

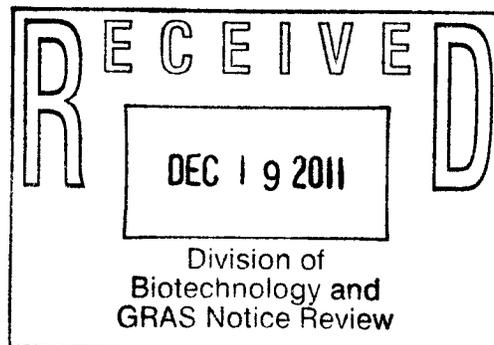
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December 15, 2011

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**Via Federal Express**

Office of Food Additive Safety (HFS-255)  
Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
5100 Paint Branch Parkway  
College Park, MD 20740



Re: REVISED GRAS Notification for peroxidase from genetically modified *Aspergillus niger*

Dear Dr. Mattia:

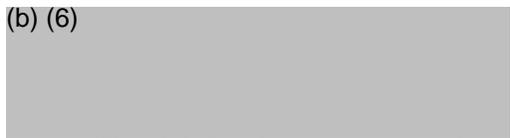
As counsel for DSM Food Specialties (DSM), we submitted in September a notification. We are now submitting three copies of a revision of that GRAS notification for a peroxidase preparation from genetically modified *Aspergillus niger*. DSM has determined through scientific procedures that the peroxidase preparation is generally recognized as safe for use as a processing aid for the bleaching of color from cheese-whey, soy milk and cream. The cheese-whey will be used in a wide range of foods, including bakery products, dairy products, beverages and infant formula.

The use of peroxidase derived from genetically modified *A. niger* is exempt from premarket approval as required by the Federal Food, Drug, and Cosmetic 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 additional information to aid its review of DSM's conclusion, please contact me at your earliest convenience.

Sincerely,

(b) (6)



Gary L. Yingling

Enclosures

cc: DSM Food Specialties

**GRAS NOTIFICATION FOR PEROXIDASE 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 the food enzyme Peroxidase, which is produced by submerged fermentation of a selected, pure culture of a genetically modified organism (“GMO”) *Aspergillus niger*. DSM produces the peroxidase preparations in a liquid form. It is stabilized with glycerol. The trade name will be MaxiBright™.

The described peroxidase preparation is intended for use in cheese-whey, soy milk and cream<sup>1</sup> as a processing aid to reduce the number of carotenoids present in, and by extension the resulting color of, the finished products. When used as proposed, peroxidase oxidizes carotenoids by reducing existing levels of hydrogen peroxide.

The treated whey is used in a variety of food products including baking and dairy products, beverages and infant formula. Due to the special nature of the target group for infant formula, special attention is given to this application throughout the dossier.

Pursuant to the regulatory and scientific procedures set forth in the Proposed Rule “Substances Generally Recognized as Safe,” 62 Fed. Reg. 18937 (April 17, 1997) (proposed 21 C.F.R. § 170.36) (“GRAS Proposed Rule”), DSM has determined that its peroxidase enzyme from a GMO *Aspergillus 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 in this Section.

The production organism, *Aspergillus niger*, has a long history of safe use and is discussed in Section 2. FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger*. See 21 C.F.R. §§ 184.1033 (Citric acid) and 184.1685 (Rennet and chymosin). In addition, FDA has subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *Aspergillus niger* strains. Carboxypeptidase, lipase, asparaginase and phospholipase A2 preparations from genetically modified *Aspergillus niger* strains derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier are examples of other such preparations to which FDA had no objections. See GRAS Notifications GRN 000345, GRN 000296, GRN 000214 and GRN000183, respectively. In a recent publication authored by FDA professionals, there was a summary of the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer et al., 2006).

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-

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<sup>1</sup> The cream thus produced can be used for the production of cheese or butter.

characterized production strain, free from known harmful sequences, for peroxidase. In Section 3, data showing peroxidase to be substantially equivalent to naturally occurring peroxidases are presented.

Section 4 discusses the safety of the materials used in the manufacturing process. Section 5 reviews the composition, specifications and general production controls. Section 6 provides information on the mode of action, application, use levels and measurable enzyme residues in the final food products in which peroxidase is to be used. Finally, the safety studies outlined in Section 7 indicate that *Aspergillus niger* and peroxidase show no evidence of pathogenicity or toxicity. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

#### 1.1 Name and Address of Notifier

##### **NOTIFIER**

**DSM Food Specialties**  
PO Box 1  
2600 MA Delft  
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 peroxidase enzyme preparation from GMO *Aspergillus niger* is produced by submerged fermentation of a selected, pure culture of *Aspergillus niger*. The common or usual name of the

substance is "peroxidase". It is produced and sold in liquid form which is standardized with glycerol. The trade name will be MaxiBright™.

### 1.3 Applicable Conditions of Use

The described peroxidase preparation is intended for use in the bleaching of color from cheese-whey soy milk and cream. Cheese-whey is used in the processing of a variety of food products including bakery and dairy products, beverages and infant formula. The peroxidase preparation oxidizes a carotenoid by reducing hydrogen peroxide, thus bleaching cheese-whey, soy or cream. In order for the enzyme to function, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is to be added as co-factor to the cheese-whey, soy milk or cream solution (see Section 3.4).

Annatto is traditionally added to milk during cheese production to make cheese more yellow in color. In practice, 90% of the added annatto ends up in the resulting cheese-whey. Cheese-whey is generally preferred however when it is less yellow in color. By adding the described enzyme to the cheese-whey, a manufacturer can reduce the overall yellow color of the cheese-whey which results in a more white color of the final food product in which the cheese-whey is applied.

Likewise, products made with soy milk are generally preferred when they are less yellow in color. The color in soy milk is determined by carotenoids present such as lutein. By adding the described peroxidase enzyme, the yellow color of the soy milk can be reduced. The same principle applies to cream where generally, less color is preferred to a more yellowish color in normal applications. In order to achieve this, the milk or cream can be treated with the enzyme. The enzyme will not be active in the final food application.

The peroxidase can be thus regarded as a processing aid in all applications.

#### 1.3.1 Substances Used In

The peroxidase preparation is to be used in cheese-whey, soy milk and cream. Cheese-whey is used in a wide range of food products, eg bakery products, dairy products and beverages. Furthermore, it is used in infant formula.

#### 1.3.2 Levels of Use

Enzyme preparations are usually used in *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used and the process conditions. The maximum dosages of the peroxidase will be 2.5 DBLU/ml soy milk, 1.0 DBLU/ml cheese-whey and 2.0 DBLU/g cream. The maximum dose of the H<sub>2</sub>O<sub>2</sub> will be 50 parts per million because above that value, the enzyme activity will be inhibited. The cheese-whey is used infant formula at a dosage of maximally 60%.

Both the amount of peroxidase and that of H<sub>2</sub>O<sub>2</sub> to be used will be optimized according to application and local process condition. As a consequence, all added H<sub>2</sub>O<sub>2</sub> is used in the reaction.

### 1.3.3 Purposes

Peroxidase can be used for the removal of color by the oxidation of carotenoids in several applications. Some of these applications are described below.

#### Cheese-whey bleaching

In the process of cheese making, annatto is added in order to create a more yellow cheese. However, in this process, only 10% of the milk used ends up as cheese, while the other 90% turns into cheese-whey. As the annatto ends up in both the cheese and the cheese-whey, so does the color. White cheese-whey has a higher commercial value than slightly colored cheese-whey. Peroxidase can be used to reduce the color of such cheese-whey.

The cheese-whey will subsequently be used in other applications, ie dairy and bakery products, beverages and infant formula.

#### Soy bleaching

Soy milk is the result of soaking and grinding soy beans in water. Its nutritional value is comparable to cow's milk. It can be used as such or further processed into cheese or other products. By appearance, soy milk differs from cow milk and has a pronounced yellow color. Some products made with soy milk are more appreciated when their color is less yellow, which is determined by carotenoids present in soy milk such as lutein for example.

Experiments have shown the effectiveness of discoloring of carotenoids by peroxidase and  $H_2O_2$ .

#### Cream bleaching

The slightly yellowish color of milk originates from the natural occurring beta-carotene in the milk fat.

For a number of dairy products, like whipped cream, a white color is preferred. There are several options for treating the milk or cream used to produce these products to obtain a whiter end product including:

- Homogenization
- Chemical bleaching
- Chemical masking with another color
- Enzymatic bleaching with peroxidase

Bleaching with peroxidase requires only a small amount of  $H_2O_2$  as a co-factor compared to the amount of chemicals needed for chemical bleaching.

In order to bring the enzyme in contact with the substrate the cream needs to be homogenized. Homogenization can cause quality defects to cheese made with such treated cream however, so

this treatment should be as moderate as possible. Lab scale experiments show the effectiveness of the bleaching of the cream with peroxidase and H<sub>2</sub>O<sub>2</sub>.

#### 1.3.4 Consumer Population

Peroxidases are abundantly present in nature as has been described in the Brenda database. They can be found among mammals (human, horse, cow), plants (*Brassicae*, barley, soja, peanut), fungi (*Marasmius*, *Arthromyces*, *Pleurotus*) and bacteria (*Streptomyces*, *Escherichia*, *Flavobacterium*) (Shindler, 1976; Mathy-Hartert, 1998; Jantschko, 2005; Singh, 2002; Rasmussen, 1998; Henriksen, 2001; Chibbar, 1984; Zorn, 2003 ; Battistuzzi, 2006; Perez-Boada, 2005; Iqbal, 1994; Baker, 2003; Cao, 1993). Therefore, the addition of the peroxidase preparation to the cheese or soy milk will not result in a reaction product different in peroxidase from normal constituents of the human diet. Because peroxidase is an enzyme protein naturally occurring in microorganisms, animals and plants, the enzyme will be digested, as would any other protein occurring in food.

Peroxidase catalyzes the reduction of peroxides (e.g. hydrogen peroxide) into water. Hydrogen peroxide is naturally produced in organisms as a by-product of oxidative metabolism. Nearly all living things (specifically, all obligate and facultative aerobes) possess peroxidases, which harmlessly and catalytically decompose low concentrations of hydrogen peroxide to water and oxygen. As a result, peroxidases are abundant in the human diet. Hence, the addition of peroxidase in any of the proposed applications will have no significant effect on the human body.

As is shown in Section 6.4 of this dossier, the total amount of enzyme TOS in the final food is expected to be about 7.6 - 117 mg/kg end product (= 0.00076 – 0.0117%).

Since peroxidase is present in food products at such low levels, and because it is a naturally occurring substance in both human cells and tissues and commonly ingested by humans, there is no data to suggest that the consumer population will be affected by the presence of the peroxidase preparation in food.

#### 1.4 Basis for GRAS Determination

Pursuant to the GRAS Proposed Rule, DSM has determined, through scientific procedures, that its peroxidase enzyme preparation from a GMO *Aspergillus niger* is GRAS for use as an oxidizing agent in cheese-whey, soy milk and cream, 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.  
K&L Gates LLP  
1601 K Street, NW  
Washington, DC 20006-1600

## 2 PRODUCTION MICROORGANISM

### 2.1 Donor, Recipient Organism and Production Strain

#### **Donor:**

The gene coding for the peroxidase (*MOX* gene) was produced synthetically based on the *Msp1* cDNA coding gene from *Marasmius scorodonius*, an edible mushroom.

#### **Recipient organism**

The recipient organism used in the construction of the peroxidase production strain is a glucoamylase (also called amyloglucosidase), protease, and amylase negative *Aspergillus niger* strain designated ISO-528 and stored in the DSM Culture Collection as DS 30829. The strain ISO-528 was declared as a suitable host strain for the construction of genetically modified organisms belonging to Group I safe microorganisms by the Dutch authorities (DSM relies on a letter from Director-General Milieu to DSM's Strain Director, dated 06-07-2005 regarding Beschikking DGM/SAS EVO 05-010/00).

The strain ISO-528 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 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) (DSM relies on a letter from CBS to Gist-Brocades, dated 29.1.1994 regarding Identification Service). This is an independent, internationally recognized laboratory.

The strain GAM-53 is being used to construct a new generation of strains according to the 'design and build' concept, in which introduced genes are targeted ('plugged') to a predetermined region of the genome. The exact technique used to construct such 'plug bugs' (designated as 'ISO-strains') and its advantages are described in literature (Selten et al., 1995 and van Dijk et al., 2003, included as Annex 2.1.1). ISO-strains, were used for the construction of production strains for phospholipase A2 (donor: porcine pancreas, see GRAS notification GRN 000183), asparaginase (donor: *Aspergillus niger*, see GRAS notification GRN 000214), lipase (donor: synthetic gene, see GRAS notification GRN 000296), arabinofuranosidase (donor: *Aspergillus niger*), phytase (donor: *Aspergillus niger*), pectin methyl esterase (donor: *Aspergillus niger*), glucoamylase (donor: *Aspergillus niger*), xylanase (donor: *Aspergillus niger*), endo-

polygalacturonase (donor: *Aspergillus niger*), carboxypeptidase (donor: *Aspergillus niger*), proline specific endo-protease (donor: *Aspergillus niger*) and amylase (donor: *Aspergillus niger*).

The recipient organism ISO-528 used in the construction of the peroxidase production strain was derived from GAM-53 as follows:

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  $\Delta$ *glaA* 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 the major protease (*pepA*) was inactivated and the major amylases (*amyA* and *amyB*) were deleted both by established rDNA techniques and the strain's capacity to secrete proteins was improved by classical mutation and selection.

