Enzyme residues in bread or other foods do not represent any unacceptable risk to consumers.

1.0. Introduction

Since the late 80'ies, and particularly since 1992 it has been repeatedly claimed that enzyme residues in foods may represent a hazard to consumers in the form of allergies, and that a certain percentage of the population are at risk of having allergicy reactions to enzymes in bread and other foods.

In particular it has been claimed that consumers were at risk of developing severe allergy symptoms caused by α-amylase. The public was somewhat alarmed and there have been complaints, questions and other reactions of concern to bakers and other suppliers.

The media's interest was based on results from a study by Schata[1], published only as a 1/2-page abstract which does not allow for scientific evaluation.

However the issue was effectively raised within the public, and industry had no data with which to make a response.

Since 1992, the issue of allergy risk in consumers have emerged from time to time on television in the TV and the printed media. The general issue as it has emerged over these years is that there is a concern in the public that enzymes are unsafe, and as far as the bakers and the flour improvers are concerned, require and request data to oppose the allegations.

An additional concern is the possible cross reaction between enzymes produced by fermentation of certain moulds which may be related to common moulds. In theory, a person with a preexisting allergy to *Aspergillus sp.* might react to enzymes from e.g. *Aspergillus niger or A. oryzae.*

2.0 Background

2.1 General

In the public mind there is some confusion about the frequency of allergy, and in particular on food allergy. However, in the scientific community there seem to be consensus of the following:

- The frequency of common allergy (all allergies included) is 20 - 30%, in most populations around the world. The figure is increasing. Part of the increase may be due to higher awareness and improved diagnostic methods, however, a true increase cannot be ruled out.
- The frequency of occupational allergy in bakers is 8 - 27%. About 30 - 35%, of the bakers with occupational allergy to flour have an additional respiratory allergy to α-amylase and/or other baking enzymes.
- There is a reasonably good documentation of the frequency of food allergy in the general population at 1 - 2%. However, the frequency of perceived food allergy allergy in the general population is 12 - 16%
- Food allergy does not differ from inhalation allergies with regard to the biological mechanisms taking place in the immune system. Any 'true' allergy is based on allergy antibodies (IgE). Allergy antibodies are produced by the white blood cells called lymphocytes after the allergen has been introduced to these cells by inhalation or by ingestion. This process is called 'sensitisation'.
- Sensitisation then, is merely the event of the body recognising the foreign aller genic protein and reacting to it by producing allergy antibodies specifically recognising the particular allergen.
- Sensitisation is not a disease.
- It only becomes an allergic disease if the person develop symptoms related to exposure to the particular allergen.
- Not all sensitised people exhibit symptoms of allergy have allergy-symptoms.

2.2 *Occupational respiratory allergy*

allergy caused by inhalation of airborne particles of proteins, incl. Enzymes

Fungal enzymes, bacterial enzymes and extracted plant and animal enzymes are equally capable of inducing respiratory allergy - Papain and Bromelain[2-4], Trypsin[5], protease's from the skin yeast Candida albicans[6], from bacteria/ subtilisins[7,8], fungal amylases[9,10], bacterial amylases[11], fungal hemicellulases[12], lipases[13], xylanases and cellulases14,15 are all examples of industrial enzymes known to induce allergic sensitisation and respiratory occupational allergy. This is a feature characterised by highly purified enzyme protein products rather than the origin or the methods of production.

They all share the structural and biological properties that may cause sensitisation when inhaled.

The classical food allergens are also capable of inducing respiratory allergy when they are brought into a dust- or aerosol form and inhaled. Soya[16], eggs[17,18], milk[19] and fish[20] are just examples. Soya may be one of the best described examples of epidemic inhalation allergy to an allergen also well recognised as a food allergen[21].

3.0. *Food allergy*

3.1. *Allergy caused by ingestion of proteins in foods*

Eight percent of children under 3 years of age are allergic to food[22]. In, and in this age group, milk, egg, fish and soya are examples of common allergens. Many of these allergies disappear with age, but food allergy is seen also in older children and in adults. The overall frequency of verified food allergy is 1 - 2% of the population[22-25].

Food allergy is the adverse reaction to food characterised by allergic sensitisation to food proteins and elicitation of symptoms by ingestion of the same food proteins.

