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October 3, 2006

REC'D OCT - 5 2006

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Via Federal Express

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

Re: GRAS Notification for Asparaginase derived from *Aspergillus niger*

Dear Sir or Madam:

As counsel for DSM Food Specialties, we are submitting under cover of this letter three copies of DSM Food Specialties' (DSM) GRAS notification of asparaginase from a genetically modified strain of *Aspergillus niger* (*A. niger*). DSM Food Specialties has determined through scientific procedures that asparaginase is generally recognized as safe for use in the food industry as a processing aid in the reduction of levels in food of free L-asparagine, a main precursor in the formation of the food contaminant acrylamide during heat treatment.

This use of asparaginase derived from *A. niger* is exempt from the premarket approval requirements of 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 any additional information to aid its review of DSM's conclusion, please contact me at your earliest convenience.

Sincerely,

Gary L. Yingling

cc: DSM Food Specialties

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**GRAS NOTIFICATION FOR ASPARAGINASE 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 asparaginase, which is produced by submerged fermentation of a selected, pure culture of *Aspergillus niger*. DSM produces the asparaginase preparations in spray-dried as well as liquid forms. The spray-dried forms are standardized with granulated wheat flour or maltodextrin and the liquid form is standardized with glycerol. The trade names will be PreventASe™ M and PreventASe™ W for the spray-dried forms containing maltodextrin and wheat flour, respectively, and PreventASe™ L for the liquid form.

The spray-dried and the liquid preparations are identical in enzyme identity, CAS number and production microorganism and intended use. Levels of use differ depending on the specific application. The manufacturing process for both forms is identical until the drying step.

These asparaginase preparations are for use in the food industry as a processing aid to reduce the levels in food of free L-asparagine, a main precursor in the formation of the food contaminant acrylamide during heat treatment. The discovery in 2002 that acrylamide is formed during heat treatment of certain foods raised concern because acrylamide is a probable human carcinogen (JECFA, 2005). At its 29th session, the Codex Alimentarius Commission therefore decided that a Code of Practice should be developed for the reduction of acrylamide in food (Joint FAO/WHO Food Standards Programme, 2006). The use of the enzyme asparaginase is one way to achieve such reduction.

In the presence of water, asparaginase catalyses the hydrolysis of the amino acid L-asparagine, giving rise to the formation of L-aspartate and ammonia. Most of the acrylamide in food heated above 120°C is formed by the reaction of L-asparagine with reducing sugars, both of which are found naturally in foodstuffs. Hydrolysis of L-asparagine by asparaginase before the heating step effectively reduces acrylamide formation. Asparaginase can thus be used in L-asparagine- and carbohydrate-containing foods that are heated above 120°C, such as bread and other baked cereal-based products, baked or fried potato-based products and reaction flavors to reduce the formation of acrylamide.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, DSM has determined that its asparaginase enzyme from GMO *A. niger* is a GRAS substance for the intended food applications and is therefore exempt from the requirement for premarket approval. Information on the enzyme and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme, its applicable conditions for use, DSM's basis for its GRAS determination and the availability of supporting information and reference materials for FDA's review can be found here in Section 1.

The production organism, *A. niger*, has a long history of safe use and is discussed in Section 2. FDA has previously affirmed as GRAS several enzyme preparations from *A. niger* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *A. niger* strains. The FDA recently summarized the safety of microorganisms, including *A. niger*, used as a host for enzyme-encoding genes (Olempska-Bier et al., 2006). In addition, a phospholipase A₂ preparation from a genetically modified *A. niger*

strain, derived from the same strain-lineage as the *A. niger* strain described in this dossier, has recently been notified as GRAS (GRN 000183).

Section 2 also describes the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from known harmful sequences, for asparaginase. In Section 3 data showing asparaginase to be substantially equivalent to naturally occurring asparaginase are presented. The safety studies outlined in Section 7 indicate that *A. niger* and asparaginase show no evidence of pathogenic or toxic substances. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

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

1.1 Name and Address of Notifier

NOTIFIER

DSM Food Specialties

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2600 MA Delft
The Netherlands

MANUFACTURER

DSM Food Specialties

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PERSON RESPONSIBLE FOR THE DOSSIER

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1.2 Common or Usual Name of Substance

DSM's asparaginase enzyme preparation from GMO *A. niger* is produced by submerged fermentation of a selected, pure culture of *A. niger*. The common or usual name of the substance is "asparaginase". It is produced and sold in spray-dried as well as liquid forms. The spray-dried forms are standardized with granulated wheat flour or maltodextrin and the liquid form is standardized with glycerol. The trade names will be PreventASe™ M and PreventASe™ W for the spray-dried forms containing maltodextrin and wheat flour, respectively, and PreventASe™ L for the liquid form.

1.3 Applicable Conditions of Use

The *A. niger* asparaginase preparation is to be used in foods that are heated above 120°C. The enzyme is added to the food before the heating step takes place. During heating, the enzyme will be completely inactivated by denaturation. The use of asparaginase can thus be regarded as a processing aid because it has no function in the finished foodstuff.

1.3.1 Substances Used In

The *A. niger* asparaginase preparation is to be used in L-asparagine- and carbohydrate-containing foods that are heated above 120°C, such as:

- bread (such as tin bread, buns and rolls, French sticks or batards, variety breads like multi grain types of bread, raisin bread, biscuits, crackers),
- other cereal-based products (cakes, Swiss rolls, Dutch honey cake, breakfast cereals),
- potato-based products (French fries, potato chips),
- reaction flavors

1.3.2 Levels of Use

Bread: The average dosage of the enzyme depends on the type and quality of the ingredients used, their asparagine content, the time length of and temperatures during the bread-making process, and the type of bakery product produced.

In the case of wheat flour, the levels of use may vary between 77 and maximal 385 ASPU/kg flour, with an average dosage of 150 ASPU/kg flour.

Cereal based products: In the case of corn masa flour, the levels of use may vary between 20 and 850 ASPU/kg, with an average dosage of 200 ASPU/kg.

Potato based products: In the case potato flour is used, the dosage may vary between 500 and 15,000 ASPU/kg, with an average dosage of 2000 ASPU/kg.