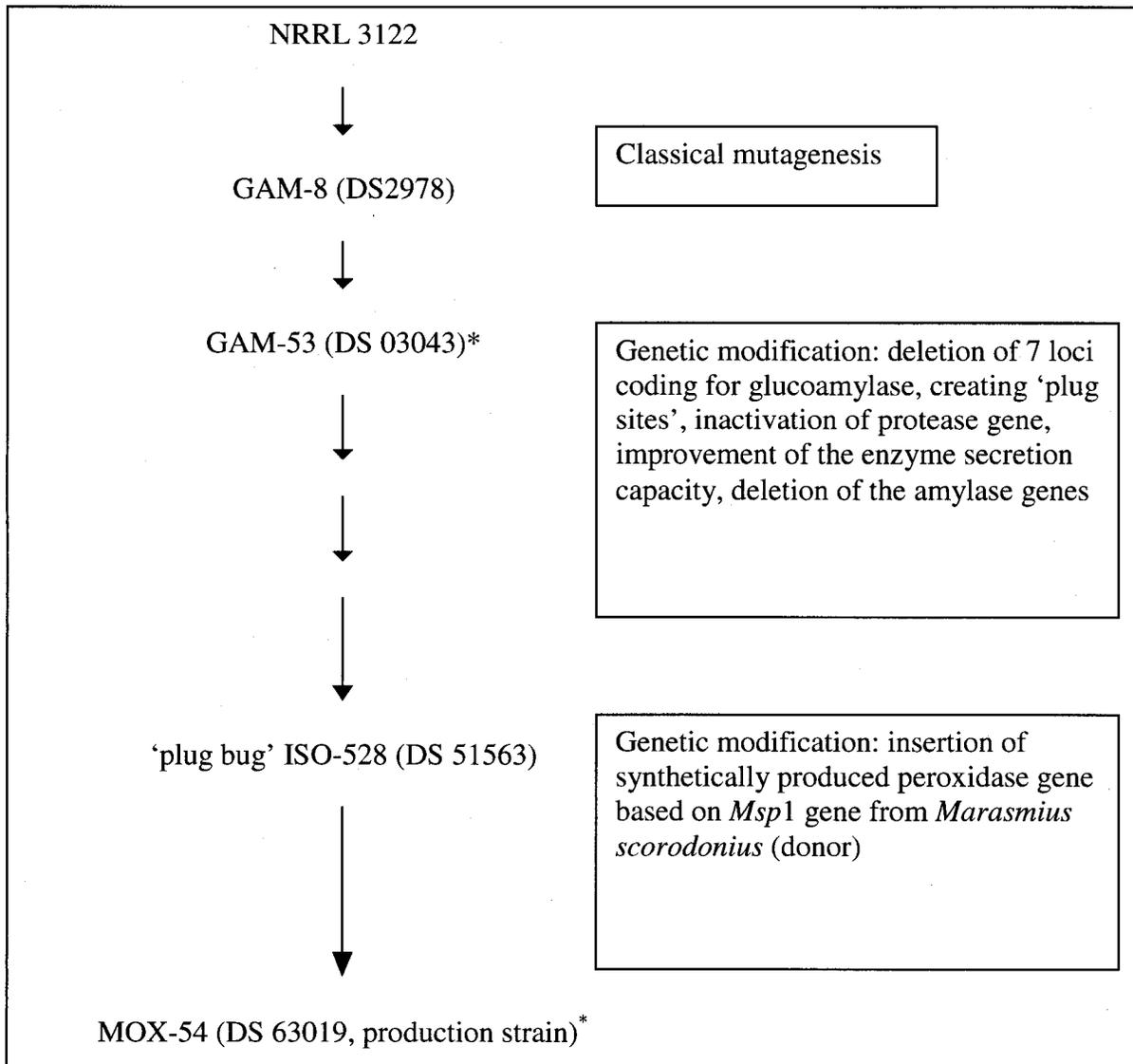
The resulting 'plug bug', ISO-528, was classified as a GMO by the Dutch competent authorities (DSM relies on a letter from Director-General Milieu to DSM's Strain Director, dated 06-07-2005 regarding Beschikking DGM/SAS EVO 05-011/00).

The ISO-528 has been used for the construction of the product strains for asparaginase (donor: *Aspergillus niger*), lipase (synthetic gene) and carboxypeptidase (donor: *Aspergillus niger*). All three enzymes are subject to GRAS Notifications (GRN 000214, GRN 000296 and GRN 000345). FDA had no questions with these GRAS notifications.

### ***Production strain***

The peroxidase production strain was obtained by further genetic modification of the *Aspergillus niger* strain ISO-528. The genetic modification techniques used are described in Section 2.2 of this dossier. The production strain was designated MOX-54 and stored in the DSM Culture Collection as DS 63019.

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



\* Strain GAM-53 and MOX-54 were 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) microorganisms. It also meets the criteria for a safe production microorganism 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 peroxidase production strain, two plasmids were used: one to derive the expression cassette, containing the peroxidase gene, and the other to derive the cassette containing a selectable marker.

### ***Donor DNA***

The MOX gene is a synthetically designed sequence, codon-optimized and based on the cDNA sequence of the msp-1 gene from *Marasmius scorodoni*. It leads to an enzyme which is identical to the enzyme from *Marasmius scorodoni*.

### ***Peroxidase expression plasmid***

The obtained fragment was ligated in the pGBTOP vector resulting in the final pGBTOPMOX-2 plasmid and propagated in *E. coli*.

Summarizing, the vector pGBTOPMOX-2 comprising the bleaching enzyme expression unit contains the following genetic elements:

- PglaA:** a 2.0 kB sized *glaA* promoter sequence of *Aspergillus niger* GAM-53 for controlling the glucoamylase and msp-1 oxidase expression.
- glaA-MOX:** the entire 1809 bp sized genomic sequence encoding the glucoamylase without its starch binding domain from ATG till the KEX2 processing site (AAGCGC) and the entire 1482 bp sized codon-optimized cDNA sequence, encoding the mature msp-1 oxidase protein (bleaching enzyme) from *Marasmius scorodoni*.
- 3'-glaA:** the 2.2 kB sized 3' flanking *Aspergillus niger* host-own *glaA* sequence for efficient termination of *glaA*-MOX gene transcription and targeting of the expression unit to the BamH1  $\Delta$ *glaA* locus.
- pTZ18R:** see Section 2.4.

The MOX expression unit is defined as the functional part of expression vector pGBTOPMOX-2. The MOX expression unit comprises the fusion construct of the *glaA* gene, without the starch binding domain, and the msp-1 oxidase gene, the expression of which is placed under control of the PglaA promoter.

### ***Selectable marker plasmid***

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

The different elements of the plasmid are:

- The *glaA* promoter (PglaA) from the parental *Aspergillus niger* strain GAM-53.
- The *gpdA* promoter (PgdpA) from *Aspergillus nidulans*.

- The *amdS* gene from *Aspergillus nidulans*.
- A 3'-flanking *glaA* terminator sequence from the parental *Aspergillus 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.

### ***Transformation and selection of the final production strain***

After both vectors had been independently propagated in *E. coli*, they were isolated and digested with restriction enzymes to remove the *E. coli* pTZ18R sequences from the vectors: PGBTOPMOX-2 was cut with the restriction enzyme *HindIII/XhoI*, whereas pGBAAS-4 was digested with *HindIII*. The resulting linearised expression units were purified by electrophoresis on agarose gels.

The MOX and *amdS* expression units – both completely free of any *E. coli* DNA – were integrated into the genome of the host ISO-528 by co-transformation. Due to the homology in the 3'-*glaA* and P*glaA* parts of the two expression units, they are preferentially targeted to one of the seven  $\Delta$ *glaA* loci, the *BamHI*  $\Delta$ *glaA* locus.

For the co-transformation, protoplasts from the host were used. The protoplasts were obtained by enzymatic removal of the cell wall. To obtain efficient uptake of the DNA by the protoplasts, co-transformation was performed in the presence of polyethylene glycol (PEG). For co-transformation, an excess of the MOX expression units was used when compared to the amount of *amdS* expression units. This was done to increase the chance that transformants, which have acquired an *amdS* expression unit, also have acquired one or more MOX expression units.

The transformants are selected on agar plates containing acetamide as the sole carbon source. Only those clones are able to grow that have acquired at least a copy of the *amdS* expression unit. A major percentage of the transformants appeared to have acquired both a number of MOX expression units and a few copies of the *amdS* expression unit.

A selection of transformants was made that contained at least one *amdS* expression unit with several in tandem integrated MOX expression units in the locus containing the *BamHI* DNA-flag. The selection of these transformants was done by PCR analyses, applying MOX and 3' *glaA* specific primers.

One of these transformants, designated as MOX528-2, contained multiple in tandem integrated MOX expression units flanked by a few *amdS* expression units, all targeted at the  $\Delta$  *glaA* locus containing the *BamHI* DNA-flag.

By counter-selection on fluoro-acetamide containing plates, a natural variant was selected in which the *amdS* selection markers were deleted as a result of a natural internal recombination event. The absence of the *amdS* marker was confirmed by Southern analysis. The resulting organism is thus not only totally free of *E. coli* DNA, but also of the *amdS* selection. This strain was designated MOX528-1 (DS61383) and was shown to contain 7 MOX gene copies. 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  $\Delta glaA$  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  $\Delta glaA$  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 containing 24 MOX gene copies which was designated as MOX528-9 (DS63009). After further testing to check the production level, the strain was renamed MOX-54.

The expression unit MOX is translated and split into a truncated glucoamylase and a peroxidase protein. They both are glycosylated and secreted into the medium as the mature active enzymes (peroxidase and glucoamylase).

### 2.3 Stability of the Transformed Genetic Sequence

The strains belonging to the *Aspergillus niger* GAM-lineage - from which both the host ISO-528 and the present recombinant peroxidase production strain MOX-54 are derived - are genetically stable strains. The whole GAM-lineage is stored for more than 30 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 production strains from this ISO strain lineage, such as the peroxidase production strain, in terms of behavior in strain management and in terms of enzyme production characteristics, does not differ from the parental GAM-strains or from production strains constructed by random integration.

Since the MOX 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 vector prior to transformation, see Section 2.5), it is not possible that the expression unit will be transferred from the *Aspergillus niger* production organism to another, non-related, organism.

### 2.4 Good Industrial Large Scale Practice (GILSP)

The peroxidase 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 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. No antibiotic resistance markers or other heterologous markers are present in the strain.

Therefore, consistent with the principles of Good Industrial Large Scale Practice (GILSP), as endorsed by the Organization of Economic Cooperation and Development (OECD), the peroxidase production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. The production organism has been approved both by the Dutch (DSM relies on a letter from Director-General Milieu to DSM Strain Director, dated 30 June 2009 regarding Beschikking DGM/RB EVO 09-004/01) and French competent authorities (DSM relies on a letter from the Comite Scientifique du Haut Conseil des Biotechnologies dated 11 January 2010 regarding AVIS 09/382) for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment. In the facilities of DSM Food Specialties, fermentations for the large-scale production of food and feed enzyme products are carried out below the GILSP level of physical containment.

#### 2.5 Absence of transferable rDNA Sequences in the Enzyme Preparation

As explained above, the expression unit contains no *Escherichia coli* origin for replication. As a result, the enzyme preparation will not contain any transferable rDNA sequences. In accordance with the rational design of the recombinant production strain, i.e., absence of any *Escherichia coli* plasmid or marker gene DNA, no transformable rDNA could be detected in the product by test.

#### 2.6 Absence of Production Organism in the Product

In accordance with the recommendations for safety evaluation by the International Food Biotechnology Committee (Coulston, 1990), all traces of the production organism are removed during the manufacturing process (see Section 4.4), ensuring that the enzyme preparations are free from the production organism *Aspergillus 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 4.6 of this dossier, quality control testing of the finished peroxidase 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 (7<sup>th</sup> 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 2006, the following is said: *“Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.”*

Instead of analyzing the final enzyme preparations for mycotoxins, DSM therefore decided it more appropriate to test whether the peroxidase-producing microorganism possesses the intrinsic capacity to produce mycotoxins. This was tested under routine conditions of industrial submerged fermentation. The test showed that the production strain does not produce any known toxins under the routine conditions of industrial submerged fermentations.

### 3 ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

#### 3.1 Enzyme Identity

- Systematic name : phenolic donor:hydrogen-peroxide oxidoreductase
- Accepted name : peroxidase
- Enzyme Commission No. : 1.11.1.7

Peroxidase belongs to the subclass of oxidoreductases.

#### 3.2 Amino Acid Sequence

The peroxidase described in this dossier is produced by *Aspergillus niger* as a homo-dimeric glycoprotein with a primary sequence of 1014 amino acids and a calculated molecular weight of 53 kDa. Based on SDS-PAGE, it can be concluded that the molecular weight of the enzyme is about 68 kDa. Annex 3.2.1 shows the amino acid sequence of the peroxidase produced by a GMO *Aspergillus niger*.

#### 3.3 Sequence Comparison to Other Peroxidases

Comparison of the peroxidase expressed by *Aspergillus niger* to *Marasmius scorodoni* peroxidase shows that both enzymes are for 94.7% similar (486 out of 513 amino acids).

In order to compare the peroxidase produced by a GMO *Aspergillus niger* with other comparable enzymes, an extensive sequence comparison study has been carried out. The amino acid sequence of the *Aspergillus niger* peroxidase was used as a query to search sequence databases using 'BLAST' software for related sequences.

About 50 different sequences with significant ( $E\text{-value} \leq 5 \cdot 10^{-38}$ )<sup>2</sup> homology were found.

The most similar sequences are peroxidases from related fungal species (Basidiomycotes) with E-values between  $2 \cdot 10^{-45}$  and 0. Basidiomycotes are mushrooms like *Marasmius* and *Termitomyces* species, but also *Polyporales* (source of single cell proteins), and others.

A second cluster of homologous fungal sequences stems from Ascomycetes. They have a E-values ranging from  $1 \cdot 10^{-38}$  to  $1 \cdot 10^{-68}$ . Ascomycetes are in general so-called 'sac-fungi', a group which is of particular relevance to humans as source for medicinally important compounds, such as antibiotics and for making bread, alcoholic beverages, and cheese, but also as pathogens of humans and plants. Familiar examples of sac fungi include morels, truffles, brewer's yeast and baker's yeast, Dead Man's Fingers, and cup fungi. Also *Aspergillus* species are part of this group.

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<sup>2</sup> E-values indicate the degree of similarity in sequences, the lower the E-value, the more similar the sequence. An E-value of 0 indicates identical sequences.

Finally, bacterial sequences show a relatively high comparison with the sequence used for the production of this peroxidase with E-values ranging from  $5 \cdot 10^{-38}$  to  $1 \cdot 10^{-47}$ . Among those species are cyanobacteria, actinobacteria and acidobacteria.

### 3.4 Enzymatic Activity

#### ***Principal Enzyme Activity***

The major enzymatic activity described in this dossier is a peroxidase produced by a GMO *Aspergillus niger*. It catalyses the following reaction:



The peroxidase activity can be determined in the presence of hydrogen peroxide, using 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) as donor. The enzyme reduces the hydrogen peroxide and oxidizes the ABTS. The green colored oxidized ABTS can be determined by measuring the increase of absorption at 405 nm in time.

The activity of peroxidase is expressed in so-called Dairy Bleaching Units (DBLU). One DBLU is defined as the amount of enzyme needed to oxidize 1  $\mu\text{mol}$  ABTS per minute under conditions described for the assay.

The molecular weight (MW) of the enzyme from *Marasmius scorodoni* as expressed in *Aspergillus niger*, deduced from the base pair sequence of the gene, is 52.9 kD. Scheibner et al. showed in 2008 the enzyme has 9 potential glycosylation sites; they also show the active enzyme presumably represents a homo-dimeric enzyme under native conditions. As is shown in Annex 3.4.1., the enzyme shows activity at a pH range between 3.0 and 5.3 and at a temperature range from 10 to 50°C. It is stable at pH 4 – 6 (remaining activity > 80%). The enzyme is stable at cold and room temperatures for several weeks; it is inactivated at 70°C within 30 minutes. Besides, Pühse et al (2009) show an optimum activity of the protein at a temperature of 55°C, with almost no activity at 70°C remaining after 6 minutes.

#### ***Subsidiary enzymatic activities***

Like any other living micro-organism, the peroxidase production organism *Aspergillus niger* produces many other enzymes needed for the breakdown of nutrients and build up of cell material.

The enzyme preparation also contains glucoamylase which has no function in any of the applications since no substrate is available. It is thus present as non-functional protein. Although peroxidase is being produced in excess, the enzyme preparation will also contain minor, non-standardized amounts of these other enzymes. These amounts do not have an effect (positive or negative) in the applications.

The expression unit MOX is translated and split into a truncated glucoamylase and a peroxidase protein. They both are glycosylated and secreted into the medium as the mature active enzymes (peroxidase and glucoamylase).

## 4 MANUFACTURING PROCESS

### 4.1 Overview

The peroxidase described in this dossier is produced by a controlled submerged fermentation of a selected, pure culture of a GMO *Aspergillus niger* (see Section 2). 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.