Symptoms

The symptoms of food allergy are gastrointestinal with vomiting and diarrhoea, sometimes accompanied by urticaria, asthma or hay-fever. Generalised very severe reactions occur in rare cases.

Many food allergies are very mild, with symptoms of itching and burning sensation in the mouth. This is also a feature of most of the well known cross-reactions between common inhalation allergens and foods. An example can be found in patients with a birch pollen allergy who also react to e.g. fresh apples, without having a specific allergy to apples. Another well known cross reaction is that of latex and bananas. There are a number of such cross reactions between common pollen allergens and certain foods.

Types of food allergens

Examples of 'true food allergens' are proteins in milk, egg, soya, wheat, fish, nuts and, peanuts and a few more. There are others, but only about 10 food allergens account for more than 95% of severe cases. However the list of food allergens is extremely long and a large number of food allergens only give rise to allergy in sporadic cases.

The common features of food allergens are largely shared by those of respiratory allergens. However, foods are very often treated by cooking and other physico-chemical means that may destroy part of the protein structure and thereby its allergenic properties.

Properties of food allergens

The molecular weights of allergens are typically in the range of 10 -70 (90) kDa.

They have a number of 'epitopes', i.e. sequences of 8 - 16 amino acids. These are the structural 'units' which can be identified by the immune system and lead to production of specific IgE (sensitisation). In the sensitised individual the specific IgE readily recognises the epitopes on the par-ticular protein, resulting in allergy symptoms. Some of these epitopes are described in literature26-28.

Food allergens are stable to digestion and most also to heating by cooking, and in most cases, food allergens can represent a very large proportion of the food itself Enzymes are not well described with regard to neither their fate after ingestion nor their allergenic properties after cooking.

The TNO Institute performed a study[58] on native α-amylase from *Aspergillus oryzae* in a gastrointestinal model simulating the physiological events in the stomach.

The results indicate that about 92%, of the epitopes of the α-amylase are destroyed and about 8%, of the epitopes on the α-amylase are intact at the delivery from the stomach to the duodenum.

However, it can be expected that the proteolytic pancreatic enzymes will reduce even further, the remaining 7 – 8%, of the α-amylase during the passage through the duodenum.

Doses at which food allergy occurs

The doses and other conditions necessary to sensitise an individual are not well known. It is believed that the sensitising doses must be considerably higher than doses required for elicitation of symptoms in patients already sensitised. There are many examples of sensitised people reacting to trace amounts of allergens in the food - some of them with fatal outcomes.

It is therefore understandable that there is some focus on hidden allergens like traces of milk, nuts and peanuts in other foods.

Steinman[29] wrote a leading article in the August 1996 issue of J. Allergy Clin. Immunol. regarding hidden allergens in food. It is representative of the concern in the medical profession and in the public. He suggested a number of preventive measures including labelling in clear language. His article does not mention enzymes.

Food produced by GMO's

Genetically Modified Organisms (GMO's), and enzymes produced by GMO's have raised concern in general and also specifically for enzymes used in food processing.

Scientists in the fields of gene technology[30-33] and allergy seem to agree that gene technology and the results thereof expressed in foods should not cause concern with regard to allergy risk. However, gene technology does bring about new proteins, and it is important to be aware that some of these new proteins may be allergenic.

Genetically modified proteins may, or may not share allergenic properties with traditional allergens. This would relate to the nature of the protein as it does in all other circumstances, and there are no examples of involuntary (or voluntary) changes of allergenicity of proteins in food.

A possibility may be that in the future, gene technology may be used as a tool to produce less allergenic proteins. This might be a future example of voluntary change of allergenicity.

Enzymes produced by GMO's have been on the market in some countries for many years. Enzyme producers have not experienced any difference in allergenicity of these enzymes as compared to traditional extracted or fermented enzymes. They appear to have the same sensitising potential as are capable of sensitising exposed employees at the same rate as traditional enzymes.

3.2. Epidemiology of Food Allergy

In a survey of 5000 households in the USA carried out in 1989, 1992 and again in 1993[25] it was found that 13.9 -16.2% of the households reported at least one member to be allergic to foods.