Reaction flavors: The levels of use of the enzyme in reaction flavors may vary between 4.7 and 6.2 ASPU/g, with an average dose asparaginase of 5.4 ASPU/g reaction flavor.

1.3.3 Purposes

The *A. niger* asparaginase preparation is intended to reduce the levels in food of free L-asparagine, a main precursor in the formation of the food contaminant acrylamide during heat treatment.

The discovery in 2002 that acrylamide is formed during heat treatment of certain foods raised concern because acrylamide is a probable human carcinogen (JECFA, 2005). At its 29th session, the Codex Alimentarius Commission therefore decided that a Code of Practice should be developed for the reduction of acrylamide in food (Joint FAO/WHO Food Standards Programme, 2006). The use of the enzyme asparaginase is one way to achieve such reduction.

The pathway that appears to account for most of the acrylamide in heat-treated food involves in particular a chemical reaction between the amino acid L-asparagine and reducing sugars, which are found naturally in foodstuffs. Removal or degradation of L-asparagine before heating can thus reduce the formation of acrylamide. Since asparaginase is able to degrade L-asparagine into L-aspartate and ammonia (see Section 3.4), the enzyme effectively reduces formation of acrylamide in heat-treated food.

1.3.4 Consumer Population

Asparaginase activity is abundantly present in nature as has been described in many publications during the last decades (Borek and Jaskólski, 2001; Pritsa and Kyriakidis, 2002; Kozak and Jurga, 2002; Wriston and Yellin, 1973). The production of asparaginase by microorganisms has already been described in 1972 (Arima et al., 1972). Asparaginase producing microorganisms include those used for the production of food and food ingredients such as *Bacillus subtilis* (Kunst et al., 1997) and bakers yeast *Saccharomyces cerevisiae* (Frederiksson et al., 2004). A GRAS notification for the use of asparaginase from *Aspergillus oryzae* in food has recently been submitted. In plants, the most studied asparaginases are from legumes where the enzyme is involved in metabolic pathways connected with the assimilation of atmospheric nitrogen (Chagas and Sodek, 2001). In animals, the enzyme has been described in chicken liver (Wriston and Yellin, 1973). Because asparaginase is an enzyme protein naturally occurring in microorganisms, animals and plants, DSM expects it will be digested, as would any other protein occurring in food.

L-asparaginase hydrolyzes L-asparagine to L-aspartate and ammonia. Both the substrate and the products of this enzymatic reaction play important roles in a number of metabolic processes in all organisms, from bacteria to mammals. As a result, asparagine, aspartate and ammonia (or its salts, 21 C.F.R. §§ 184.1133-184.1143) are quite abundant in the human diet. Hence, there is no basis to believe that conversion of asparagine to aspartate and ammonia will have a significant effect, if any, on processed foods or on the human body.

As is shown in Section 6.4 of this dossier, the amount of enzyme TOS in the final food is expected to be about 2 – 13 mg/kg (= 0.0002 – 0.0013%) for bread, 0.2 – 30 mg/kg (=0.00002 – 0.0030%) for cereal based products, 4 – 562 mg/kg (=0.0004 – 0.0562%) for potato based products, and 3-5 mg/kg (=0.0003-0.0005%) for savory ingredients.

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

1.4 Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, DSM has determined, through scientific procedures, that its asparaginase enzyme preparation from GMO *A. niger* is GRAS for use as an enzyme for the hydrolysis of asparagine in L-asparagine containing, carbohydrate-rich foods that are heated above 120°C, such as bread and other baked cereal-based products, baked or fried potato-based products and reaction flavors, in levels not to exceed good manufacturing practices.

1.5 Availability of Information for FDA Review

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

Gary L. Yingling, Esq.
Kirkpatrick & Lockhart, Nicholson Graham LLP
1601 K Street, NW
Washington, DC 20006-1600

2. PRODUCTION MICROORGANISM

2.1 Donor, Recipient Organism and Production Strain

Donor:

The gene coding for asparaginase was derived from *Aspergillus niger* strain GAM-8. This strain belongs to the same strain-lineage (i.e. the GAM lineage) as the recipient organism (see schematic presentation of the genealogy given under the heading *Production strain*).

Recipient organism

The recipient organism used in the construction of the asparaginase 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 suitable host strain for the construction of genetically modified organisms belonging to Group I safe microorganisms by the Dutch authorities.

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). 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 Dijck et al., 2003, included as Annex 2.1.1). One of these ISO-strains, ISO-502, was used for the construction of the production strain for phospholipase A2 (donor: porcine pancreas), an enzyme which has recently been notified as GRAS (GRN 000183). ISO-strains were also used for the construction of production strains for arabinofuranosidase (donor: *A. niger*), phytase (donor: *A. niger*), pectin methyl esterase (donor: *A. niger*), glucoamylase (donor: *A. niger*), xylanase (donor: *A. niger*), endo-polygalacturonase (donor: *A. niger*), proline specific endo-protease (donor: *A. niger*) and amylase (donor: *A. niger*).