The fermentation medium used has been developed for optimum production of enzymes (in this case peroxidase) by the DSM *Aspergillus niger* hosts.

### 4.3 Fermentation Process

The peroxidase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *Aspergillus niger* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

The fermentation process consists of three steps: pre-culture fermentation, seed fermentation and main fermentation. The whole process is performed in accordance with current Good Manufacturing Practices.

Biosynthesis and excretion of peroxidase occur during the main fermentation. To produce the enzyme of interest, a carefully controlled, submerged, aerobic fed batch fermentation process is employed under aseptic conditions, using a stirred tank fermentor.

Growth of the production organism and increase of enzyme production are checked at the end of the main fermentation by analysis of aseptically collected samples. After the fermentation is stopped, downstream processing begins.

### 4.4 Recovery Process

The fermentation is stopped by addition of methyl ethyl paraben under conditions that effectively kill off the production organism.

The cell material is separated from the enzyme by means of a simple solid-liquid filtration process. Subsequently, the remaining particles are removed with a polish filtration and a germ reduction filtration, and then concentrated by ultrafiltration (UF).

#### 4.5 Stabilization, Formulation and Standardization Process

After ultrafiltration, the purified peroxidase (eluate) is again filtered with a polish and a germ reduction filtration and finally, it is formulated to a liquid preparation.

The product is stabilized with glycerol to reach a peroxidase activity of  $5000 \pm 10\%$  DBLU/ml, in accordance with the product specification. Finally the product is filter sterilized.

#### 4.6 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 2006 and the FCC (7<sup>th</sup> edition), the final peroxidase preparation from a GMO *Aspergillus niger* meets the following specifications:

<u>ITEM</u>	<u>NORM</u>
Lead	$\leq 5$ mg/kg
Cadmium	$\leq 0.5$ mg/kg
Mercury	$\leq 0.5$ mg/kg
Arsenic	$\leq 3$ mg/kg
Standard plate count	$\leq 5 \times 10^4$ /g
Coliforms	$\leq 30$ /g
<i>Salmonella</i>	0/25
<i>Escherichia coli</i>	0/25 g
<i>Staphylococcus aureus</i>	0/g
Antimicrobial activity	Absent by test
Mycotoxins	Absent by test

The additional characteristics for the liquid formulation are:

<u>ITEM</u>	<u>NORM</u>
Peroxidase activity	$5000 \pm 10\%$ DBLU/g
Appearance	Green to brownish
Glycerol	$> 45\%$
pH	5.8 – 6.2

## 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:

<u>Item</u>	<u>Value</u>	<u>Unit</u>
Enzyme activity	9000 - 15000	DBLU/g
Water	84 - 89	%
Ash	0.5 - 1.1	%
Total Organic Solids	11 - 16	%

Apart from the enzyme complex, the peroxidase preparations will also contain some substances derived from the microorganism and the fermentation medium. These harmless contaminations consist of polypeptides, proteins, carbohydrates and salts.

The Total Organic Solids ("TOS") of the peroxidase preparations were calculated from 3 different batches of the UF concentrate:

Calculation of the TOS					
Batch nr	Dry matter (% w/w)	Ash (% w/w)	TOS (% w/w)	Activity (DBLU/g)	DBLU / g TOS
(b) (4)	17.2	1.1	16.1	13400	83230
	12.4	0.62	11.8	9230	78220
	16.4	0.49	15.91	15000	94280
MEAN					85243

Based on the above figures it can be calculated that the formulated commercial products with activities of 5000 DBLU/g will have a TOS value between 60.1 and 69.1 mg/g enzyme preparation.

### 5.2 General Production Controls and Specifications (Good Manufacturing Practice)

Commercial demands require a strictly controlled fermentation process.

The enzyme fermentation factory at Seclin, France, which has fermentation experience since 1922, 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 labeled and strict aseptic techniques are applied during the recovery of the culture.

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

The fermentor is a contained system. Only sterilized 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 sterilized 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. 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 and microbial quality are monitored. If microbial controls show that significant 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 significant contamination has occurred, the downstream processing will be discontinued.

The finished product is subjected to extensive controls and complies with JECFA and Food Chemical Codex specifications. See Section 4.6: Quality Control of Finished Product.

## 6 APPLICATION

### 6.1 Mode of Action

The function of peroxidase in living cells is to reduce peroxides (e.g. hydrogen peroxide) into water (see Section 1.2). For this reaction, a variety of electron donors can be used including aromatic amines, phenols, and enediols like ascorbic acid. Also carotenoids, known as effective antioxidants, can be used as electron donor. In contrast to the reduced form, the oxidized form of relevant carotenoids is colorless (see sections below for more detail).

In the presence of hydrogen peroxide, peroxidase can consequently be used for the removal of color by the oxidation of carotenoids in several food ingredients. By reducing the color, the commercial value of these ingredients is increased as they can be applied in a wider range of final food products.

So-called creamer agents are widely used in the food industry for their color reducing effect on final food products. The use of peroxidase is a valuable alternative as it is a natural derived enzyme.

### 6.2 Application

The peroxidase in question is to be used in cheese-whey, soy milk or cream.

#### Cheese-whey

In the process of cheese making, the carotenoid annatto is added in order to create a more yellow cheese. However, in this process only 10% of the milk used ends up as cheese, while the other 90% turns into cheese-whey. As the annatto ends up in both the cheese and the cheese-whey so does the color. White cheese-whey has a higher commercial value than slightly colored cheese-whey. The effectiveness of the decolorization of the cheese-whey by the addition of peroxidase and  $H_2O_2$  is shown in Annex 6.2.1.

Cheese-whey is used in a wide range of food products. In bakery products, it is used in the production of flat breads, crackers, biscuits, baking mixes and tortillas. In the dairy industry, it is applied in cheeses, yoghurts, ice cream and desserts. Other applications include beverage mixes, snacks, soups, nutritional products, sports and nutritional bars and flavourings. Furthermore, it is used in infant formula.

#### Soy Milk

Soy milk is the result of soaking and grinding soy beans in water. Its nutritional value is comparable to cow's milk. It can be used as such or further processed to cheese or other products. By appearance, soy milk differs from cow milk and has a pronounced yellow color. Some products made with soy milk are more appreciated when their color is less yellow, which is determined by carotenoids naturally present in soy milk such as for example lutein.

Experiments have shown the effectiveness of discoloring of carotenoids in soy milk by peroxidase and H<sub>2</sub>O<sub>2</sub>. The results can be found in Annex 6.2.2.

### Cream

The slightly yellowish color of milk originates from the natural occurring beta-carotene in the milk fat.

For a number of dairy products, like whipped cream, a white color is preferred. There are several possibilities to treat the milk or cream that are used to produce these products in order to obtain a whiter end product:

- Homogenization
- Chemical bleaching
- Chemical masking with another color
- Enzymatic treatment with peroxidase

Treatment with peroxidase requires only a small amount of H<sub>2</sub>O<sub>2</sub> as co-factor compared to the amount of chemicals needed for chemical bleaching.

In order to bring the enzyme in contact with the carotenoid, the cream needs to be homogenized. Laboratory-scale experiments that show the effectiveness of discoloring cream with peroxidase and H<sub>2</sub>O<sub>2</sub> are presented in Annex 6.2.3.

## 6.3 Use Levels

Enzyme preparations are usually used in *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used and the process conditions. The maximum dosages of the peroxidase will be 4.5 DBLU/ml soy milk, 1.0 DBLU/ml cheese-whey and 2.0 DBLU/g cream. The maximum dose of the H<sub>2</sub>O<sub>2</sub> will be 50 parts per million since when using more than that, the enzyme activity will be inhibited. . The cheese-whey will be used in the infant formula at a dosage of maximally 60%.

Both the amount of peroxidase and H<sub>2</sub>O<sub>2</sub> to be used will be optimized according to application and local process condition. For each customer, the amount of added H<sub>2</sub>O<sub>2</sub> used is such that it is totally consumed during the enzymatic reaction (see Annex 6.2.1), with a maximum of 50 ppm.

## 6.4 Enzyme Residues in the Final Food

### 6.4.1 Residues of inactive enzyme

Based on the information given in Sections 5.1 and 6.3, and assuming that all the TOS of the peroxidase preparation ends up in the cheese, the following calculation can be made:

Final food	Enzyme use level in food ingredient	Amount of ingredient in final food	Residual amount of (inactivated) enzyme in final food	Amount of TOS in final food
Cheese-whey products in dairy, bakery, and beverages <sup>1</sup>	1.0 DBLU/ml cheese-whey	up to 100%	100 DBLU/ml cheese-whey protein	1.17 mg TOS/ml cheese-whey protein
Cheese-whey in Infant Formula <sup>2</sup>	1.0 DBLU/ml cheese-whey	up to 60%	600 DBLU/kg infant formula powder	7.02 mg TOS/kg infant formula powder
Soy milk	4.5 DBLU/ml soy milk	up to 100%	4500 DBLU/l soy milk	52.79 mg TOS/l soy milk
Cream	2.0 DBLU/g cream	up to 100%	2000 DBLU/kg cream	23.46 mg TOS/kg cream

<sup>1</sup>: 1 ml cheese-whey contains 1% cheese-whey-protein.

<sup>2</sup>: Mentioned separately here because of the specificity of the consumer group.

### Fate of peroxidase in the end products

In the end products, the enzyme will not have a function because it needs the co-factor (H<sub>2</sub>O<sub>2</sub>) to function, and, as indicated in Section 6.3, that is no longer present. Inactivation of the enzyme by heat treatment requires a pasteurization time of 1 minute at 80°C (when bleaching with 2DBLU/ml). At 75°C a pasteurization time of 4 minutes leads to total inactivation of the enzyme. It can thus be considered a processing aid.

#### 6.4.2 Possible Effects on Nutrients

Peroxidase enzymes are very widespread. They are found in mammals, plants as well as microorganisms. As a consequence, they also naturally occur in food. It is therefore not expected that the enzyme will create reaction products that are not normal constituents of the diet.

The enzyme activity is very widespread. As can be seen in the Brenda enzyme database, it can be found among mammals (human, horse, cow), plants (*Brassicae*, barley, soja, peanut), fungi (*Marasmius*, *Arthromyces*, *Pleurotus*) and bacteria (*Streptomyces*, *Escherichia*, *Flavobacterium*). (Shindler, 1976; Mathy-Hartert, 1998; Jantschko, 2005; Singh, 2002; Rasmussen, 1998; Henriksen, 2001; Chibbar, 1984; Zorn, 2003; Battistuzzi, 2006; Perez-Boada, 2005; Iqbal, 1994; Baker, 2003; Cao, 1993).

Peroxidase from a GMO *Aspergillus niger* will be used to oxidize carotenoids such as lutein and annatto present in soy milk and dairy products.

As neither lutein nor annatto are a source of vitamin A for the body, the oxidation of these carotenoids by peroxidase from a GMO *Aspergillus niger* does not affect the nutritional value of the end products.

As a consequence, the nutritional value of the soy-milk, infant formula, baking, beverages and dairy products is not changed or impaired by the application of peroxidase from *Aspergillus niger*.

## 7 SAFETY EVALUATION

### 7.1 Safety of the Donor and 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 International Food Biotechnology Council ("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 (Coulston, 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).

*Aspergillus niger* 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, *Aspergillus 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 *Aspergillus 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 *Aspergillus niger* and understanding of the metabolic reactions.

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

Even though products from *Aspergillus niger* have been used in food for many decades, there is no evidence that the industrial strains used produce toxins under the routine conditions of industrial submerged fermentations. The safety has been confirmed by a large amount of toxicological tests, as well as batch testing of the various end products for toxins.

The toxicological studies performed on various enzyme preparations from *Aspergillus niger* provided the basis for a safety evaluation by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO in 1988 (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 *Aspergillus 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 *Aspergillus 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 *Aspergillus niger* in a number of food applications.

Strains belonging to the *Aspergillus niger* GAM-lineage as well as the host (recipient) strain ISO-528 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 *Aspergillus niger* GAM-53 strain, which was 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. DSM uses these strains on industrial scale since 1991 and 1996, respectively.

The recombinant peroxidase strain MOX-54 (DS 63019) has been classified by both the Dutch and French competent authorities as a Group I safe micro-organism. Consequently, the strain was approved for large scale production of peroxidase 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 3045) 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 peroxidase production strain is not able to produce any toxins under the routine conditions of industrial submerged fermentations. The results of these tests showed that the production strain does not produce any known toxins under these conditions.

## 7.2 Safety of the Peroxidase Enzyme

As noted above, enzymes produced by *Aspergillus niger* have already been used for food production for several decades. In the USA, FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *Aspergillus niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132), and lipase (GRN 000111 and GRN 000158). A recent publication authored by FDA professionals included a summary of the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer et al., 2006). In addition, a phospholipase A<sub>2</sub> (GRN 000183), an asparaginase (GRN 000214), a lipase (GRN 000296) and a carboxypeptidase (GRN 000345) preparation from genetically modified *Aspergillus niger* strains, which were derived from the same strain-lineage

as the *Aspergillus niger* strain described in this dossier, have been notified as GRAS. FDA had no objections to each of these notifications.

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 (see also Section 7.1).

Since it is generally known that commercial enzyme preparations of *Aspergillus niger* are not toxic and since peroxidase is a natural constituent of many organisms, including microorganisms, used for food (see Sections 6.4.2 and 7.2.2), it is not expected that peroxidase will have any toxic properties.

The enzyme preparation peroxidase produced by *Aspergillus niger* strain MOX-54, over-expressing the peroxidase gene from *Marasmius scorodoni*, 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. Based on the Pariza and Johnson decision tree analysis, DSM concludes that the peroxidase preparation is safe.

To confirm the above assumption that the peroxidase preparation does not have any toxic properties and to further establish the toxicological safety of the use of peroxidase from *Aspergillus niger* in food, the following studies were performed:

- Sub-chronic (90 day) oral toxicity study
- Ames test
- Chromosomal aberration test, *in vitro*

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

#### 7.2.1 Allergenicity

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.

Because they are proteins, enzymes could 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 it is described why ingestion of enzymes used as food processing aids is unlikely to elicit an allergic response.

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.

Enzymes have a long history of safe use in food. Since new enzymes are generally (based on) existing enzymes, it is very unlikely that a new enzyme would be a food allergen. Moreover, exposure to the enzyme associated with ingestion is typically very low and residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system. To our knowledge no reports exist on sensitization to enzyme products after ingestion.

The absence of food allergenicity has been confirmed by an extensive literature search and survey of producers' files, which has not revealed any cases of sensitization or an allergic response upon ingestion of food prepared with various enzymes (see Annex 7.2.1). Even when high daily doses of enzymes as digestive aids are ingested, there have not been any reports on gastrointestinal allergy to enzymes, after many years of daily intake. Recently, it was concluded that ingestion of food enzymes in general is not considered to be a concern with regards to food allergy (Bindslev-Jensen et al, 2006).

For the purpose of this dossier and in accordance with the requirements of FDA, the amino acid sequence of peroxidase has been compared with the amino acid sequences of known (food) allergens. In addition, as described in Section 2.4, a truncated glucoamylase promoter sequence was introduced in the host strain ISO-528. Therefore, an amino acid sequence comparison of the truncated glucoamylase with known allergens has also been performed.