A study of food allergy in a random sample of 1483 adults in Holland[23] showed that 12.4% reported allergy to foods, but by controlled tests only 2.4% could be confirmed by Double Blind Placebo Controlled Food Challenge (DBPCFC).

In Spain, 3034 patients from the outpatient allergy clinics at two hospitals were tested for food allergy[24]. The patients were tested by skin prick, RAST and open food challenge. They found 0.98% positive to one or more foods.

When looking at food additives, the same pattern emerges. In a survey of a population sample in the UK, 7% claimed to have reactions to food additives. Double blind challenge tests could verify only 0.01 - 0.23% to be true reactions to food additives[34].

The frequencies of confirmed food allergy in different countries in Europe and the USA are quite uniform at 1 - 2.5% of the populations.

A number of explanations to the discrepancy of perception and verified cases has been offered. There are indications that the public attribute a number of conditions to 'something in the food' and consider themselves allergic without ever having it tested.

A certain number of perceived food allergy may be induced by members of the medical profession, conducting less efficiently controlled test programs. In some cases, patients are declared food allergic solely based on skin prick tests -which may well over-diagnose food-reactions. High focus on food allergy in the media combined with personal and psychological conditions may also play a role. Actually some specialists in food allergy consider the psychological disorders the most important differential- diagnosis from food allergy.

A diagnosis must rest upon a combination of a medical history and objective tests to confirm or reject the tentative diagnosis. In the field of food-related allergies, the diagnostic test systems have been difficult to establish. However, the Double Blind Placebo Controlled Food Challenge (DBPCFC)[35,36], is the method of choice to confirm or reject indications of food allergy that may derive from the patient's perception and in many cases also from skin prick testing.

The experience from food allergy centres is that objective test programs to confirm or reject a suspected 'food allergy', requires skin- and blood tests and up to 6 placebo controlled challenges to be reliable.

Therefore a diagnosis of food-related allergy, based solely on medical history and a skin prick test is not good clinical practice and must be regarded un-ethical

3.3. Enzymes in food

In theory, enzyme sensitisation and allergy symptoms may be induced by direct ingestion of consumer products containing enzyme residues may occur

The tendency in recent years to focus on allergy and food allergy in particular may explain part of the marked discrepancy between the public perception of allergy to food - and the relatively few cases that can be verified in controlled clinical tests.

Papain is relatively widely used as a meat tenderiser, often supplied in a powder form to apply to the meat before cooking.

In 1983 Mansfield and co-workers[37] published a case story of a person who had allergicy symptoms after ingestion of papain used as a meat tenderiser. - Later, in 1985 they reported a study of 475 patients[38] with allergy of which 5 had a positive skin prick test to Papain.

The 5 papain positive were subjected to oral challenge with papain and all had positive reactions to the challenge.

Unfortunately, the challenge was only single blinded, and there is no report of occupational exposure or the use of powdered meat tenderisers that may have caused respiratory sensitisation.

In one other case story by Binkley[39], described below in the section 3.6.2, it can't be totally excluded that sensitisation took place by ingestion of a food product containing relatively high amounts of industrial produced enzymes.

A recent review by Wûthrichl[40] of enzymes in food concluded that orally ingested enzymes are not potent allergens and that sensitisation to ingested enzymes is rare as is also the case of reactions to bread in bakers with occupational allergy to enzymes.

The member companies of AMFEP have not registered, experienced or heard of consumers that have become sensitised to enzymes or enzyme residues in consumer products by ingestion.

It has not been possible to verify the claims in the media of such cases, and they seem as yet un-substantiated as examples of enzyme allergies in consumers. The patients presented and the symptoms and tests described are not documented, merely describing sensations and feelings,however presented as facts.

A large proportion of adverse reactions to food must be ascribed to digestive disorders such as intolerance to for example gluten and lactose, which are not allergic reactions.

3.4. The Theory of cross reactions

people sensitised with common moulds might react to enzymes produced in related moulds

The theory that people with allergy to common moulds which are related to those used for the fermentation of enzymes might react to enzyme residues in food was one of Schata's[1] claims and was given relatively high coverage in the media.

The theory could not be readily rejected as cross-reactions are relatively common in allergy. A number of food allergy reactions are merely cross reactions than caused by primary sensitisation.