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

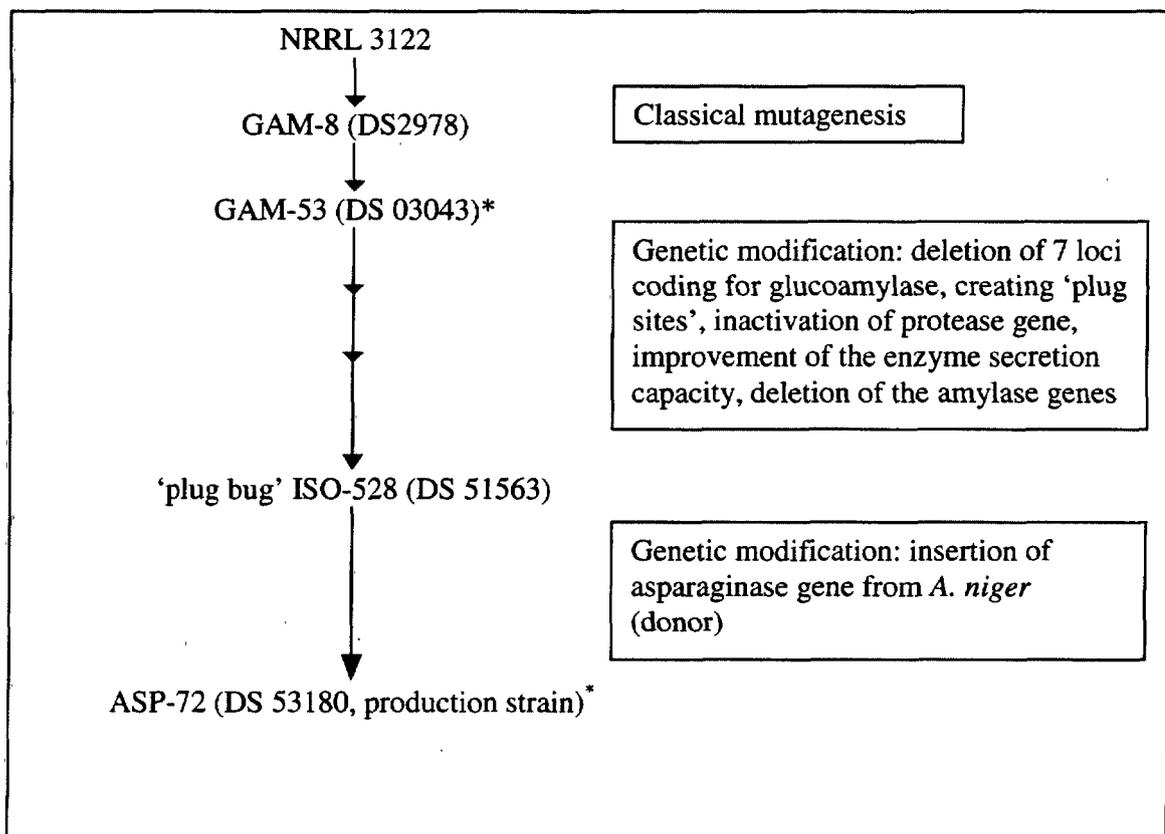
The strain GAM-53 contains 7 loci (i.e. the promoter and coding sequences) for the glucoamylase gene. These 7 loci were removed, creating so-called 'plug-sites' (also called $\Delta glaA$ loci) into which expression units containing various genes can be integrated ('plugged'). The 7 'plug-sites' were 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 selfcloned GMO by the Dutch competent authorities.

Production strain

The asparaginase production strain was obtained by further genetic modification of the *A. niger* strain ISO-528. The genetic modification techniques used are described in Section 2.2 of this dossier. The production strain was designated ASP-72 and stored in the DSM Culture Collection as DS 53180.

Below, a schematic presentation of the genealogy of the production strain is given. The donor strain GAM-8 has also been included, to show its relationship with the production strain.



* Strain GAM-53 and ASP-72 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 asparaginase production strain, two plasmids were used: one to derive the expression cassette, containing the asparaginase gene, and the other to derive the cassette containing a selectable marker.

Asparaginase expression plasmid

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

The different elements of the plasmid are:

- 1.5 kb DNA from the *glaA* promoter from the parental *A. niger* strain GAM-53.
- The entire 1254 bp sized genomic sequence encoding the asparaginase protein of *A. niger* GAM-8 from the ATG initiation codon to the ATG termination signal. The sequence of the *aspA* gene results in an asparaginase enzyme of 378 amino acids in size.
- 4.5 kb DNA from 3' flanking *glaA* terminator sequence from the parental *A. niger* strain GAM-53 for efficient termination of *aspA* gene transcription and targeting of the expression unit to the Δ *glaA* loci.
- DNA sequences from the *E. coli* plasmid pTZ18R. These sequences are removed prior to transformation of the asparaginase expression cassette into the host.

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

Selectable marker plasmid

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

The different elements of the plasmid are:

- 1.5 kb DNA from the *glaA* promoter from the parental *A. niger* strain GAM-53.
- 0.9 kb DNA from the *gpdA* promoter from *A. nidulans*.
- 2.2. kb DNA from the *amdS* gene from *A. nidulans*
- 4.5 kb DNA from the 3'-flanking *glaA* terminator sequence from the parental *A. niger* strain GAM-53.
- DNA sequences from the *E. coli* plasmid pTZ18R. These sequences are removed prior to transformation of the *amdS* selection cassette into the host.

The plasmid map is shown in Annex 2.2.3.

Transformation and selection of the final production strain

The asparaginase expression cassettes and the selection marker cassettes, both completely devoid of any *E. coli* DNA sequences, are integrated into the genome of the recipient organism ISO-528 by co-transformation following standard methodology. Due to the homology in the *glaA* promoter and 3'-*glaA* terminator parts of the two expression units, they are targeted to one of the seven Δ *glaA* loci.

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

By counter-selection on fluoro-acetamide containing plates, a natural variant of a transformant was selected in which the *amdS* selection marker was 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 marker.

Starting with such a natural variant it is possible to multiply the region comprising the expression unit(s) and the "DNA-flag" into the other Δ *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 Δ *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 sufficient gene copies to allow for commercial attractive expression levels of the asparaginase enzyme: this strain was designated ASP-72.

The nucleotide sequence of the final asparaginase expression unit as present in the ASP-72 production organism has been determined.

Translation of this *aspA* expression unit and subsequent secretion by the microbial cell into the fermentation broth results into a fully functional, biologically active, asparaginase protein of 378 amino acids.

2.3 Stability of the Transformed Genetic Sequence

The strains belonging to the *A. niger* GAM-lineage - from which the host ISO-528 and the recombinant asparaginase production strain is derived - are genetically stable strains. The whole GAM-lineage is stored at the DSM laboratory since 1976. New cultures are frequently derived from stock material and tested after many generations on morphological-, growth-, production- and product characteristics. These characteristics remain stable except that after plating out a low frequency of morphologic dissimilar colonies are found. This, however, is a normal phenomenon observed for the parental as well as the highly selected industrial strains. The stability of the ISO-strain and the asparaginase production strain does not differ from the parental GAM-strains.