It has to be noted that allergenicity is not expected from peroxidase since the gene coding for the enzyme is based on the cDNA sequence of the *msp-1* peroxidase gene from *Marasmius scorodoni*, an edible mushroom with a safe history of human consumption. Neither *Marasmius scorodoni*, nor any member of the *Marasmiaceae* family is listed as an allergen in the SwissProt and WHO-IUIS databases.

#### Amino acid sequence comparison with known (food) allergens

For the comparison use was made of the databases Allermatch™ and SDAP™ (Structural Database of Allergenic Proteins). Allermatch™ allows the search in the SwissProt database and the WHO-IUIS database using a FASTA algorithm. SDAP™ allows the search in SwissProt, PIR, GenBank, PDB and the WHO-IUIS databases also using a FASTA algorithm. The WHO-IUIS list is set up by the IUIS Allergen Nomenclature Sub-committee operating under the auspices of the International Union of Immunological Societies and the World Health Organization. The objectives of the IUIS Allergen Nomenclature Sub-committee are to maintain a unique and unambiguous nomenclature for allergen molecules and maintain the 'official list of allergens'.

The searches were performed following the guidelines from the FAO/WHO. According to the guideline cross-reactivity between the expressed protein and a known allergen has to be

considered when there is more than 35% identity in the amino acids sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty

The amino acid sequence comparison with peroxidase and with the truncated glucoamylase (promoter sequence) did not show 35% or more overlap with known allergens using a window of 80 amino acids. At least two IgE binding sites and high affinity are required to effectively cross-link mast cells and trigger an allergic response (Goodman et al, 2006; 2008). This is clearly not the case here.

Considering the absence of a relevant match with known (food) allergens by screening the amino acid sequences of peroxidase and truncated glucoamylase, and due to the high level of fragmentation of glucoamylase expected in pepsin degradation conditions, it was concluded that the peroxidase described in this dossier – including its carrier protein – is not likely to produce any allergenic or sensitization reactions by oral consumption.

#### 7.2.2 Leading Enzyme Publications on the Safety of Peroxidase 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 *Aspergillus niger*, has been demonstrated to be 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, the authors include a list of the organisms being used in the industry of which *Aspergillus niger* is one.

The FDA has also accepted GRAS Notifications from the Enzyme Technical Association and DSM Food Specialties stating that carbohydrase, pectinase, protease, glucose oxidase and catalase (GRN 000089), lipase (GRN 000111, GRN 000158 and GRN 000296), lactase (GRN 000132), phospholipase A2 (GRN 000183), asparaginase (GRN 000214), lipase (GRN 000296) and carboxypeptidase (GRN 000345) enzyme preparations from *Aspergillus niger* are generally recognized as safe. *Aspergillus 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 *Aspergillus 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 1991, 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.

Peroxidases are ubiquitous in nature and can be found in fungi, plants and animals (including humans) (see Section 6.4.2). They have been recognized as an individual category by the International Union of Biochemistry and Molecular Biology (IUBMB) since 1961.

Peroxidases (of which catalase is one) are oxidoreductases that act on a peroxide as receptor. Catalase has been affirmed as GRAS by FDA in 2002 (GRN 000089) and it has been included in the safety evaluation by Pariza and Johnson (2001).

As Jantschko described in 2005, Henriksen in 2001 and Rasmussen in 1998, cows, soy and peanuts all contain peroxidases and as such, naturally-occurring peroxidases are consumed as they will be in the applications described here.

### 7.2.3 Substantial Equivalence

Several expert groups 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 appears to have accepted 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-directed mutagenesis, would not raise safety concerns.

There are no agreed-upon criteria by which substantial equivalence is determined. Considering enzymes produced by micro-organisms the enzyme activity and intended use, the production organism and the process conditions should be taken into account.

As indicated in Section 3.3, close similarities exist between the peroxidase that is the subject of the present GRAS notification and other oxidoreductases that have been safely marketed for years, including those that are the subject of GRAS notifications.

Peroxidases (IUB 1.11.1) are part of the oxidoreductases (IUB 1.11). An other peroxidase has been notified as GRAS by the FDA: Catalase enzyme preparation derived from *Aspergillus niger* (GRN 000089; IUB 1.11.1.6). To this notification, FDA responded with a letter stating they had no questions.

In addition to the safety of the peroxidase enzyme itself, the current production strain is derived from a safe strain lineage of *Aspergillus niger*. *Aspergillus niger* is a common food constituent of products like rice, seeds, nuts, olives and dried fruits. In addition, *Aspergillus niger* has been used for several decades for the production of organic acids and enzymes to be used in the food

industry. The FDA recently summarized the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer et al., 2006).

Other food substances from *Aspergillus niger* were previously affirmed as GRAS. See 21 C.F.R. §§ 184.1033 (Citric acid); 184.1685 (Rennet and chymosin). Also, the FDA subsequently received GRAS notifications for additional enzyme preparations from *Aspergillus niger*, including several produced from genetically modified *Aspergillus niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132), and lipase (GRN 000111 and GRN 000158). FDA has no questions with these GRAS notifications.

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 (see also Section 7.1).

Finally, the safety of the *Aspergillus niger* strain lineage that is used to produce DSM’s peroxidase has been extensively described by van Dijck et al. (2003). The publication describes the standardized method of producing food enzymes. The production process, the production strain, the construction of the production strain, and the raw materials used in the fermentation and down stream processing are kept the same. Only the gene encoding the enzymatic activity is changed. Extensive toxicological studies of a number of strains built and processed according to this method demonstrate the safety of the enzymes produced this way. The peroxidase-producing strain described in this dossier was built and produced according to this method.

Moreover, a phospholipase A<sub>2</sub> (GRN 000183), an asparaginase (GRN 000214), a lipase (GRN 000296) and a carboxypeptidase (GRN 000345) preparation from genetically modified *Aspergillus niger* strains, derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier and processed according to the mentioned concept, were the subject of GRAS notifications to which FDA has no questions.

Thus, the *Aspergillus niger* production strain used to produce peroxidase is as safe as the production strains that have produced other GRAS enzymes subject to GRAS notifications. Accordingly, it can be concluded that the resulting enzyme product from the production strain is as safe as other enzymes produced by strains from the same safe strain lineage and processed the same way.

Also the use of the peroxidase described here is substantially equivalent to other, already described enzymes like catalase. This can be seen in peer-reviewed articles like Kowallis (1929), Beckhorn *et al.* (1965), Bossuyt and Weckx (1975) and Adler-Nissen (1987).

Since the production strain and production process are as safe as those used to produce other GRAS enzymes, and the peroxidase itself is substantively similar to other GRAS peptidase enzymes in terms of activity and intended use, it can be concluded that the peroxidase is GRAS.

### 7.3 Safety of the Manufacturing Process

Peroxidase 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 5.2, the peroxidase 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 peroxidase preparation.

#### 7.4.1 Safety Studies in Summary

All safety studies were performed in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

The studies were performed with batch number DBL.GRZ.0914, referred to as 'tox-batch'. The tox-batch was produced according to the procedure used for commercial production and represents the commercial product. After the UF-concentration step, the batch was freeze dried to produce the final, non-formulated tox-batch with an activity of 65280 DBLU/g and a TOS content of 86.38%. The Certificate of Analysis is included in the sub chronic oral toxicology report.

#### **1 90-day oral toxicity**

A sub-chronic oral toxicity study with the tox-batch was conducted at TNO, The Netherlands, in accordance with the following guidelines:

- OECD Guideline for the Testing of Chemicals 408. Repeated Dose 90-day Oral Toxicity Study in Rodents, adopted 21st September 1998.

The study consisted of four groups of 10 male and 10 female Wistar rats. For a period of 90 days, the control group was fed with unsupplemented diet; the three test groups received diets containing 0.7% (low-dose), 2% (mid-dose) and 4% (high-dose) of the tox-batch, corresponding with 6, 17 and 35 mg TOS- or 457, 1306 and 2611 DBLU/g feed, respectively.

General clinical observations, neurobehavioral testing, ophthalmoscopic examination, body weight, food consumption, intake of test substance, water consumption, hematology, clinical chemistry, renal concentration test and urinalysis, organ weights of principal organs, macroscopic examination, histopathology of organs (control and high-dose group only) and microscopy of all lesions were studied.

## Results

The administration of the tox-batch at dietary levels up to 4% (w/w) did not lead to any toxicologically relevant findings. The NOAEL is therefore 4% of the test substance in the diet, the highest dose level tested.

This leads to an overall NOAEL of 2.3 g/kg bw/day (i.e. approximately 2.0 g TOS or 150,000 DBLU/kg bw/day).

## **2 Mutagenicity tests**

### AMES test

A bacterial reverse mutation test was performed with the tox-batch at TNO, the Netherlands, in order to assess its mutagenic activity in four selected strains of *Salmonella typhimurium*, TA 1535, TA 1537, TA 98 and TA 100, as well as in the *Escherichia coli* mutant WP2 *uvrA*. All were tested in both the absence and presence of a metabolic activation system (S9-mix).

The study was conducted in accordance with the OECD guideline no. 471, Genetic toxicology: Bacterial Reverse Mutation Test, adopted 21<sup>st</sup> July 1997.

One bacterial reverse mutation test was performed in the absence and presence of S9-mix with five concentrations of the tox-batch, ranging from 62 to 5000 mg/plate. Negative controls (water) and positive controls were run simultaneously with the tox-batch.

The negative control values were within the acceptable range and strain-specific positive controls gave the expected increase in the mean number of revertant colonies.

The tox-batch was not toxic to any strain, in both the absence and presence of S9-mix. A slightly more dense background lawn of bacterial growth was observed in strain TA 98 in the presence of S9-mix at and above 1667 mg/plate, as well as in strain WP2 *uvrA* in both the absence and presence of S9-mix at and above 556 mg/plate. This was most likely caused by the proteinaceous nature of the test substance.

In all strains and in both the absence and the presence of S9-mix, the test substance did not cause a significant dose-related increase in the mean number of revertant colonies compared with the negative control.

It was concluded that the tox-batch is not mutagenic under the conditions employed in this study.

### Chromosomal aberration test

A chromosomal aberration test *in vitro* was performed with the tox-batch at TNO, the Netherlands, in order to assess its ability to induce structural chromosomal aberrations in cultured human lymphocytes in the absence and presence of a metabolic activation system (S9-mix).

The study was conducted in accordance with the OECD guideline 473, Genetic toxicology: *In vitro* Mammalian Chromosome Aberration Test, adopted 21<sup>st</sup> July 1997.

Two separate tests were conducted. Dose levels of the tox-batch ranging from 10 to 5000 mg/ml were tested. Negative controls and adequate positive controls were run simultaneously.

In the first test cells were treated/harvested for 4/24 hours (pulse treatment) in both the absence and presence of S9-mix. Three dose levels of the tox-batch were tested: 625, 1250 and 2500 µg/ml.

In the second test, the treatment/harvesting times were 4/24 hours (pulse treatment) and 24/24 hours (continuous treatment) respectively in the presence and absence of S9-mix. Three dose levels of the tox-batch were tested: 1000, 3000 and 5000 mg/ml.

The incidence of structural chromosomal aberrations found in the negative (vehicle) controls, was within the historical range. The positive control substances mitomycin C (in the absence of the S9-mix) and cyclophosphamide (in the presence of the S9-mix) induced the expected increases in the incidence of structural chromosomal aberrations.

The tox-batch did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations in the absence and presence of S9-mix in either of the two tests.

It was therefore concluded that the tox-batch is not clastogenic to cultured human lymphocytes under the conditions employed in this study.

## 7.5 Estimates of Human Consumption and Safety Margin

The estimation of human consumption is split into two separate sections because of the differences in the groups. The first section contains intake estimates for people with specific nutritional requirements like allergic people or infants, while the second section regards the intake of the general population.

### Specific groups of population

- An EDI of peroxidase was calculated for an exclusive consumption of soy milk instead of milk by persons allergic to dairy products or lactose intolerant. The EDI was calculated based on consumption data in the USA (USDA 1994 and 1995 food intake survey, Wilson et al., 1997). The highest consumption of milk products is found in the age category 1-2 years old children (400 mL/day) and although DSM does not see soy in infant foods as a realistic market, since this is a worst case approach, we did calculate the safety of this application. A 90<sup>th</sup> percentile intake level of milk would correspond to 800 mL/day<sup>3</sup>, or 84.2 mL/kg bw/day for a child weighing 9.5

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<sup>3</sup> 90<sup>th</sup> percentile is 2 times the intake level (CFSAN, 2006).

kg bw. With an enzyme level of 4.5 DBLU/ml soy milk, this would lead to a peroxidase maximal intake of 379 DBLU/kg bw/day.

- An EDI of peroxidase was also calculated through the consumption of cheese-whey in infant formula. Infant formula contains 0.8 g cheese-whey proteins per 13.5 g powder in 100 ml water<sup>4</sup>. Dry weight powder consists of 10% proteins. Assuming that all proteins come from the cheese-whey, infant formula would therefore contain maximally  $0.8 / 13.5 \times 10 \times 100 = 60\%$  cheese-whey. The highest consumption of infant formula is 1000 ml/day for a 6 kg baby, equivalent to 135 g formula powder. This would represent a maximal daily intake of  $0.6 \times 135 / 6 = 13.5$  g cheese-whey/kg bw/day. With an enzyme level of 1.0 DBLU/ml cheese-whey, this would lead to a peroxidase maximal intake of 13.5 DBLU/kg bw/day.

Food product	Residual amount of (inactivated) enzyme in final food	High intake level (g food/person/day)	Estimated daily intake of (denatures) enzyme (DBLU/kg bw)	Estimated daily intake of (denatured) enzyme (mg TOS/kg bw)
Cheese-whey products in infant food	1.0 DBLU/ml cheese-whey	1,000	13.5	0.16
Soy milk	4500 DBLU/l soy milk	800	379	4.45

### General population

On the basis of information given in section 6.1, an estimation of the human consumption of peroxidase was made for the whole population. The EDI was calculated based on consumption data in the USA (USDA 1994 and 1995 food intake survey, Wilson et al., 1997). Moreover, FDA estimates a daily consumption of 3.8 g cheese-whey products/person in the USA (GRN 000037).

Food product	Residual amount of (inactivated) enzyme in final food	90 <sup>th</sup> percentile intake level (g food/person/day)	Estimated daily intake of (denatured) enzyme (DBLU/kg bw) <sup>5</sup>	Estimated daily intake of (denatured) enzyme (mg TOX/kg bw)
Cheese-whey products in dairy, bakery, beverages...	100 DBLU/ml cheese-whey protein	7.6	12.7	0.15
Soy milk <sup>6</sup>	4500 DBLU/l soy milk	46 <sup>7</sup>	3.45	0.04
Cream <sup>8</sup>	2000 DBLU/kg cream	8 <sup>5</sup>	0.27	0.00
Total			17.0	

<sup>4</sup> Data from Nutrilon from Nutricia

<sup>5</sup> Calculated for a person of 60 kg.