The most commonly used moulds for fermenting enzymes are *Aspergillus oryzae* or *A. niger*.

According to the theory, people with allergy to *Aspergillus*-moulds would be a high risk population. *Aspergillus* allergy occurs in less than 0.5%, of the population.

A study by Cullinan[41] was conducted with the objective of testing if patients with a well-documented allergy to the widely distributed common mould *Aspergillus fumigatus* reacted upon the ingestion of bread prepared with enzymes of *Aspergillus* origin. The study was a double blind placebo controlled food challenge study on 17 *Aspergillus* allergic people.

The 17 test persons all had allergy antibodies to *Aspergillus fumigatus*, but in addition, 6 also reacted at the skin prick test to the enzymes produced in *A. oryzae* or *A. niger*.

Each patient was challenged with bread baked with the 2 enzymes in standard doses and with placebo bread baked without enzymes. Allergy symptoms and a number of general physiological parameters were monitored before, during and for 24 hours after the challenge.

No allergicy reactions were seen upon ingestion of enzyme containing bread as compared to placebo bread.

This study clearly demonstrates that patients who must be considered at the highest risk for cross reactions to baking enzymes do not react with clinical symptoms when they eat enzyme containing bread containing enzymes.

It is a general experience that once a person is sensitised, even very small amounts of the allergen can elicit allergy symptoms.

In the case of baking enzymes it seems well documented that even patients with severe asthma caused by *Aspergillus fumigatus* did not react to the baking enzymes produced in *A. oryzae* and *A. niger*.

3.5. *Food related reactions in occupationally sensitised people*

The situation of possible reactions to enzymes in bread in patients with occupational allergy to enzymes

There are a few papers describing cases of allergy symptoms elicited by the ingestion of enzymes in people who have occupational allergy to enzymes:

Kanny & Moneret-Vautrin,.[42] and Baur & Czuppon[43] each describes one patient who since late childhood, has had asthma and occupational asthma with allergy to flour and enzymes for several years. Both patients were tested for elicitation of symptoms by ingestion of bread baked with and without enzymes. Kanny & Moneret-Vautrin's patient was tested in a blinded design, Baur's patient in an open, non-controlled programme. In both cases the result was elicitation of respiratory symptoms after challenge with bread baked with enzymes. Baur's patient also had a slight reaction to bread without enzymes, however not as pronounced as the reaction after the enzyme containing bread.

Losada et al[44] investigated occupational allergy to α-amylase in a pharmaceutical plant and found a number of employees sensitised to α-amylase. None reported reactions related to ingestion of bread. Five patients, all positive to α-amylase were given oral doses of native α-amylase in doses up to 10 mg.

At this dosage, one of the 5 test persons reacted with respiratory- and generalised allergy symptoms. Four did not react.

Baur et al[45] described the possible background for consumer sensitisation to α-amylases in bread. 138 subjects, of which 98 were allergic, and 11 bakers with occupational allergy were tested. The bakers reacted to α-amylase as may be expected. None of the atopics and none of the control persons reacted to skin prick test with α-amylase. Two atopics had weak RAST to native α-amylase and one reacted also to heated ce-amylase. Reactions to other related compounds, for example *Aspergillus* was not tested.

Tarlo and co-workers[46] reported results of testing for papain allergy in 330 allergy patients. - Seven had positive RAST and Skin prick test but none of them had any gastrointestinal or other allergic symptoms to papain.

The elicitation of gastrointestinal symptoms upon respiratory sensitisation is also reported for flours. One example is reported by Vidal et al[47] and describes a man with occupational asthma after exposure to flours and other grain dusts. He was sensitised to barley, and experienced gastrointestinal reaction upon ingestion of foods and beverages made from barley.

Enzyme producers and other companies handling concentrated enzymes do see cases of employees being sensitised to baking enzymes. These would be the people at the highest risk of reacting to enzyme residues in bread.

However, none of the members of AMFEP had any reports of sensitised employees who had experienced allergy symptoms in connection to ingestion of bread, and there are no reports of α-amylase sensitised employees avoiding bread.