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

2.4 Good Industrial Large Scale Practice (GILSP)

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

The host organism is non-pathogenic, does not produce adventitious agents and has an extended history of safe industrial use (see Section 7.1). The ancestor of the host, GAM-53 (see Section 2.1) has been shown to have a limited survival outside the optimal conditions of the industrial fermentor (see Annex 2.4.1). From the genetic modification performed, there are no reasons to believe that the survival of the genetically modified production organism would be different when compared to its ancestor. The DNA insert is fully characterized and is free from known harmful sequences. No antibiotic resistance markers or other heterologous markers are present in the strain. Both the host and its ancestor GAM-53 also have the ability to produce asparaginase, albeit in less efficiently than the asparaginase production strain.

Therefore, the asparaginase production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. This is the concept of Good Industrial Large Scale Practice (GILSP), as endorsed by the Organization of Economic Cooperation and Development (OECD). The production organism has been approved both by the Dutch and French competent authorities for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment. In the facilities of DSM Food Specialties for the large-scale production of food and feed enzyme products only fermentations are carried out not exceeding the GILSP level of physical containment.

2.5 Absence of transferable rDNA Sequences in the Enzyme Preparation

As explained above, the expression unit only contains host-own DNA and no *E. 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

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

2.7 Absence of Antibiotic Resistance Gene

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

2.8 Absence of Toxins

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

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

In the General Specifications for enzyme preparations laid down by JECFA in 2006, the following is said: "*Although nonpathogenic and nontoxic 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 asparaginase-producing microorganism possesses the intrinsic capacity to produce mycotoxins. This was tested under fermentation conditions, as well as conditions that induce toxin production. The test showed that the production strain does not produce any known toxins under these conditions. Test results are discussed in more detail in Section 7 of this dossier.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

- Systematic name : L-asparagine amidohydrolase
- Common name : Asparaginase
- Other names : asparaginase II; L-asparaginase; colaspase; elspar; leunase; crasnitin; α -asparaginase
- Enzyme Commission No. : 3.5.1.1
- CAS number : 9015-68-3

Asparaginase belongs to the subclass of enzymes that hydrolyze linear amides.

3.2 Amino Acid Sequence

Asparaginase from *Aspergillus niger* is a glycoprotein with a primary sequence of 378 amino acids and a calculated molecular weight of 39583 Da. Annex 3.2.1 shows the amino acid sequence of asparaginase from *Aspergillus niger*.

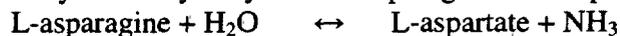
3.3 Sequence Comparison to Other Asparaginase Enzymes

In order to compare the asparaginase from *Aspergillus niger* with other presently known asparaginases, an extensive sequence comparison has been carried out. The amino acid sequence of the *Aspergillus niger* asparaginase was used as a query to search sequence databases using 'BLAST' software for related sequences. About 300 different sequences with significant homology were found. Clustering of the amino acid sequences based upon their homologous sequence patterns revealed that fungal and yeast asparaginases are very closely related. *Aspergillus niger* asparaginase belongs to the fungal cluster that also contains asparaginase from *Aspergillus oryzae* and *Aspergillus nidulans*. The cluster with yeast asparaginases contains the *Saccharomyces cerevisiae* (Bakers' Yeast) asparaginases I and II and *Kluyveromyces lactis* asparaginase.

3.4 Enzymatic Activity

Principal Enzyme Activity

Asparaginase catalyses the hydrolysis of L-asparagine to L-aspartic acid and ammonia:



The activity of asparaginase can be determined by measuring the release of ammonia using L-asparagine as substrate. From various existing ammonia detection methods, DSM uses the so-called Berthelot method. This method is based on a reaction of ammonia with phenol nitroprusside and alkaline hypochlorite, resulting in the formation of a blue color. The ammonia concentration is subsequently determined using an ammonium sulfate standard.

The activity of asparaginase is expressed in so-called ASPU (asparaginase units). One ASPU is defined as the amount of enzyme required to produce 1 micromole of ammonia from L-asparagine per minute under the conditions described for the asparaginase assay.

The biochemical properties of asparaginase from *A. niger* have been investigated extensively (see also Annex 3.4.1). The enzyme has shown to exhibit activity over a pH range between 4 and 8. The pH optimum is between pH 4 and 5. The temperature optimum for asparaginase activity is around 50°C. The enzyme is inactivated (denatured) at temperatures above 70 °C.

Subsidiary enzymatic activities

Like any other living organism, the asparaginase production organism produces many other enzymes needed for the breakdown of nutrients and build up of cell material. Although asparaginase 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 in the applications.

4. MANUFACTURING PROCESS

4.1 Overview

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

4.2 Raw Materials

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

4.2.1 Raw materials for the fermentation process

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

- Yeast extract, Dextrose
- Inorganic salts (potassium chloride, manganese sulfate, zinc sulfate, copper sulfate, magnesium sulfate, iron sulfate)
- Phosphoric acid, citric acid, sodium hydroxide, ammonium hydroxide
- Antifoam: ethoxylated propoxylated glycerol oleate (CAS 78041-14-2)¹
- Water

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

4.2.2 Raw materials for the recovery process

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

- Sodium benzoate
- Filter aid (Europerl E400C, dicalite BF)
- Granulating agents (maltodextrin, flour)
- Salt (CaCl₂)
- Phosphoric acid, ammonia
- Water

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

4.3 Fermentation Process

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

Pre-culture fermentation

Conserved mycelium of a pure culture of *Aspergillus niger* is aseptically transferred to a sterile fermentation medium. The pre-culture is grown for 32 hours at 30°C, after which it is used as inoculum for the seed fermentation.

Seed fermentation

Before the pre-culture is aseptically transferred to the seed fermentor, the fermentor containing the fermentation medium is sterilized. Growth of the microorganism takes place during approximately 45 hours at a constant temperature of 30°C, a fixed pH and dissolved oxygen concentration. At the end of the fermentation, the complete content of the fermentor is aseptically transferred as inoculum to the main fermentation.