<sup>6</sup> Mean intake for category 'legumes' (includes cooked dry beans, peas, and lentils; mixtures having legumes as main ingredient, such as baked beans or lentil soup; soybean-derived products, such as soy-based baby formulas,

The Margin of Safety (MoS) can be calculated by dividing the NOAEL by the EDI. With an overall NOAEL of 150,000 DBLU/kg bw/day, the MoS was calculated for an intake of peroxidase (1) by lactose-intolerant people through the consumption of soy milk, (2) through the consumption of infant formula and (3) in the general population:

MoS (soy milk)	MoS (infant formula)	MoS (general population)
150,000 / 379 = 396	150,000 / 13.5 = 11,111	150,000 / 17.0 = 8,818

Regarding the height of the MoS, which is based on worst-case intake calculation, it was concluded that further testing of the safety of the product is not meaningful.

## 7.6 Results and Conclusion

Results of the toxicity, mutagenicity and clastogenicity tests described in Section 7.4.1 demonstrate the safety of DSM's peroxidase preparation, which showed no toxicity, mutagenicity or clastogenicity across a variety of test conditions. The data resulting from these studies are consistent with the long history of safe use for *Aspergillus niger* in food processing, the natural occurrence of peroxidase in foods, and data presented in relevant literature. Based upon these factors, as well as upon the limited and well-characterized genetic modifications allowing for safe production of the peroxidase preparation, it is DSM's conclusion that peroxidase preparation from *Aspergillus niger* is GRAS for the intended conditions of use.

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tofu, soy sauce, and soy-based meal replacement; and meat substitutes that are mainly vegetable proteins). The intake of soy milk is therefore highly overestimated by considering this food category.

<sup>7</sup> Combined results from USDA's 1995 and 1995 continuing survey of food intakes by individuals and 1994 and 1995 diet and health knowledge survey (Wilson et al, 1997). Results of the mean intake are used; 90<sup>th</sup> percentile is 2 times the intake level (CFSAN, 2006).

<sup>8</sup> Mean intake for category 'table fats' (includes butter, margarine, imitation margarine, margarine-like spreads, blends of butter with margarine or vegetable oil, and butter replacement).

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- 3.2.1 Amino acid sequence of peroxidase from *Aspergillus niger*
- 3.4.1 Biochemical characterization of peroxidase from *Aspergillus niger*
- 4.1.1 Flow diagram of manufacturing process
- 6.2.1 The use of peroxidase from *Aspergillus niger* on colored cheese-whey
- 6.2.2 The use of peroxidase from *Aspergillus niger* on soy milk
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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.

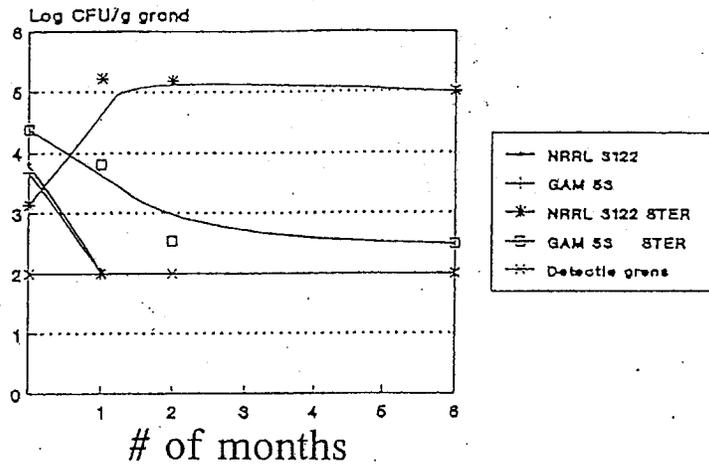
In all sterile environments at 25°C inoculated with *A. niger* spores, having a titre between  $10^4$ - $10^5$ , colony forming units/gram sample were found. At 8°C the wild-type strain was surviving well in soil as well as in surface water, whereas in waste water it was decreased beneath the detection 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.

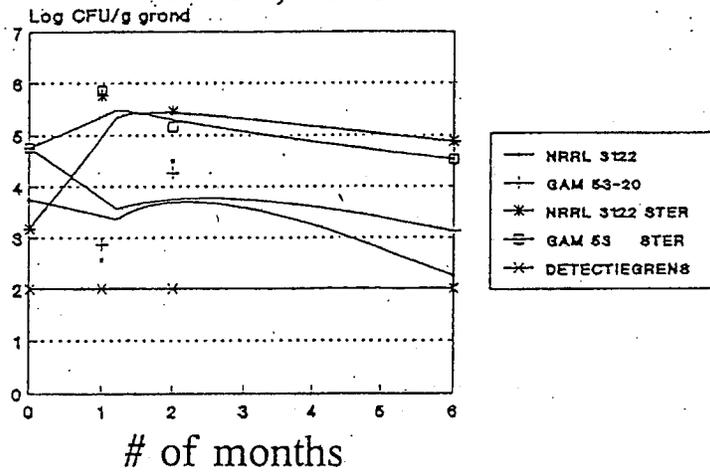
In natural environments and in the presence of competitive micro-organisms surviving *Aspergillus niger* strains were found after 6 month only in soil samples at 25°C, inoculated with at least  $10^3$  spores/g. In both aqueous environments at 8°C and 25°C and in soil of 8°C a fast decline of surviving 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°C

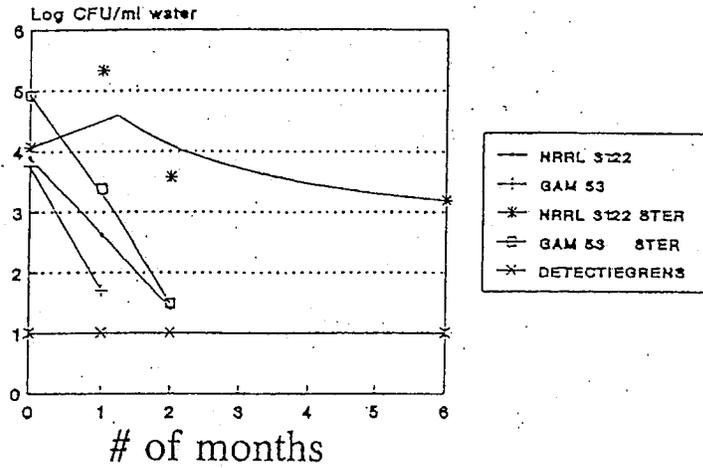


Incubation of *A. niger* mycelium  
in soil, 25°C

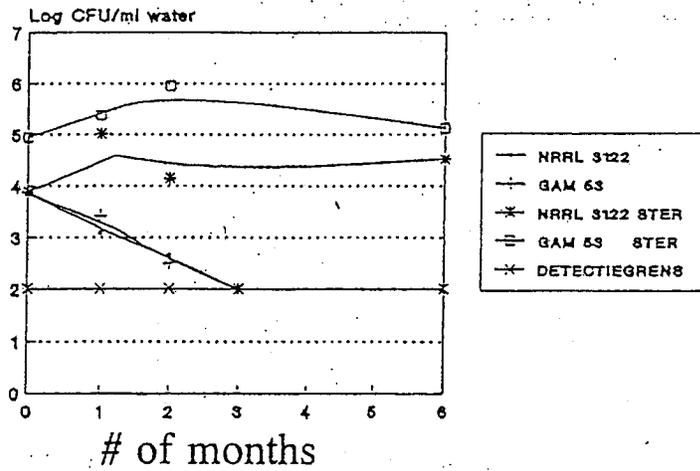


Surviving of *Aspergillus niger* I

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

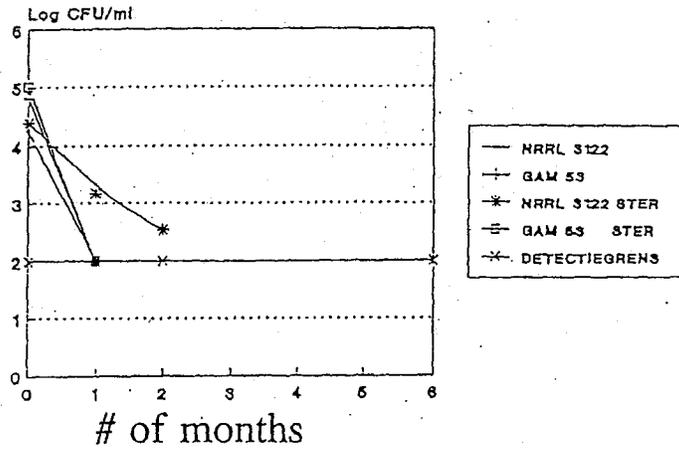


Incubation of *A. niger* mycelium  
in surface water, 25°C

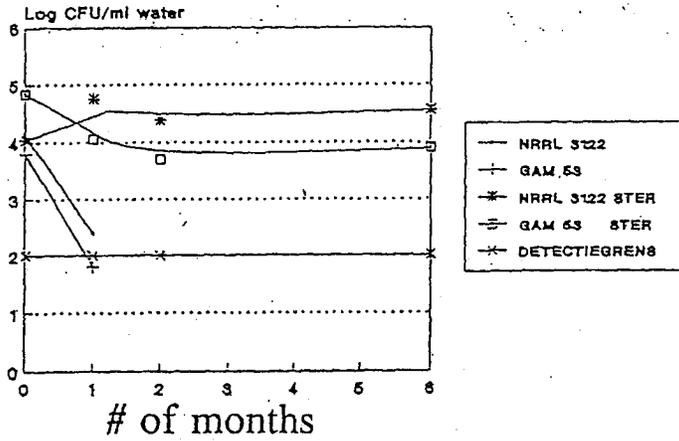


Surviving of *Aspergillus niger* II

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



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



Surviving of *Aspergillus niger* IH.



Amino Acid Sequence of Peroxidase from *Aspergillus niger*

MSFRSLALSGLVCTGLANVISKRATLDSWLSNEATVARTAILNNGADGAWVSGADSG  
IVVASPSTDNPDYFYTWTRDSGLVCLKTLVDLFRNGDTSLLSTIENYISAQAIVQGISNP  
SGDLSSGAGLGEPKFNVDEYAYTGSWGRPQRDGPALRATAMIGFGQWLLDNGYTSTATD  
IVWPLVRNDLSYVAQYWNQTYDLWEEVNGSSFFTIAVQHRALVEGSAFATAVGSSCSW  
CDSQAPEILCYLQSFWTGSFILANFDSSRSGKDANTLLGSIHTFDPEAACDDSTFQPCS  
PRALANHKEVVDSEFRSIYTLNDGLSDSEAVAVGRYPEDTYNGNPWFLLCTLAEEQLYD  
ALYQWDKQGSLEVTDVSLDFFKALYSDAATGTYSSTSSSTYSSIVDAVKTFADGFVSIVE  
THAASNGSMSEQYDKSDGEQLSARDLTWSYAALLTANNRRNSVVPASWGETSASSVPGT  
CAATSAIGTYSSVTVTWPSIVATGGTTTTATPTGSGSVTSTSKTTATKRTAHIRAPNV  
KPRRTNSLLITPPQPPLPSAQQAASASSAGLNLTDIQGDILIGMKNKELFFFSVT  
DAATFKAKLGS DILGLITSTDQLLANDTQPVTAVNVAFSSTGLKALGITDDLKDPVFEA  
GMLSNAVSDLSDPGTGNWVPGFVGTSVHGVFLASDTIDNVNTELANIQTILNGSITEI  
HRLQGEARPGDQQGHEHFGFMDGISNPAVDGFTPPAEIRPGQALIPPGIMLLGEANDTF  
QNDRPPWAKDGSFLVFRQMQORAFENKFLQDHALNMPNMTSEQGADLLGARIVGRWKS  
GAPIDLTPLVDDPVLAADNQRNNNFDFSDATNQTRCPFSAHIRKANPRGDLGGINKFPN  
QHIIIRAGIPYGPEVTDAEKASNSSSTDP SLERGLAFVAYQSNIQNGFVFLQKNWVDNTN  
FFRPGTGVDPLIGTNSRNSGTDAPNTPRVVSGLDPNNATSTIEIDIDFVVSARGGEYFFS  
PSLSAIRTVLSV



# Biochemical characterisation of peroxidase from a GMO *Aspergillus niger*

## 1. Introduction

The following biochemical characteristics of peroxidase from a GMO *Aspergillus niger* were investigated:

- pH profile
- Temperature profile
- Molecular weight (MW) of the enzyme protein
- Iso-electric point (IEP) of the enzyme protein
- Amino acid sequence

## 2. Materials and Methods

pH and temperature profiles were made using the standard protocol for measuring peroxidase activity. In order to cover a pH range from 3 to 7, the standard buffer was replaced by a range of pH buffers containing Tris and phosphoric acid. For the pH range the activities were measured at 37°C at pH 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7. For the temperature profile the activities were measured at pH 3.5 at temperatures of 7, 20 and 35°C, during several minutes. Activities were measured by measuring the absorption at 405 nm on the Konelab Arena 30 analyzer.

The MW of the enzyme protein was measured by subjecting the ccUF as well as the chromatographically purified enzyme to an SDS-PAGE electrophoresis.

The IEP of the enzyme protein was measured by subjecting the chromatographically purified enzyme to Iso-electro Focussing (IEF).

## 3. Results and Conclusions

### 3.1 pH profile

As can be seen in Figure 1, peroxidase is active in the pH range 3.0 – 7.0, with an optimum between 4.5 and 6.5.

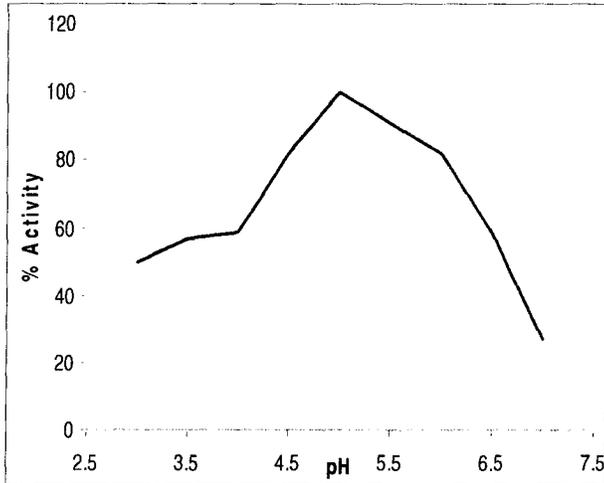


Figure 1: Relative activity of peroxidase between pH 3.0 – 7.0

### 3.2 Temperature profile

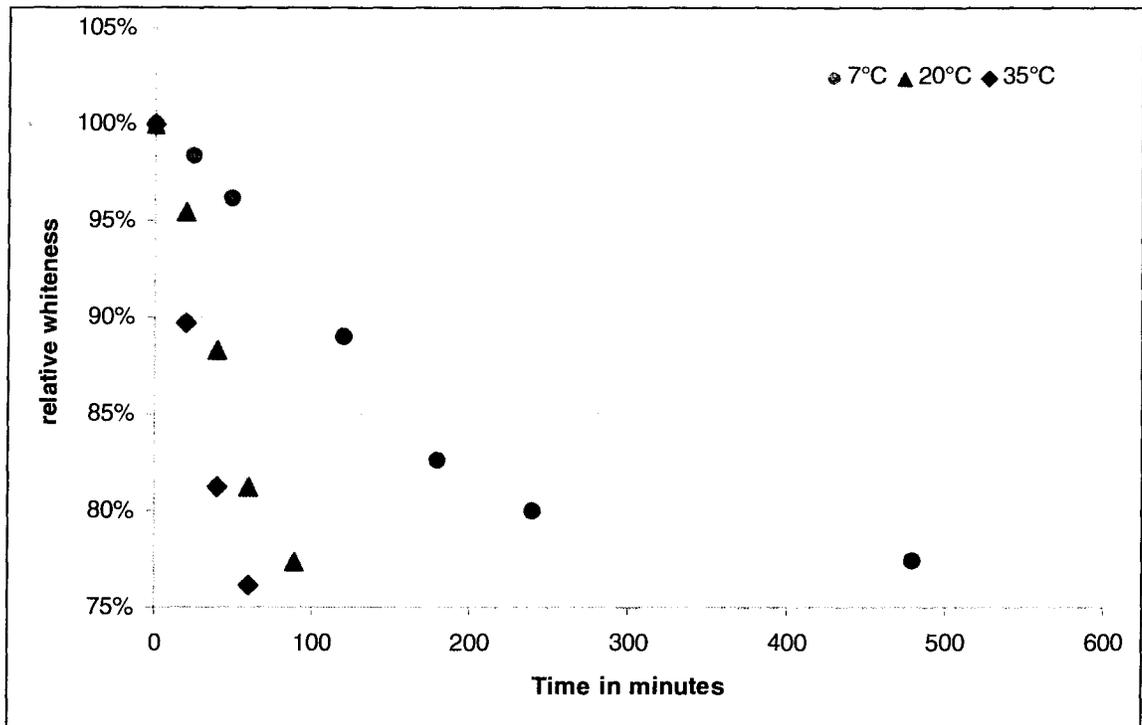


Figure 2: relative whiteness of peroxidase-treated whey at different temperatures

### 3.3 MW of the peroxidase enzyme protein

Figure 3 shows that the apparent MW of the chromatographically purified peroxidase protein (Lane 1) is 68.3 kDa. This is more than the predicted value of 52.9 kDa based on the amino acid sequence, due to the fact that the enzyme is glycosylated. In Lane 2, the MW of amyloglucosidase side activity is shown. As can be seen in the Lane called 'Feed', the ccUF consists mainly of peroxidase and amyloglucosidase.