Cases of people with occupational allergy to flours and food-related reactions to ingestion of flours/bread do occur. One case report describes a person with asthma to barley dust and also with reaction to beverages and foods produced from barley.

The conclusion from these reports of people with pre-existing occup. allergy to α-amylase is:

- Allergic reactions after ingestion of enzyme containing foods are described in 3 individuals.
- The 3 cases are people with definite occupational respiratory allergy to flour and an additional sensitisation to α-amylase. It means they are most probably sensitised by inhalation of flour dust and enzyme dust and not by eating bread or other foods with enzyme residues in it.

3.6 The consumption of enzymes for medical purposes and as digestive aids:

Many people around the world eat enzymes for medical purposes or for convenience as digestive aids.

In many countries enzymes are used routinely as digestive aids by healthy people. The number of people in the world, frequently eating enzyme preparations must be counted in millions.

A number of diseases require the daily addition of enzyme preparation to the food to compensate the patient's insufficient production of digestive enzymes.

3.6.1. Medical uses:

Medical use of enzyme preparations are subject to clinical trials, the results of which are normally reported to the health authorities, and such adverse effects are described in the pharmacopoeia/registry of drugs.

Patients with chronic pancreatitis suffer from insufficient production of digestive enzymes from the pancreas. They are dependent on daily intake of enzymes, some of these produced from *Aspergillus* and other moulds, some extracted from animal glands. The doses of these enzymes are in the order of gram's a day. - we have not been able to identify published documentation of allergy to enzymes in these patients, and the drug registry's does not even mention allergy as an adverse effect.

Proteolytic enzymes and mixtures of different enzymes are commonly used for treatment of a number of physical lesions and also for a number of more special conditions[48-50].

The enzymes are administered in the form of tablets with mixtures of enzymes and in doses of 6 to 600 mg per day, in some cases several times more.

We have not been able to find any evidence of sensitisation or allergy symptoms caused by the ingestion of enzymes from these enzyme preparations. One example is the use of enzymes given as tablets for the treatment of non-articular rheumatism. Uffelmann[51] describes a double blind study of 424 patients, of which 211 received enzyme treatment. The daily doses of the mixed enzyme preparations was 240 mg Lipase, 240 mg Amylase, 1,44 g Papain, 1,08 g Bromelain and 2.4 g Pancreatin,. This dosage was given for 8 weeks and no serious adverse effects and no allergy reactions were reported.

Patients with Cystic Fibrosis suffer a hereditary disease characterised by severe lung symptoms and insufficient production of digestive pancreatic enzymes. They too are dependent of daily intake of grain-doses of enzymes. - There are a few reports of parents

and hospital staff who have become sensitised by inhalation of dust from these enzyme preparations[52-54]. This of course might also happen to the Cystic fibrosis patients when they handle the enzyme preparations themselves. However no cases of enzyme allergy in Cystic Fibrosis patients have been described, but there are reports of allergy to common food allergens[55].

An informal telephone survey on unpublished cases of enzyme allergy to European Cystic fibrosis Centres, resulted in only one possible case. The patient was a boy who reacted with vomiting after administration of the enzyme preparation containing amylase, protease and lipase. - The enzyme treatment had been stopped because of suspected allergy to the enzymes. However, testing for specific allergy antibodies by Maxisorp RAST[56] did not confirm sensitisation to any of the enzymes. Challenge tests have not been performed[57]

3.6.2. Digestive aids one possible case of allergy to digestive aid enzymes

In some cultures the use of digestive enzymes after large meals is very common. Enzymes for this purpose are 'over the counter' (OTC) drugs. We have found no studies of possible allergy to enzymes in these populations. That may be irrelevant if no-one ever thought of the possibility that enzymes might be the cause of allergicy symptoms had not been considered. - However, with millions of people using enzymes frequently, some cases of adverse effects in the form of allergic symptoms would be expected to emerge and be described in the literature. In most patients with allergic reactions, symptoms would appear immediately or very shortly after the intake.