Main fermentation

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

Before transfer of the inoculum, the fermentor and the fermentation medium are sterilized. Directly after inoculation till a few hours before the end of fermentation (duration approximately 72 hours), the fermentor is continuously fed with aseptically introduced sterilized fermentation medium. The medium flow rate is used to keep the residual sugar concentration in the broth between 10 and 40 g/kg, in order to prevent carbon limitation.

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

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

4.4 Recovery Process

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

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

4.5 Formulation and Standardization Process

In order to obtain a liquid enzyme preparation, the UF concentrate is standardized with glycerol to an average enzyme concentration of 2500 ASPU/ml, followed by a polish filtration and another germ reduction filtration.

In order to obtain a dry enzyme preparation, the UF concentrate subjected to a polish filtration, followed by another germ filtration. Subsequently, the UF concentrate is spray-dried and granulated with either maltodextrin or wheat flour and subsequently standardized to an activity of 10,000 ASPU/g with the same carrier.

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 (5th edition), the final asparaginase preparations from *Aspergillus niger* meet the following specifications:

<u>ITEM</u>	<u>NORM</u>
Lead	< 5 mg/kg
Antimicrobial activity	Absent by test
Coliforms	< 30/g
Salmonella	0/25g
Escherichia coli	0/25g
Anaerobe sulfite reducing bacteria	< 30/g
Staphylococcus aureus	0/g
Listeria monocytus	0/25g
Yeasts	<100/g
Moulds	<100/g
Total viable count	< 5*10 ⁴ /g

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

Liquid preparations:

<u>ITEM</u>	<u>NORM</u>
-------------	-------------

Appearance	Brown, clear liquid
Asparaginase activity	2375 – 2625 ASPU/ml
PH	4.3 – 4.7

Dry preparations:

<u>ITEM</u>	<u>NORM</u>
Appearance	Off white granulates
Asparaginase activity	9500 – 10500 ASPU/g*
Dry matter	> 90%
Particle size	63 < 90% < 224 µm

* activity depending on the formulation

5. COMPOSITION AND SPECIFICATIONS

5.1 Formulation

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

<u>Item</u>	<u>Value</u>	<u>Unit</u>
Enzyme activity	2700-5500	ASPU/g
Dry matter	13-19	%
Ash	0-1	%
Proteins (N x 6.25)	6 - 12	%

Apart from the enzyme complex, the asparaginase 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 asparaginase preparations were calculated from 3 different batches of the UF concentrate:

Calculation of the TOS					
Batch number	Water (%)	Ash (%)	TOS (%)	Activity (ASPU/g)	ASPU/g TOS
APE0503	86.7	0.8	12.5	2900	23200
APE0505	85.2	0.2	14.6	5460	37397
APE0602	85.7	0.3	14	2721	19436
MEAN	85.9	0.4	13.7	3694	26678

In the case of the dry enzyme preparations, the UF concentrate is spray-dried and formulated with either maltodextrins or granulated wheat flour (see also Section 4.5). In the case of the liquid, the UF concentrate is formulated with glycerol (see also Section 4.5).

The TOS values of the final standardized enzyme preparations can be easily calculated on basis of the above values, taking the dilution factor into account. For instance, a standardized enzyme preparation containing 2668 ASPU/g will have a TOS value of about 100 mg/g enzyme preparation.

5.2 General Production Controls and Specifications

Commercial demands require a strictly controlled fermentation process.

The enzyme fermentation factory at Seclin, France, which has fermentation experience 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 FCC specifications: see Section 4.7: Quality Control of Finished Product.

6. APPLICATION

6.1 Mode of Action

The enzyme asparaginase hydrolyses the amino acid L-asparagine naturally present in foodstuffs, resulting in the formation of ammonia and L-aspartate. The chemical reaction between L-asparagine and reducing sugars during heat treatment of foods is seen a major cause of the formation of the food contaminant acrylamide. Enzymatic hydrolysis of L-asparagine before the heating step therefore effectively reduces acrylamide formation. During heating, the enzyme will be completely inactivated by denaturation. The use of asparaginase can thus be regarded as a processing aid, having no function anymore in the finished foodstuff.

6.2 Application

6.2.1 Bread

Bread is usually prepared with wheat flour, but potato and corn flour may also be added. All these flours contain the main precursors for acrylamide, i.e. L-asparagine and carbohydrates. Although baker's yeast produces asparaginase (Frederiksson et al., 2004), the yeast fermentation of dough is usually too short for effective hydrolysis of L-asparagine. As a result, acrylamide is formed during baking (Mottram et al., 2002).

The results of laboratory scale bakery trials show that, depending on the type of bread and amount of enzyme added, the acrylamide formation in the bread crust can be reduced from 36% to 75% by the addition of asparaginase to the dough. The results of these trials are given in Annex 6.2.1.

6.2.2 Other cereal-based products

Apart from bread, cereals are used for many other baked products, such as crackers, cakes, cookies, Dutch honey cake and tortilla chips. The presence of L-asparagine and high sugar levels in many of these products results in acrylamide formation during the baking step.

The results of laboratory scale bakery trials show that the acrylamide formation in above-mentioned products can be effectively reduced with more than 80% by the addition of asparaginase before baking. The results of these trials are given in Annex 6.2.2.

6.2.3 Potato-based products

In potato products, very high levels of up to 2510 ppb acrylamide have been found (U.S. FDA, 2006). Also in this case, the L-asparagine and carbohydrates present in potato are responsible. Addition of asparaginase to potato doughs has shown to reduce the level of free L-asparagine more than 80%. The results of these trials are given in Annex 6.2.3.

6.2.4 Reaction flavors

Reaction flavors – also called 'thermal process flavors' – may be defined as 'products obtained after heat treatment from a mixture of ingredients not necessarily having flavoring properties

themselves, of which at least one contains nitrogen (amino) and another is a reducing sugar'. Reducing sugars, which are typically used in reaction flavors, include ribose, xylose, arabinose, glucose, fructose, lactose and sucrose. Meat extracts, hydrolyzed vegetable proteins (HVPs) and yeast extracts are excellent sources of free amino acids and peptides for use as nitrogen (amino) precursors for reaction flavors. As these precursors also contain the amino acid L-asparagine, heat-treatment in the presence of reducing sugars will consequently lead to the formation of acrylamide.