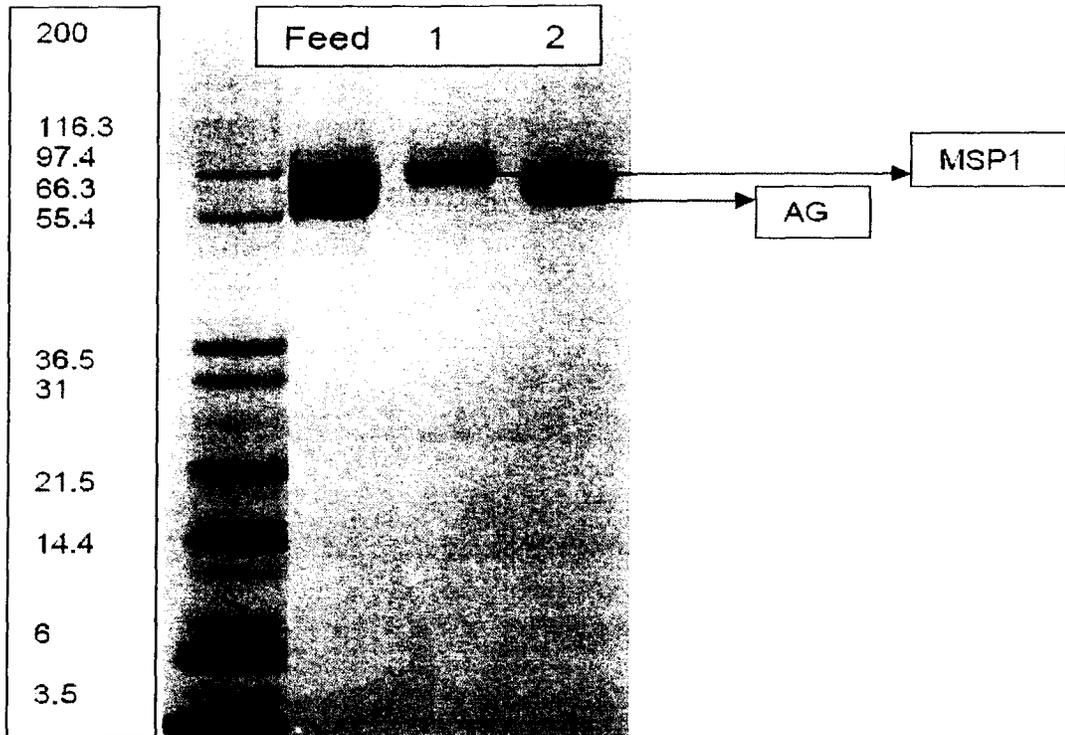


Figure 3: MW of the peroxidase enzyme protein

#### 3.4 IEP of the peroxidase enzyme protein

As can be seen in Figure 4, the IEP of the peroxidase protein (called 'DBE') is 3.65. This is lower than the predicted value of 4.83 based on the amino acid sequence, due to the fact that the enzyme is glycosylated.

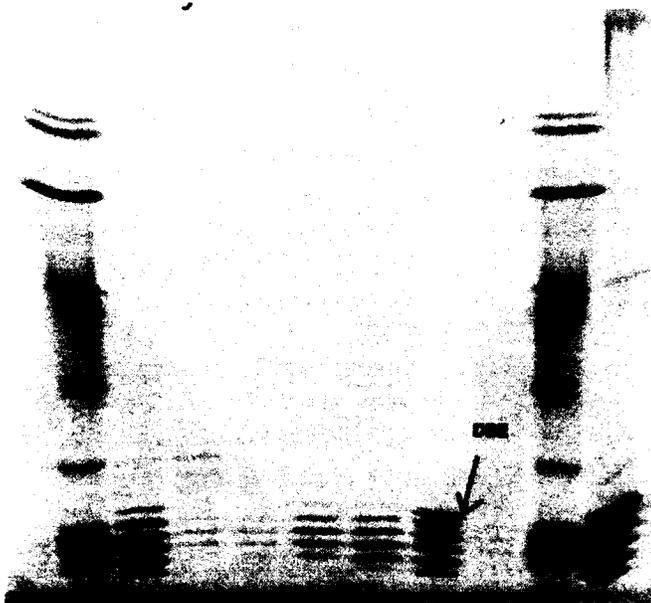
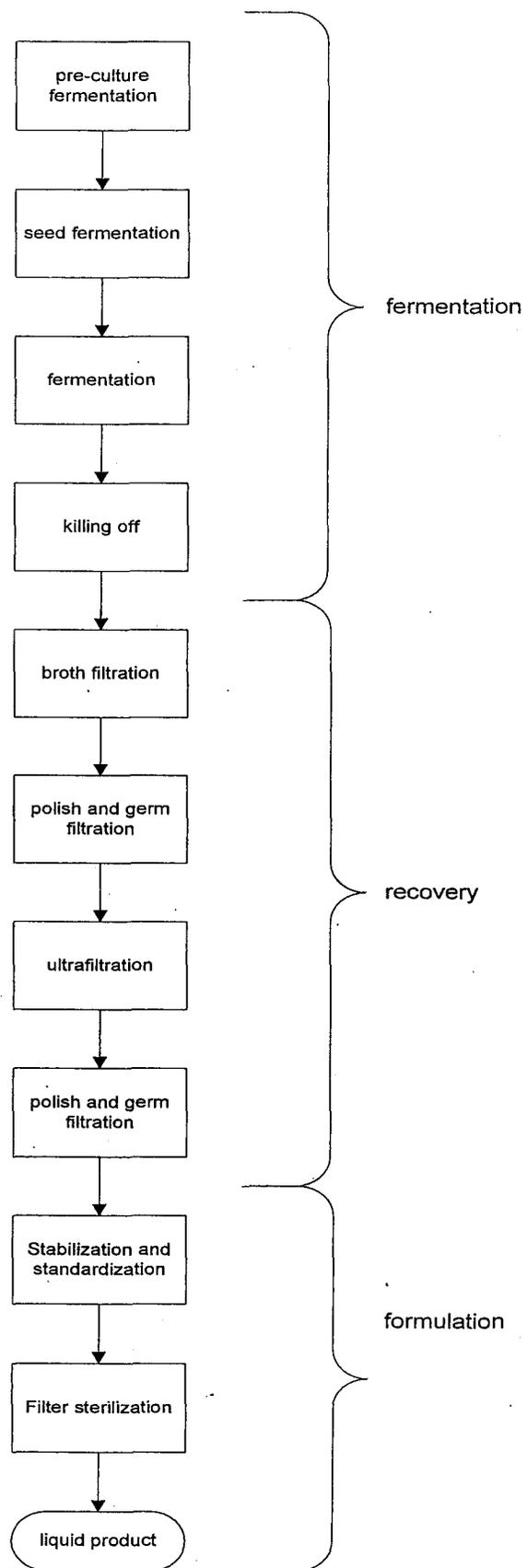


Figure 4: IEP of the peroxidase enzyme protein

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# Peroxidase production scheme



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# The use of peroxidase from a GMO *Aspergillus niger* on colored cheese-whey

## Results from laboratory scale experiments

### Introduction

The effect of peroxidase from a GMO *Aspergillus niger* on the color of Annatto-containing cheese-whey was measured in time, in the presence of various concentrations of  $H_2O_2$ . At the end of the incubation with peroxidase, the residual amount of  $H_2O_2$  was measured.

### Materials and Methods

Milk containing 300 mg/l Annatto was used to prepare Cheddar cheese-whey at the DSM R&D cheese application laboratory. After separation of the cheese-whey from the cheese curd, the cheese-whey was incubated with 1 DBLU peroxidase/ml and various concentrations  $H_2O_2$ . The experiments took place in the dark at a temperature of 20°C.

The decrease of the color of the cheese-whey was followed in time by measuring the yellow/blue components (the so-called b-values) by means of an X-Rite 968 reflection spectrophotometer.

After 45 minutes incubation time, residual  $H_2O_2$  was measured with  $H_2O_2$  strips (Merckoquant® 1.10081), with a detection limit of 1 mg/l.

### Results and Conclusions

As can be seen in Figure 1, the effect of peroxidase on the cheese-whey color depends on the concentration of  $H_2O_2$  present. The peroxidase is active at  $H_2O_2$  concentrations between 0.3 and 0.7 mM. Optimal decrease of cheese-whey color was found at a  $H_2O_2$  concentration of 0.4 mM. At higher concentrations, the presence of  $H_2O_2$  starts to inhibit the peroxidase activity.

At the optimal  $H_2O_2$  concentration, the decrease of the cheese-whey color is not only maximal, but the decrease also occurs the fastest. Within only 20 minutes, the maximal decrease is obtained.

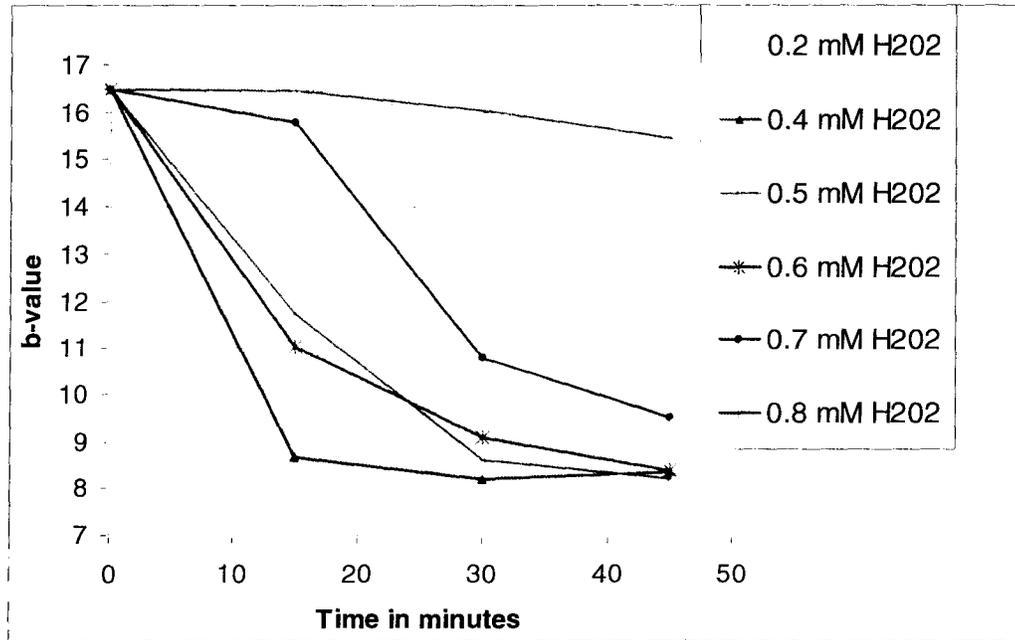


Figure 1: Effect of peroxidase on cheese-whey color in time at various H<sub>2</sub>O<sub>2</sub> concentrations

Figure 2 shows that at the optimal H<sub>2</sub>O<sub>2</sub> concentration of 0.4 mM, the peroxidase has converted all H<sub>2</sub>O<sub>2</sub> after 45 minutes, as no residual can be measured. It can thus be concluded that in practice the use of peroxidase will not result in measurable amounts of H<sub>2</sub>O<sub>2</sub> in the final foodstuff.

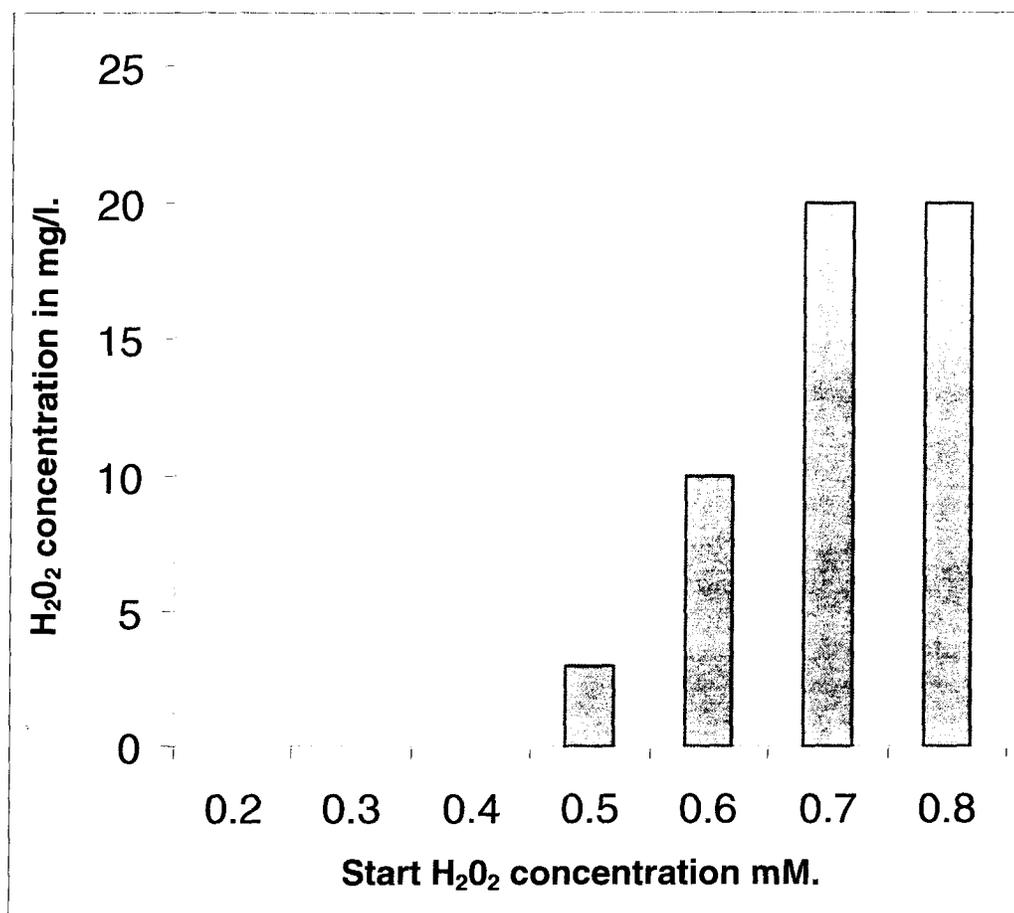


Figure 2: Residual amount of H<sub>2</sub>O<sub>2</sub> after 45 minutes incubation with peroxidase



# The use of peroxidase from GMO *Aspergillus niger* on soy milk

## Results from laboratory scale experiments

### Introduction

The effect of peroxidase from GMO *Aspergillus niger* on the color of soy milk was measured using various peroxidase and H<sub>2</sub>O<sub>2</sub> concentrations.