Binkley[39], described a case of allergic reaction to ingested lactase. This patient had a respiratory allergy with positive skin prick test reaction to *Aspergillus sp.*

He had had two incidents with allergic reactions in the form of swelling and burning s tion of Lactaid tablets. The lactase was produced from fermentation of Aspergillus oryzae. Skin prick test with extracts of Lactase tablets gave a very strong positive reaction. He had not taken Lactaid tablets previous to the first experience of symptoms, but he had taken milk products containing lactase from *Saccharomyces fragilis* and from *Kluyveromyces lactis*. Although highly unlikely, it may be speculated if these may cross react with Lactaid. In this case it seems unlikely that sensitisation was caused by the Lactaid tablets as the symptoms appeared the first time he ever took Lactaid. It could be a 'cross reaction' based on sensitisation to yeast-produced lactase and symptoms elicited by the ingestion of Lactaid. Another possibility may be a cross reaction from his pre-existing *Aspergillus sp.* allergy.

This case may be regarded a possible but not verified case of oral sensitisation to enzymes in food.

A few other consumers haves claimed allergy to these OTC drugs but thorough testing has not verified allergy to enzymes in any of these cases.

With the background of the very high awareness of food related allergy in the populations, the widespread use of digestive aid and medical uses of enzymes should have attracted interest if allergy to ingested enzymes were of importance. However, up to now, only the single case mentioned above have been described.

To evaluate the risk of sensitisation from ingestion of enzymes and eventually experience of symptoms, we are aware of only the one case that may have become sensitised by ingestion.

This has to be related to the total number of people world-wide who ingest enzymes for short periods of time as part of a medical treatment, and to those who are dependent of daily intake of high amounts of digestive enzymes.

4.0. Conclusion

The working group has studied the available literature on these subjects and came to the conclusion that from a scientific point of view there is no indication that enzyme residues in bread or in other foods may represent an unacceptable risk for consumers.

Lack of scientific data is not evidence of lack of risk, and the working group realises that evidence of 'no risk' is extremely difficult or impossible to generate.

The group wish to stress that a 'zero-risk' can never be proved by science, and it must be anticipated that even an extremely low risk (e.g. 1 in 50 or 100 millions) of verified allergy to enzymes in food may well be perceived as a significant and unacceptable risk by the public in which more than 10% believe they are allergic to food.

Scientific data are of high value as the credible background for promotion to the public, to trade organisations and individual customers and for an ongoing dialogue with opinion leaders and consumer organisations.

It is the opinion of the group that many cases of perceived allergy to enzymes may be attributed to insufficient diagnostic procedures employed by members of the medical profession.

A minimum requirement for establishing a diagnosis of food related enzyme allergy should be a well conducted DBPCFC.

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SUBMISSION END

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Reference List for Industry Submission, GRN 000183

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000067 - 000068	Committee on Food Chemicals Codex	Phospholiphase A2 Activity	July 1, 1996	Food Chemicals Codex	Fourth Edition, pg 808
000098 - 000107	Schuster, E.; Dunn-Coleman, N.; Frisvad, J.C.; vanDijck, P.W.M.	On the safety of Aspergillus niger - a review	June 20, 2002	Applied Microbiology and Biotechnology	Volume 59, pgs 426 - 435
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000130 - 000134	Joint FAO/WHO Expert Committee on Food Additives	3.2 Enzyme preparations: Enzyme preparations derived from Aspergillus niger	1990	World Health Organization Technical Report: Evaluation of certain food additives and contaminants	Series 789, pgs 15, 44-46

NA- Not applicable

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REC'D JAN 30 2006

Re: *Aspergillus niger* Expressing a Gene Encoding Porcine Phospholipase A_2 GRN No. 183

Dear Mr. Merker:

As we discussed in our telephone conversation on January 9, 2005, FDA is seeking clarification of some points arising from the agency's review of the DSM Food Specialties, Inc. GRAS Notification for phospholipase A_2. This letter responds to each of FDA's inquiries.

1. **FDA asked for the name of the antifoam used in the fermentation process.**

   The name of the antifoam presently in use in the manufacture of DSM's phospholipase A_2 product is [redacted] (b)(4) [redacted]

2. **FDA requested clarification of an apparent inconsistency in the measurement of phospholipase activity in EYU, as described in Section 4.7 Quality Control of Finished Product (page 17) as compared to Section 5.1 Formulation (page 18).**

   The activity of the UF concentrate, which is the common starting material for all formulations, is lower than both the final dried as well as the final liquid formulation, because the downstream processing (described on page 16) concentrates the final formulations.