The results of laboratory scale trials show that the treatment of yeast extract with asparaginase results in complete removal of L-asparagine in the yeast extract. Moreover, the acrylamide formation in yeast extract-based reaction flavors can be effectively reduced with more than 70% by the treatment of yeast extract with asparaginase. The results of these trials are given in Annex 6.2.4.

6.3 Use Levels

Enzyme preparations are generally 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. Section 1.3.2 describes the levels of use expected to result in beneficial effect.

6.4 Enzyme Residues in the Final Food

6.4.1 Residues of inactive enzyme in various applications

In all applications, the action of asparaginase takes place before the heating step of the food. As mentioned in Section 3.4, the enzyme is inactivated (denatured) at temperatures above 70°C. Because all intended applications involve heating above 120°C no enzyme activity is expected to remain in the finished product.

To verify this assumption, experiments were conducted to measure the level of asparaginase in baked bread. The results of these experiments, as given in Annex 6.4.1, show that no asparaginase activity is present anymore in the finished product.

Based on the information given in Sections 1.3.2 and 5.1, the following calculation can be made:

Final food	Enzyme use level in food ingredient	Amount of ingredient in final food	Residual amount of (denatured) enzyme in final food	Amount of TOS in final food
Bread ¹	77-385 ASPU/kg flour	67-91%	52-350 ASPU/kg bread	2-13 mg/kg
Cereal based products ²	20-850 ASPU/kg flour	25-95%	5-808 ASPU/kg	0.2-30 mg/kg
Potato based products	500-15,000 ASPU/kg flour	20-100%	100-15000 ASPU/kg	4-562 mg/kg
Savory ingredients ³	4700-6200 ASPU/kg reaction flavor	≤ 2%	94-124 ASPU/kg	3-5 mg/kg

¹ Includes 3 categories: 1) Yeast bread and rolls, 2) Quick breads, pancakes and French toast, and 3) Cakes, cookies, pastries and pies.

² Crackers, popcorn, pretzels and corn chips.

³ Includes soups and bouillon, sauces and gravy, mixed dishes, processed cheese.

6.4.2 Possible Effects on Nutrients

L-asparaginase hydrolyzes L-asparagine to L-aspartate and ammonia. Both the substrate and the products of this enzymatic reaction play important roles in a number of metabolic processes in all organisms, from bacteria to plants and mammals.

As a result, asparagine, aspartate and ammonia (or its salts) are quite abundant in the human diet. Hence, there is no basis to believe that conversion of asparagine to aspartate and ammonia will have a significant effect, if any, on processed foods or on the human body.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the IFBC, food or food ingredients are safe to consume if they have been produced, according to current Good Manufacturing Practices, from a nontoxigenic and nonpathogenic organism (IFBC, 1990). A nontoxigenic organism is defined as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances" (Pariza and Foster, 1983).

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

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

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

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

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

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

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

Strains belonging to the *Aspergillus niger* GAM-lineage as well as the host (recipient) strain ISO-528 from DSM were declared suitable host strains for the construction of genetically modified organisms belonging to Group I safe microorganisms by the Dutch authorities.

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

The recombinant asparaginase production strain ASP-72 (DS53180) has been classified by the Dutch authorities as well as the French Genetic Committee as a Group I safe microorganism. Consequently, the strain was approved for large-scale production of asparaginase in the DSM factory in Seclin, France. In addition it was recognized in both the Netherlands as well as in France as a genetically modified microorganism obtained through self-cloning.

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

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

Specific tests have been performed to confirm that the recombinant asparaginase production strain is not able to produce any toxins under fermentation conditions, nor under conditions that are known to induce toxin production in general. The results of these tests showed that the production strain does not produce any known toxins under these conditions.

7.2 Safety of the Asparaginase 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 *A. niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132), and lipase (GRN 000111 and GRN 000158). The FDA recently summarized the safety of microorganisms, including *A. niger*, used as a host for enzyme-encoding genes (Olempska-Beer et al., 2006). Even more recently, a phospholipase A₂ preparation from a genetically modified *A. niger* strain, derived from the same strain-lineage as the *A. niger* strain described in this dossier, has been notified as GRAS (GRN 000183).

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 asparaginase is a natural constituent of many organisms, including microorganisms, plants and animals used for food (see Section 6.4), it is not expected that asparaginase would have any toxic properties.

The enzyme preparation asparaginase derived from *Aspergillus niger* strain ASP-72, over-expressing the asparaginase gene from *Aspergillus niger*, was evaluated according the Pariza & Johnson Decision Tree. The decision tree is based on the safety evaluation methodology published by Pariza and Foster in a 1983 article, which was extended by the IFBC into the decision tree format and published in 1991. In 2001, Pariza and Johnson published an update. DSM's decision tree analysis, based on the most recent update of the decision tree, is described in Annex 7.2.

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

- Subchronic (90-day) oral toxicity study,
- Prenatal development toxicity study,
- Ames test,
- Chromosomal aberration, *in vitro*,
- Mouse lymphoma assay, *in vitro*.

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

7.2.1 Allergenicity

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens. Below we describe briefly why ingestion of enzymes used as food processing aids is unlikely to elicit an allergic response after consumption.

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

The unique role of enzymes in food processing is as a catalyst. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a

food. The use levels are based on the activity of the enzyme, not the amount of the enzyme product. Under these circumstances, the enzyme used is classified as an incidental additive, and where it has no functional effect on the finished food and is present in an insignificant amount, it need not be included in the ingredient label.

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

7.2.2 Leading Enzyme Publications on the Safety of Asparaginase Enzymes or Enzymes that are Closely Related

The safety of the production organism is the point of focus as to the safety of the enzyme which will be used in food processing. In this case, the production organism *A. niger*, has been demonstrated to be nontoxigenic and nonpathogenic and any food ingredient (enzyme) from that organism will exhibit the same safety properties if manufactured under current Good Manufacturing Practices ("cGMPs"). Pariza and Foster (1983) noted that a nonpathogenic organism was very unlikely to produce a disease under ordinary circumstances. In their publication, *A. niger* is included in the authors' listing of the organisms being used in the industry.