### Materials and Methods

Soy milk (pH 6.9) was prepared from de-hulled and hydrated soy beans. The soy milk was incubated at room temperature in the dark with different concentrations of peroxidase and H<sub>2</sub>O<sub>2</sub>.

The decrease of the color of the soy milk was followed in time by measuring the yellow/blue components (the so-called b-values) by means of an X-Rite 968 reflection spectrophotometer.

### Results and Conclusions

As can be seen in Figure 1, the presence of H<sub>2</sub>O<sub>2</sub> alone results in some decrease of the soy milk color (lines 5 and 6). When peroxidase is added as well, further decrease of the soy milk color was obtained. Whilst the higher peroxidase concentration (lines 3 and 4) resulted in a faster colour decrease during the first hour than the lower peroxidase concentration (line 1), the final decolouration effect was more dependent on the ratio between the peroxidase and H<sub>2</sub>O<sub>2</sub> concentration. In the presence of a too high relative H<sub>2</sub>O<sub>2</sub> concentration, the peroxidase activity was partly inhibited (line 2). No decrease in the color of peroxidase treated soy milk could be obtained in the absence of H<sub>2</sub>O<sub>2</sub> (lines 7 and 8).

The results indicate that the ratio of the peroxidase versus H<sub>2</sub>O<sub>2</sub> concentration is important for obtaining optimal decrease of color. Depending on the specific process conditions (pH, temperature), the optimal ratio will have to be determined case by case.

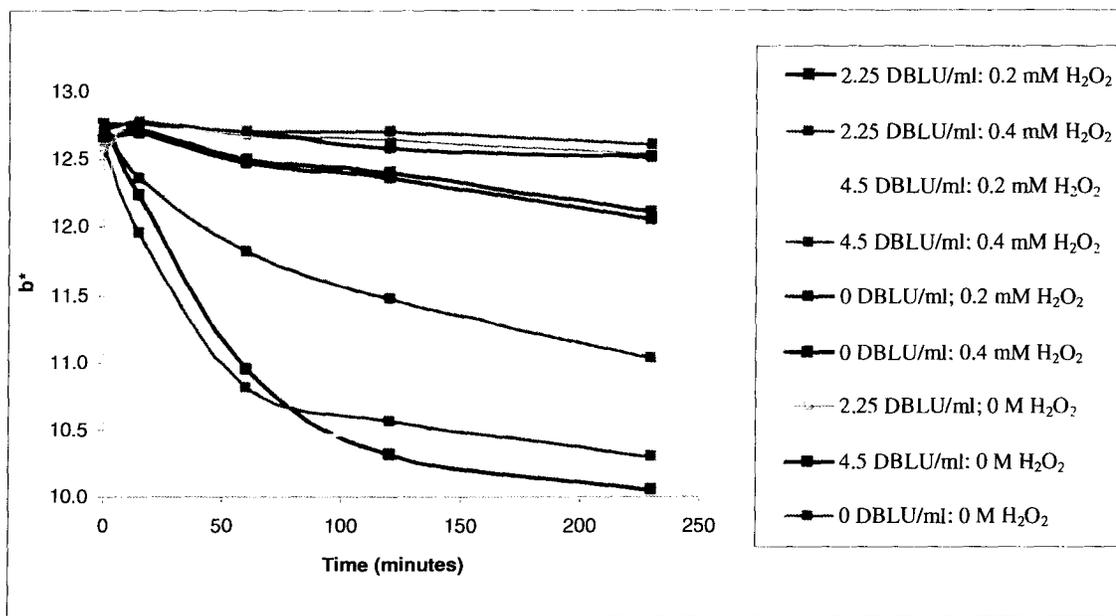


Figure 1: Effect of various peroxidase and  $H_2O_2$  concentrations on the color of soy milk at room temperature



# The use of peroxidase from GMO *Aspergillus niger* on fresh cream

## Results from laboratory scale experiments

### Introduction

In the presence of  $H_2O_2$ , the effect of peroxidase from GMO *Aspergillus niger* on the yellowish color of fresh cream was measured at various homogenisation conditions.

### Materials and Methods

To fresh cream (38% fat; 50°C) 2000 DBLU peroxidase/l and 0.7 mM  $H_2O_2$  was added. Directly after addition, the cream was homogenized at 0; 50; 100 and 200 bars and kept in the dark during 10 minutes at 50°C. As a control, untreated cream was homogenized at the same pressure levels.

The decrease of the colour of the cream was determined by measuring the yellow/blue components (the so-called b-values) by means of an X-Rite 968 reflection spectrophotometer.

### Results and Conclusions

As can be seen in Figure 1, no decrease in color was observed without homogenisation (i.e. 0 bars), independent whether peroxidase and  $H_2O_2$  were present or not. Homogenisation as such resulted in some color decrease in the control samples. In the samples treated with peroxidase and  $H_2O_2$ , however, further decrease of the color was observed. The total color decrease was independent of the degree of homogenisation. Consequently, a slight homogenisation of 50 bars is sufficient to achieve the required decolouration effect.

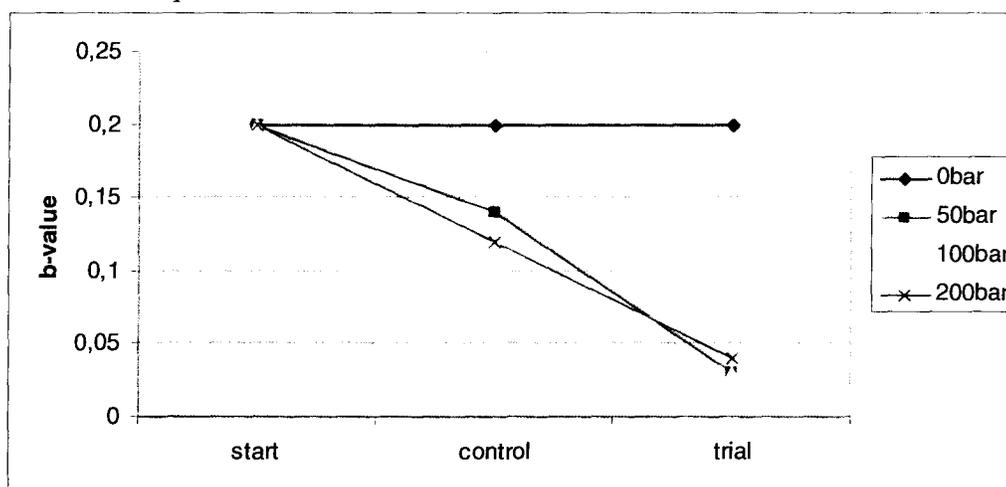


Figure 1: Effect of peroxidase on color of fresh cream at various degrees of homogenisation



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ENZYMES DERIVED FROM *ASPERGILLIS NIGER*

## EXPLANATION

*A. niger* is a contaminant of food and was not considered in the same light as those organisms regarded as normal constituents of food. It is necessary to show that the strains used in enzyme preparations do not produce mycotoxins.

Microbial carbohydrases prepared from some varieties of *A. niger* were evaluated at the fifteenth meeting of the Committee, at which time a temporary ADI "not limited" was established (Annex 1, reference 26). A toxicological monograph was prepared (Annex 1, reference 27). An adequate 90-day study in rats was requested. Since the previous evaluation, additional data have become available on a number of carbohydrases, which are summarized and discussed in the following monograph. These enzymes were considered by the Committee to encompass the carbohydrases previously considered. The previously published monograph has been expanded and reproduced in its entirety below.

## AMYLOGLYCOSIDASES (E.C. 3.2.1.3)

## BIOLOGICAL DATA

## Biochemical aspects

No information available.

## Toxicological studies

## Special studies on aflatoxin-related effects

## Ducklings

Four groups of 5 ducklings received in their diet 0, 1, 5, or 10% enzyme preparation for 29 days. Growth, feed consumption, survival, behaviour, and mean liver weights were comparable, in all groups. No gross or histopathological lesions of the liver were seen (FDRL, 1963a).

Four groups of 5 ducklings received in their diet 0, 1, 5, or 10% enzyme preparation for 29 days. Growth, feed consumption, survival, behaviour, and development were comparable in all groups. No gross liver lesions were seen at autopsy and mean liver weights of treated animals were similar to those of controls. Histopathology of the livers was normal. No toxic elements were noted (FDRL, 1963b).

Acute toxicity<sup>1</sup>

000239

Species	Route	LD <sub>50</sub> (mg/kg b.w.)	Reference
Mouse	oral	> 3,200	Hunt & Garvin, 1963
		> 4,000	Hunt & Garvin, 1971
		> 3,200	Willard & Garvin, 1968
		> 4,000	Garvin et al., 1966
Rat	oral	10,000	Gray, 1960
		31,600	Kay & Calendra, 1962
		> 3,200	Willard & Garvin, 1968
		> 4,000	Garvin et al., 1966
		12,500 - 20,000	Kapiszka & Hartnage, 1978
Rabbit	oral	> 4,000	Garvin et al., 1966
Dog	oral	> 4,000	Garvin et al., 1966

<sup>1</sup> These data were obtained with several different commercial enzyme preparations.

#### Short-term studies

##### Rats

Three groups of 10 male rats received 0, 0.5, or 5% enzyme preparation in their diets for 30 days. No adverse effects related to treatment were observed regarding growth, appearance, behaviour, survival, food consumption, haematology, organ weights, or gross pathology (Garvin *et al.*, 1966).

Two groups of 10 male and 10 female rats received either 0 or 5% enzyme preparation in their diets daily for 91 days. No differences from controls were observed regarding appearance, behaviour, survival, weight gain, haematology, organ weights, or gross pathology (Garvin & Merubia, 1959).

Two groups of 10 male and 10 female ARS Sprague-Dawley rats were fed diets containing 5 or 10% of the test enzyme preparation (equivalent to 3.5 or 7 g enzyme preparation/kg b.w./day) for 90 to 94 days. A control group of 20 male and 20 female rats were maintained on the diet alone. No signs of toxicity were observed during the test period. Body-weight gain and food consumption were similar between test and control groups. Differential blood counts were within the normal range at weeks 4 and 8 of the study in both test and control animals. At the end of the study serum clinical chemistry parameters, organ weight analyses, and gross and microscopic pathology showed no compound-related effects (Garvin *et al.*, 1972).

##### Long-term studies

No information available.

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## Observations in man

No information available.

## COMMENTS

Several short-term feeding studies in rats on amyloglucosidase preparations from *A. niger* have been performed. One study, in which the preparation was fed at up to 10% of the diet, was considered to be acceptable by current standards. No compound-related effects were observed in this study or in duckling tests that were performed to investigate potential aflatoxin-related effects.

The evaluations by the Committee of the carbohydrates and the protease from *A. niger* are summarized at the end of this section.

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$\beta$ -GLUCANASE (E.C. 3.2.1.6)

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## BIOLOGICAL DATA

## Biochemical aspects

No information available.

## Toxicological studies

(The TOS of the enzyme preparation used for toxicity studies was 49%).

## Special Studies on mutagenicity

The enzyme preparation was tested for mutagenic activity using 5 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, and TA1538 both with and without metabolic activation (S-9 fraction). The preparation was not mutagenic or toxic at concentrations up to 40 mg/ml (McConville, 1980).

A cytogenic bone marrow study was performed using adult male Chinese hamsters. Groups of adult male hamsters received up to 5000 mg/kg b.w./day of the enzyme preparation for 5 consecutive days. Treatment did not result in an increased frequency of chromosomal aberrations in bone marrow (McGregor & Willins, 1981).

## Acute toxicity

Species	Route	Sex	LD <sub>50</sub> (ml/kg b.w.)	Reference
Mouse (NMRI)	oral	M & F	30	Novo, 1978a
Rat (Wistar)	oral	-	28.1	Novo, 1978b

## Short-term studies

## Rats

Three groups, each containing 5 male and 5 female Wistar/Mol SPF rats, were dosed orally by gavage once a day for 14 days with enzyme preparation at dose levels equivalent to 2.5, 5.0, or 10 ml/kg b.w. No clinical changes were observed. Body-weight gains of test and control animals were similar. At termination of the study, measurements of organ weights showed no compound-related effects (Novo, 1978c).

In another study, 4 groups, each containing 15 male and 15 female Wistar/Mol SPF rats, were dosed by gavage once a day for 90 days with enzyme preparation at dose levels equivalent to 0, 2.5, 5.0, or 10 ml/kg b.w. Deaths, primarily in the high-dose group, appeared to be related to injury during dosing. No clinical signs were observed in the other test animals. Male rats in the high-dose group showed decreased weight gain and marked decrease in food intake. Haematology studies showed increased platelet counts and decreased clotting times

in the high-dose group at week 6, but this effect was not apparent at week 12. No other effects were reported. Clinical chemistry and urinalysis values at weeks 6 and 12 were within the normal range. At termination of the study, organ weight analysis showed a marked increase in relative weights of the spleen and testes of the males in the high-dose group. Gross and histopathological examination of the principal organs and tissues showed no compound-related effects (Perry *et al.*, 1979).

#### Dogs

Three groups, each containing one male and one female beagle dog, received single doses of 5, 10, or 15 ml/kg b.w. of the enzyme preparation over a 4-day period. Following a 7-day observation period the dogs were sacrificed and subjected to macroscopic post-mortem examination. No compound-related effects were observed, with the exception of vomiting during the first 4 days of the study. In another study, dogs were administered consecutive doses of 15 ml/kg b.w./day for 9 days, and 10 ml/kg b.w./day for 5 days. No deaths occurred during the course of the study. The only clinical sign noted was excessive salivation and emesis shortly after dosing. Body weights, electrocardiograms, haematological parameters, blood serum chemistry, organ weights, gross pathology, and histopathology showed no compound-related effects (Osborne *et al.*, 1978).