   In the case of drying, the UF concentrate of course loses water and thus becomes more concentrated. Nevertheless, the ratio between activity and TOS will remain the same, as both the enzyme as well as other TOS components will remain in the dried product.

   In the case of the liquid formulation, the UF concentrate is purified by column chromatography. This also results in a concentration of the enzyme activity. In this case, however, the ratio between activity and TOS will also change, because other TOS components are removed from the enzyme during the column chromatography. In other

words, the product becomes more pure. The purity is about a factor 30 higher, as explained on the top of page 19.

The above explains why there is a difference between the activity levels of the UF concentrate and the final liquid and dry products. However, the difference is not as high as it appears from the values given because, as noted, a range is mentioned at the top of page 18 (2200-3000 EYU/g) that is actually based on the first few batches (including the tox-batch) produced some time ago on lower scale. Nowadays, the production is at higher scale and automatically more efficient. This results in higher activities in the UF concentrate, as can be seen in the Table on page 18, where the activities of three batches are mentioned.

With the present range of 4000-7000 EYU/g for the UF concentrate, this means that the maximal difference between the UF concentrate and the dried and the liquid product is a factor of 6 and 3, respectively.

3. **FDA asked to see residual amount of enzyme information consistently expressed in TOS.**

As explained above (question 2), the TOS values of the dried and liquid preparation differ from the UF concentrate and from each other. Moreover, in the case of the liquid preparation also the ratio between activity and TOS differs from the one of the UF concentrate.

For this reason, the figures in Tables on page 23 were expressed in activity and not in TOS. In order to express these figures in TOS, one can make the following calculations:

<u>Dried product:</u>

- Residual activity in final bread and fine bakery wares: 875-9100 EYU/kg.
- Ratio activity/TOS = same as UF concentrate (page 18) = 25285 EYU/g TOS (or 25.3 EYU/mg TOS)
- Thus TOS/kg bread and fine bakery wares: 875/25.3 to 9100/25.3 = **35-360 mg TOS/kg**

<u>Liquid product:</u>

- Residual activity in final dressings and sauces: 330-1300 EYU/kg.
- Ratio activity/TOS = factor 30 higher than UF concentrate (page 19) = 759 EYU/mg TOS

- Thus TOS/kg dressings and sauces: 330/759 to 1300/759 = **0.4 – 1.7 mg TOS/kg**

Based on the above, the estimated daily intake (page 32) can also be expressed in TOS:

- Bread: 4.8/25.3 – 26.1/25.3 EYU/kg bw/day = **0.2 – 1.0 mg TOS/kg bw/day**
- Fine bakery wares: 1.3/25.3 – 2.6/25.3 EYU/kg bw/day = **0.05 – 0.1 mg TOS/kg bw/day**
- Dressings and sauces: 0.2/759 – 0.8/759 EYU/kg bw/ day = **0.0003 - 0.001 mg TOS/kg bw/day**

It should be noted that the tox-studies have been performed with the UF concentrate, and not with the 30-fold purified product. If expressed on basis of TOS, the safety margin for the dressings and sauces would become a factor 30 higher than when expressed on basis of activity as it is done on page 32. Therefore, the firm chose to express the safety margin on basis of activity.

We hope that these responses address the questions.

Sincerely,

(b)(6)

Gary L. Yingling

GLY

AM

Merker, Robert I

From: Yingling, Gary L. [gyingling@klng.com]
Sent: Thursday, April 06, 2006 9:46 AM
To: Merker, Robert I
Subject: RE: GRN 183 - Response to Questions

Thanks. gary

From: Merker, Robert I [mailto:robert.merker@fda.hhs.gov]
Sent: Wednesday, April 05, 2006 3:07 PM
To: Yingling, Gary L.
Subject: RE: GRN 183 - Response to Questions

Gary,
I think we'll be fine with the email.