The FDA has also accepted the Enzyme Technical Association's GRAS Notifications stating that carbohydrase, pectinase, protease, glucose oxidase and catalase (GRN 000089), lipase (GRN. 000111 and GRN 000158), lactase (GRN 000132) and phospholipase A2 (GRN 000183) enzyme preparations from *A. niger* are generally recognized as safe. *A. niger* is listed as a production organism for enzymes (Pariza and Johnson, 2001) and has a long history of safe use (see Annex 7.1.1).

As is clear from the information provided in this notification, there have been genetic modifications to the *A. niger* used by DSM, but these genetic modifications are thoroughly well characterized and specific in that the DNA encoded is host-own and 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.

The activity of asparaginase is very comparable with the one of glutaminase (see Section 7.2.4), an enzyme that was included in the safety evaluation by Pariza and Johnson (2001). Moreover, asparaginase has shown to be produced by the bakers yeast and GRAS organism *Saccharomyces cerevisiae* during dough fermentation (Fredriksson et al., 2004). Prolonged fermentation of dough has shown to reduce the free asparagine content (Collar et al., 1991).

LD50s are available of asparaginase from *Escherichia coli* or *Erwinia carotovora*, showing oral toxicity (LD50 >5000-7500 kU/kg bw) of at least a factor 10 less than parental toxicity (intravenous) (LD50 50-750 kU/kg bw) (Ohguro et al., 1969; Lorke and Tettenborn, 1970). The rabbit seems to be exceptionally sensitive with a LD50 (iv) of 286 U/kg bw. Effects observed in subchronic toxicity studies in rats, dogs, rabbits and guinea pigs mirror the effects observed in human, i.e. reduction of weight gain and food consumption, nausea, diarrhea and hypersensitivity, hematological effects and changes in some blood chemical parameters. In addition, fatty deposition in liver cells was observed (Ohguro et al., 1969). Several poorly reported prenatal development toxicity studies have been performed (Ohguro et al., 1969; Lorke and Tettenborn, 1970; Adamson and Fabro, 1968; Adamson et al., 1970). Rats and mice were administered intravenously or intraperitoneal with asparaginase at different periods during pregnancy. Observed effects include increased number of resorptions, reduced fetal weight, brain hernius, skeletal malformations, microphthalmia and retardation of ossification. However, all effects were observed in the presence of maternal toxicity (reduced weight gain). Asparaginase dosed intravenously at gestation day 7 and 8 at levels of 100 and 1000 U/kg bw in rats did not show any maternal toxic or fetotoxic effect.

When evaluating the relevance of the above studies for the intended application in food, the following facts have to be taken into account:

First, the route of administration in the studies described in literature is mainly parenteral as this is the route used in therapeutics, whereas the intended application is food. In general, toxic effects are less in case of oral exposure compared to parenteral administration due to decreased bioavailability and chemical interactions (deactivation) in the stomach.

Second, the enzyme is inactivated in the intended food applications, resulting in a normal protein (see 6.4.4), whereas for therapeutic use the enzyme is active. The therapeutical use is based on depletion of asparagine. Although asparagine is not an essential amino acid it was observed that certain tumors are dependent on an external source of asparagine for viability. The high requirement for asparagine in fast growing tissues like an embryo is probably also the ground for the embryotoxic properties of intravenously administered asparaginase. Again, users of asparaginase treated food products are only exposed to inactive enzyme via the oral route. Thus, effects that are observed in studies with asparaginase of *Escherichia coli* and *Erwinia carotovora* administered intravenously are not to be expected after oral consumption of processed food treated with asparaginase from *Aspergillus niger*.

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 noted 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.

DSM has shown that asparaginase (EC 3.5.1.1) and glutaminase (EC 3.5.1.2) are substantially equivalent:

1. The similarity of the chemical conversions is quite obvious: asparagine and glutamine are very similar molecules, only differing in the length of the side chain (by one carbon atom). Asparagine and glutamine are abundant and universally occurring amino acids.
2. The chemical conversion is quite simple: hydrolysis of the amide side chain, to generate the acid side chain and free ammonia. The only reagents are the respective amino acids (asparagine or glutamine) and water.
3. Many (if not all) glutaminases will also have some asparaginase activity and vice versa. The extent to which they hydrolyze their preferred substrate compared to the non-preferred ones varies widely. Some enzymes even show so little preference, that they are termed glutaminase-asparaginase (EC 3.5.1.38). For instance, an enzyme from *Pseudomonas* is described in an article by Davidson et al. (1977). This enzyme has a hydrolysis ratio of asparagine to glutamine of 1 : 1.45, and similar (and low) K_m values for both substrates.
4. Asparagine/aspartate and glutamine/glutamate are metabolically closely connected: they all are linked to the TCA cycle, which is a universal pathway in respiratory organisms, including moulds (where DSM's enzyme originates from) and humans. Consequently, in humans, none of these four amino acids is an essential nutrient, due to their easy synthesis from central metabolites. They are, however, quite abundant in the human diet. The overall picture is that humans are always exposed to these four amino acids, and are fully capable of converting one into the other. Hence, there is no basis to believe that conversion of asparagine to aspartate will have significantly different effects on the human body compared to the conversion of glutamine to glutamate.

7.3 Safety of the Manufacturing Process

Asparaginase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 4, the asparaginase 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 asparaginase preparation.

7.4.1. Safety Studies in Summary

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

The safety studies have been performed on batch APE0501 –referred to as “tox-batch” (containing 4,160 SPU/g and 11.8 %TOS). The strain used to produce this batch was a predecessor of the current production strain, i.e., the gene coding for asparaginase was integrated in a random matter. The relationship with the current production strain is shown in Annex 7.4.1.

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

1 90-days oral toxicity

The sub-chronic oral toxicity of the tox-batch was examined in a 90-day toxicity study with groups of 10 male and 10 female Wistar rats. The rats received daily the tox-batch by gavage. The control group received the vehicle (water) alone. In addition, one group of rats received a heat-inactivated form of tox-batch at a dose level 2300 mg/kg bw/day. Clinical signs, body weight, pre-terminal deaths, food intake, hematology and clinical biochemistry parameters, functional performance, organ weight, macroscopy and histopathology of the organs were studied.