In another study, three groups, each containing 3 male and 3 female beagle dogs, were dosed with the enzyme preparation by gavage once a day, seven days a week, for 13 weeks, at dose levels equivalent to 2, 5, or 9 ml/kg b.w./day. Two dogs in the high-dose group died during the course of the study, which the authors concluded was due to respiratory distress as a result of foreign material in the lungs. Vomiting was reported after dosing in the high-dose group. Haematological parameters at weeks 6 and 12 were within normal limits, with the exception of a significant increase in WBC count, specifically in the group mean neutrophil counts, in the high-dose group. Clinical chemistry values were within the normal range at weeks 8 and 12, with the exception of slight increases in blood glucose and cholesterol in the high-dose group. Urinalysis showed no compound-related effects. At termination of the study, organ-weight analyses and gross and histopathological examination of the principal organs and tissues showed no compound-related effects (Greenough *et al.*, 1980).

#### Long-term studies

No information available.

#### Observations in man

No information available.

#### COMMENTS

This enzyme preparation was not genotoxic in microbial or in mammalian test systems. Short-term studies in rats and dogs resulted in no observed compound-related effects at levels up to 5 ml/kg b.w./day of enzyme preparation.

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The evaluations by the Committee of the carbohydrases and the protease from *A. niger* are summarized at the end of this section.

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## HEMI-CELLULASE

## BIOLOGICAL DATA

## Biochemical aspects

No information available.

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## Toxicological studies

### Special studies on mutagenicity

The enzyme preparation was tested for mutagenic activity using *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 both with and without metabolic activation (S-9 fraction). The test substance was not mutagenic or toxic at concentrations up to 5 mg/plate (Clausen & Kaufman, 1983).

In an *in vitro* cytogenetic test using CHO-K1 cells, both with and without metabolic activation (S-9 fraction), the enzyme preparation at test levels up to 2.5 mg (dry wt)/ml did not induce chromosomal aberrations (Skovbro, 1984).

### Acute toxicity

No information available.

### Short-term studies

#### Rats

Four groups, each containing 5 male and 5 female Wistar MOL/W rats, were dosed by gavage once a day for 90 days with the enzyme preparation at doses equivalent to 0, 100, 333, or 1000 mg/kg b.w./day. No significant clinical changes were observed. Body-weight gain and food intake were similar among test and control animals. Haematologic and clinical chemistry measurements at termination of the study were within normal ranges. Post-mortem examinations, measurements of organ weights, and histopathology showed no compound-related effects. Slight increases in kidney and adrenal weights in the mid-dose group were not associated with histopathological effects, and did not show a dose response (Kallesen, 1982).

### Long-term studies

No information available.

### Observations in man

No information available.

### COMMENTS

This enzyme preparation was not genotoxic in microbial or in mammalian test systems. In a limited 90-day study in rats, no effects were observed at the highest dose administered (1 g/kg b.w./day). This enzyme preparation contained high levels of pectinase. The pectinase enzyme preparation summarized below may be identical to this hemi-cellulase preparation, which provides added assurance of the safety of this preparation.

The evaluations by the Committee of the carbohydrases and the protease from *A. niger* are summarized at the end of this section.

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PECTINASE (E.C. 3.1.1.11; 3.2.1.15; 4.2.2.10)

## BIOLOGICAL DATA

## Biochemical aspects

No information available.

Toxicological studies (The TOS of the commercial preparation is approximately 5%).

## Acute toxicity

Species	Route	LD <sub>50</sub> (ml/kg b.w.)	Reference
Rat	oral	18.8-22.1	Porter & Hartnagel, 1979

## Short-term studies

## Rats

Two groups of 10 male and 10 female ARS Sprague-Dawley rats were fed diets containing 5 or 10% of the test enzyme preparation (equivalent to 3.5 or 7 g of the enzyme preparation/kg b.w./day), for 90 to 94 days. A control group of 20 male and 20 female rats was maintained on the diet alone. No signs of toxicity were observed during the test period. Body-weight gain and food consumption were similar among test and control groups. Differential blood counts at weeks 4 and 8 of the study were within the normal range in test and control animals. At the end of the study serum clinical chemistry analyses, organ weight analyses, and gross and microscopic pathology showed no compound-related effects (Garvin *et al.*, 1972).

## Long-term studies

No information available.

000246

## Observations in man

No information available.

## COMMENTS

In a short-term study in rats, no adverse effects were observed at dietary levels of the enzyme preparation up to the equivalent of 7 mg/kg b.w./day. This enzyme preparation may be identical to the hemi-cellulase preparation summarized above. The hemi-cellulase enzyme preparation summarized above also contained high levels of pectinase, which provides added assurance of the safety of this preparation.

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## PROTEASE

No information available.

GENERAL COMMENTS ON ENZYMES FROM A. NIGER

Aspergillus niger is a contaminant of food. Although there may be possible strain differences in A. niger, and different cultural conditions might be used to prepare the various enzymes, the available toxicity data, which consist primarily of short-term feeding studies in rats and some studies in dogs, show that all the enzyme preparations tested were of a very low order of toxicity. The enzyme preparations tested were non-mutagenic in bacterial and mammalian cell systems. Studies on some strains of A. niger used to prepare carbohydrases showed no aflatoxin or related substance production. These studies provide the basis for evaluating the safety of enzyme preparations derived from A. niger. It was also noted that the enzyme preparations tested exhibit a number of enzyme activities, in addition to the major enzyme activity. Thus, there may be considerable overlap of the enzyme activities of the different enzyme preparations so that safety data from each preparation provides additional assurance of safety for the whole group of enzymes.

Since the enzyme preparations tested were of different activities and forms, and most of the organic materials in the preparations are not the enzyme per se, the numerical ADI is expressed in terms of total organic solids (TOS) (see introduction to enzyme preparations section).

## EVALUATION

**Level causing no toxicological effect**

All enzyme preparations tested showed no-observed-effect levels greater than 100 mg TOS/kg b.w./day in 90-day studies in rats.

**Estimate of acceptable daily intake**

0-1 mg TOS/kg b.w. for each of the enzyme preparations.

See Also:

Toxicological Abbreviations



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.  
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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, all 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 B<sub>1</sub>, ochratoxin A, sterigmatocystin, 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 all 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<sub>1</sub>, 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<sub>1</sub>, sterigmatocystin, T-2 toxin, or zearalenone.

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Sep. 1988

THE OCCURRENCE AND SIGNIFICANCE  
OF MYCOTOXINS

000258

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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  $\beta$ -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<sub>1</sub>, 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	<u>Aspergillus flavus, A. parasiticus</u>
OCHRATOXIN	<u>Aspergillus ochraceus,</u> <u>Penicillium viridicatum</u>
CITRININ	<u>Penicillium citrinum</u>
PENICILLIC ACID	<u>Penicillium spp., Aspergillus spp.</u>
PATULIN	<u>Penicillium expansum,</u> <u>Aspergillus clavatus</u>
STERIGMATOCYSTIN	<u>Aspergillus versicolor</u>
MYCOPHENOLIC ACID	<u>Penicillium roquefortii</u>
PENITREM A	<u>Penicillium aurantiogriseum</u>
P R TOXIN	<u>Penicillium roquefortii</u>
VIOMELLEIN	<u>Aspergillus ochraceus</u> <u>Penicillium viridicatum</u>
CYTOCHALASIN E	<u>Aspergillus clavatus</u>
CITREOVIRIDIN	<u>Penicillium citreonigrum</u>
CYCLOPIAZONIC ACID	<u>Aspergillus flavus,</u> <u>Penicillium aurantiogriseum</u>
ROQUEFORTINE	<u>Penicillium roquefortii</u>
ISOFUMIGACLAVINE	<u>Penicillium roquefortii</u>
ZERALENONE	<u>Fusarium spp</u>
ZEARALENOL	<u>Fusarium spp</u>
TRICHOHECENES	<u>Fusarium spp</u>
MONILIFORMIN	<u>Fusarium spp</u>
TENUAZONIC ACID	<u>Alternaria spp</u>
ALTERNARIOL	<u>Alternaria spp</u>
ALTENUENE	<u>Alternaria spp</u>
ERGOT ALKALOIDS	<u>Claviceps spp</u>

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

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

\* recognised as mycotoxins

## APPENDIX 1

Search Strategy Used

Set	Items	Description
S1	5329	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	22846	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 Bio- technology 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 Microbio- logy, Microbiological abstracts
Chemcial Exposure	Dialog	Databank
Martindale on line	Dialog	Databank
Medline	Dialog	Index Medicus (3000 Internatio- nal 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





**SAFETY EVALUATION using the PARIZA & JOHNSON DECISION TREE of Peroxidase from a genetically modified strain of *Aspergillus niger***

**Introduction**

The "Decision Tree for evaluation of the relative safety of food and food ingredients derived from genetically modified organisms" from the International Food Biotechnology Council (IFBC) was published in 1990<sup>1</sup>. This publication was an extension based on an earlier publication by Pariza and Foster in 1983<sup>2</sup>. Recently, an update of the 1991 IFBC Decision Tree was prepared by Pariza and Johnson and is published in the April issue of the Regulatory Toxicology and Pharmacology of 2001<sup>3</sup>.

The enzyme preparation of peroxidase from a genetically modified strain of *Aspergillus niger* MOX-54 was evaluated according the Pariza and Johnson Decision Tree. The result is described below.

**Decision Tree**

1. Is the production strain genetically modified?  
**YES**  
The strain *Aspergillus niger* MOX-54 is derived from host ISO-528, which is a genetically modified strain (GMO self-clone) derived from the DSM GAM lineage of *A. niger* strains.  
If yes, go to 2. ~~If no, go to 6.~~
2. Is the production strain modified using rDNA techniques?  
**YES**  
If yes, go to 3. ~~If no, go to 5.~~
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?  
**NO**  
~~If yes, go to 3e.~~ If no, go to 3b
- 3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?  
**YES**  
If yes, go to 3c. ~~If no, go to 12.~~  
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 lineage<sup>4</sup>; a 90-day study on the current peroxidase ccUF did not show any effects resulting in a sufficient high NOAEL in relation to the proposed use.

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<sup>1</sup> IFBC (International Food Biotechnology Committee), Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification, Regulatory Toxicology and Pharmacology. Vol. 12:S1-S196 (1990).

<sup>2</sup> Pariza M.W. and Foster E.M. J. Food Protection Vol. 46. (1983), 453-468

<sup>3</sup> Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. Vol. 33 (2001) 173-186

<sup>4</sup> P.W.M. van Dijk et al. Reg. Toxicol. Pharmacol. Vol. 38 (2003) 27-35.

- 3c. Is the test article free of transferable antibiotic resistance gene DNA?  
**YES**  
 If yes, go to 3e. ~~If no, go to 3d.~~
- 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**  
~~If yes, go to 12. If no, go to 3e.~~
- 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?  
**YES**  
 If yes, go to 4. ~~If no, go to 12.~~  
 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 yes, go to 5.~~ If no, go to 6.  
 The introduced DNA is targeted when 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 ancestor strain MOX528-2 has been analyzed with respect to its potential to produce secondary metabolites, including mycotoxins. It showed no potential to produce secondary metabolites or mycotoxins of importance in food. This was supported by analyses on broth and concentrated UF samples (REG#55724).  
 If yes, go to 6. ~~If no, go to 7.~~
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 P&J Decision Tree evaluation scheme.  
**If yes, the test article is ACCEPTED.** ~~If no, go to 7.~~
7. Is the organism nonpathogenic?  
**NA**  
~~If yes, go to 8. If no, go to 12.~~
8. Is the test article free of antibiotics?  
**NA**  
~~If yes, go to 9. If no, go to 12.~~
9. Is the test article free of oral toxins known to be produced by other members of the same species?

**NA**  
~~If yes, go to 11. If no, go to 10.~~

10. Are the amounts of such toxins in the test article below levels of concern?

**NA**  
~~If yes, go to 11. If no, go to 12.~~

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?

**NA**  
~~If yes, the test article is ACCEPTED. If no, go to 12.~~

12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.

**NA**



# WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

AMFEP

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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 - 1 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  $\alpha$ -amylase, and wheat flour who developed symptoms after the ingestion of bread. The symptoms were somewhat more pronounced after bread prepared with  $\alpha$ -amylase than bread without. One case with occupational allergy to  $\alpha$ -amylase reacted upon ingestion of a very high test-dose of pure  $\alpha$ -amylase, but not at lower doses. Four other persons with occupational  $\alpha$ -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  $\alpha$ -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's, 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 allergic 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  $\alpha$ -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<sup>1</sup>, 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  $\alpha$ -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 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 **a l l e r g y                      a n t i b o d i e s                      ( I g E )**. 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 allergenic 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<sup>2-4</sup>, Trypsin<sup>5</sup>, protease's from the skin yeast *Candida albicans*<sup>6</sup>, from bacteria/ subtilisins<sup>7,8</sup>, fungal amylases<sup>9,10</sup>, bacterial amylases<sup>11</sup>, fungal hemicellulases<sup>12</sup>, lipases<sup>13</sup>, xylanases and cellulases<sup>14,15</sup> 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<sup>16</sup>, eggs<sup>17,18</sup>, milk<sup>19</sup> and fish<sup>20</sup> 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<sup>21</sup>.

## 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<sup>22</sup>. 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<sup>22-25</sup>.

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 literature<sup>26-28</sup>.

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<sup>58</sup> on native  $\alpha$ -amylase from *Aspergillus oryzae* in a gastrointestinal model simulating the physiological events in the stomach.

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The results indicate that about 92%, of the epitopes of the  $\alpha$ -amylase are destroyed and about 8%, of the epitopes on the  $\alpha$ -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  $\alpha$ -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<sup>29</sup> 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<sup>30-33</sup> 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<sup>25</sup> 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<sup>23</sup> 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<sup>24</sup>. 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<sup>34</sup>.

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

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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)<sup>35,36</sup>, 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<sup>37</sup> published a case story of a person who had allergy symptoms after ingestion of papain used as a meat tenderiser. - Later, in 1985 they reported a study of 475 patients<sup>38</sup> 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<sup>39</sup>, 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 Wüthrich<sup>40</sup> 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*

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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<sup>1</sup> 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<sup>41</sup> 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 allergic 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,<sup>42</sup> and Baur & Czuppon<sup>43</sup> 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<sup>44</sup> investigated occupational allergy to  $\alpha$ -amylase in a pharmaceutical plant and found a number of employees sensitised to  $\alpha$ -amylase. None reported reactions related to ingestion of bread. Five patients, all positive to  $\alpha$ -amylase were given oral doses of native  $\alpha$ -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<sup>45</sup> described the possible background for consumer sensitisation to  $\alpha$ -amylases in bread. 138 subjects, of which 98 were allergic, and 11 bakers with occupational allergy were tested. The bakers reacted to  $\alpha$ -amylase as may be expected. None of the atopics and none of the control persons reacted to skin prick test with  $\alpha$ -amylase. Two atopics had weak RAST to native  $\alpha$ -amylase and one reacted also to heated ce-amylase. Reactions to other related compounds, for example *Aspergillus* was not tested.

Tarlo and co-workers<sup>46</sup> 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<sup>47</sup> 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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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<sup>48-50</sup>.

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<sup>51</sup> 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<sup>52-54</sup>. 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<sup>55</sup>.

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<sup>56</sup> did not confirm sensitisation to any of the enzymes. Challenge tests have not been performed<sup>57</sup>.

### **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 allergic 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<sup>39</sup>, 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 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 have 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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*NA- Not applicable*