Bob

From: Yingling, Gary L. [mailto:gyingling@klng.com]
Sent: Wednesday, April 05, 2006 1:24 PM
To: Merker, Robert I
Subject: FW: GRN 183 - Response to Questions

Bob: Attached is a memo with the response to the questions FDA had concerning the GRAS Notification. Would it be helpful to submit these in hard copy or is the email acceptable. You can reach me at 202-778-9124. gary

000164

Answers to questions from FDA (email 27 March 2006)

Question 1:

Has the notifier confirmed that the pH optima and other characteristics of the porcine enzyme and enzyme produced in *A. niger* are the same? Or does the information only apply to the porcine enzyme?

Answer:

As the nucleotide sequence (see Chapter 2.2 of the GRAS notification dossier) of the DNA coding for both enzymes is exactly the same, it is to be expected that the enzymes themselves as well as their characteristics are the same. This has been confirmed for the pH- as well as temperature optima.

The GRAS notification dossier describes some other characteristics that confirm that both enzymes are the same:

- The specific enzyme activity (see Chapter 3.4, page 13)
- The Molecular Weight (see Chapter 3.3, page 12)
- The N-terminal amino acid sequence of the protein (see Chapter 3.3., page 12)

Question 2:

Please provide information on the meaning of the units of the glucoamylase and alpha-amylase activity units for which limits are provided in the specs and reference to assay conditions. [The alpha amylase units were available pretty easily from sources we could access over the Internet, but should be described in the notice.]

Answer:

Glucoamylase or amyloglucosidase:
DSM developed its own method for measuring glucoamylase activity. The method is based on the use of soluble starch as substrate. The enzyme activity described in this method is expressed in so-called "Amyloglucosidase Units" (AGI/g). One AGI is defined as the quantity of enzyme which produces 1 µmole of glucose per minute at pH 4.3 and 60°C from a soluble starch substrate.

Alpha-amylase:
DSM developed its own method for measuring alpha-amylase activity. The method is based on the use of soluble starch as substrate. The enzyme activity described in this method is expressed in so-called "Fungal Alpha-amylase Units" (FAU/g). One FAU is defined as the quantity of enzyme that transforms one gram of soluble starch in one hour under the conditions of the assay into a product having an equal absorption to a reference color at 620 nm after reaction with a known concentration of iodine at pH 5.0 and 30^0C and a reaction time between 15-25 minutes. The reference colour is defined as the absorbance of a $CoCl_2$ colour standard consisting of 25.0 g $CoCl_2.H_2O$ and 3.84 potasium dichromate in 100 ml of 0.01 N hydrochloric acid.

Answers to questions from FDA (email 27 March 2006)

Question 3:

On page 26 of the notice, it says that the toxicological studies were performed on the "UF concentrate." However, page 29 refers to the use of the "native active preparation." Elsewhere the term "tox batch" is used, which seems to refer to the information for batch PLA 9901 on page 18. Do these all refer to the same thing? Please clarify.

In section 7.4.1 on page 29, it says:

both an EDTA inactivated tox batch (batch PLA9901-inactivated; 70 EYU/g) and a phospholipase **A2** enriched tox-batch (batch PLA9901-enriched; 9750 EYU/g) were prepared and tested at a single high oral dose in the rat. Since no adverse effects were observed it was decided to perform the necessary toxicity studies with the native, active preparation (batch PLA9901; 2230 EYU/g, TOS 13.5% (values same as those on page 18). In essence, is the native active preparation the same as the UF concentrate?

Answer:

The 'tox-batch' (PLA 9901 with an activity of 2230 EYU/g) was used for all the toxicity studies except for the acute studies.

For the two acute studies the tox-batch was inactivated using EDTA (called batch PLA9901-inactivated with an activity of 70 EYU/g) or enriched, which means additional pure enzyme was added (called batch PLA9901-enriched with an activity of 9750 EYU/g). These studies were performed to check for possible irritating effects due to bioactivity of the PLA2 preparation. As these studies did not show any adverse effects, it was decided to perform the main toxicity studies with the normal tox-batch (PLA 9901).

On page 29 this is called the native active preparation to discriminate between this batch and the inactivated and enriched batches mentioned on this page. The tox-batch is the most concentrated form of the enzyme preparation in our production process and sampled after ultrafiltration. Therefore, it is also called UF-concentrate (p.26). This also refers to the tox-batch (PLA 9901). We apologize for any misunderstanding the wording has caused.