Results

As no treatment-related changes were observed in the above-mentioned parameters, for the active as well as the heat-inactivated form of the tox-batch, a No Observed Adverse Effect Level (“NOAEL”) of 2300 mg/kg bw/day could be established.

2 Prenatal developmental toxicity

The teratogenic, embryotoxic and/or fetotoxic potential of the tox-batch was examined in a prenatal developmental toxicity study in Wistar rats. The rats received the tox-batch by gavage from gestation day 6 to 19. The control group received only the vehicle (i.e. distilled water). Parameters that were studied included number of pregnant animals, clinical signs, feed intake, weight gain, number of resorptions, live and dead fetuses, fetal weight, sex ratio and fetal external, visceral and skeletal changes.

Results

No treatment related effects were observed in the above mentioned parameters up to a level of 8500 mg/kg bw/day.

3 Mutagenicity tests

(a) AMES test

The mutagenic potency of the tox-batch was studied in four histidine-dependent *Salmonella typhimurium* mutant strains TA 98, TA 100, TA 1535 and TA 1537 and one tryptophan-dependent *Escherichia coli* mutant WP2uvrA strain. Experiments were performed in the absence and presence of S9-mix, a rat liver-derived metabolic activation system.

Tester bacteria were exposed to five concentrations ranging from 62 to 5000 µg dry matter/plate in the absence and presence). Negative (i.e. milli-Q water) and positive controls were run simultaneously with the test substance.

Results

The tox-batch was slightly cytotoxic to strain TA 1537 at 5000 µg/plate, in the absence of S9-mix, as was evidenced by a decrease in the number of revertants and a slightly less dense background lawn compared to the negative control. The tox-batch did not induce a dose-related or more than two-fold increase in the number of revertant colonies in any of the five tester strains both in the absence and presence of S9-mix as compared to the negative control. The positive control substances gave the expected strong increase in the number of revertants.

Based on the results of this study it is concluded that the tox-batch is not mutagenic in the *Salmonella typhimurium* reverse mutation assay nor the *Escherichia Coli* reverse mutation assay.

(b) Chromosomal aberration test, in vitro

The tox-batch was examined for its potential of induction of chromosomal aberrations in cultured human peripheral lymphocytes in the presence and absence of S9-mix. Two independent chromosomal aberration experiments were conducted. For both chromosomal tests, the same donor was used. Negative (i.e. RPMI-1640 medium) and positive controls were run simultaneously with the test substance.

(i) Experiment 1

In both the absence and presence of S9-mix, the tox-batch was incubated with the cells during 4 hours (pulse treatment) in concentrations 1250-5000 µg/ml. The harvesting time was 24 hours.

(ii) Experiment 2

In the presence of S9-mix, the tox-batch was incubated with the cells during 4 hours (pulse treatment). In the absence of S9-mix, the tox-batch was incubated with the cells during 24 hours (continuous treatment). In both cases, the harvesting time was 24 hours. In the pulse-treatment group three concentrations were tested, i.e. 3000, 4000 and 5000

µg/ml. While in the continuous-treatment group the concentrations 1000, 1500 and 2000 µg/ml were tested for chromosomal aberrations.

Results

In the first experiment, the group treated with S9-mix showed a reduction in mitotic indices at all analyzed concentrations, compared to the control treated group. Although the tox-batch was cytotoxic for the cells, an increase in the number of aberrant cells at any of the concentrations was not observed. Without S9-mix the tox-batch was not toxic and did not induce a reduction in mitotic index or induced an increase in the number of aberrant cells.

In the 2nd experiment, the tox-batch reduced the mitotic indices and was toxic both in the absence and presence of S9-mix. However, an increase in the number of cells with chromosome aberrations in any of the concentrations and time points analyzed was not observed. In both experiments the negative control values were within the historical range. The positive control substances gave the expected statistically significant increase in the incidence of structural chromosomal aberrations.

Based on the results of this study it is concluded that the tox-batch is cytotoxic, but not clastogenic for cultured human lymphocytes.

(c) Gene mutation test at the TK-locus of L5178Y cells (Mouse Lymphoma Assay)

The tox-batch was examined for its potential to induce gene mutations at the Thymidine Kinase (TK)-locus of cultured mouse lymphoma L5178Y cells. This experiment was conducted in the presence and absence of S9-mix. Three independent experiments were conducted in which the potential mutagenicity of the tox-batch was evaluated after 4 and 24 hours exposure in the absence of S9-mix and after 4 hours in the presence of S9-mix. Negative (i.e. RPMI-1640 medium) and positive controls were run simultaneously with the test substance.

In the absence of S9-mix, the highest concentration tested was 1200 and 30 µg/ml after 4 and 24 hours treatment, respectively. In the presence of S9-mix the highest concentration tested was 2450 µg/ml.

Results

In the presence and absence of S9-mix, the tox-batch was cytotoxic to the cells. In all assays, the relative total growth (RTG) at the highest concentration tested for mutagenicity was between 10 and 20%. In addition, in both the presence and absence of S9-mix no increase of the mutant frequency was observed at any dose level causing less than 90% cytotoxicity, i.e. RTG>10%. This means that there is no indication for a mutagenic potential.

Conclusion

Based on the results of this study it is concluded that the tox-batch is cytotoxic, but not mutagenic at the TK-locus of mouse lymphoma L5178 cells.

7.5 Estimates of Human Consumption and Safety Margin

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

Final food	Residual amount of (inactive) enzyme in final food (ASPU/kg)	90 th percentile intake level (g food/per person/day) ¹	Estimated daily intake of (inactive) enzyme (ASPU/kg bw/day) ²	Estimated daily intake of TOS (mg/kg bw/day)
Bread ³	52-350	260	0.2-1.5	0.007-0.056
Cereal based products ⁴	5-808	28	0.002-0.38	0-0.014
Potato based products	100-15000	58	0.1-14.5	0.004-0.537
Savory ingredients ⁵	94-124	540	0.9-1.1	0.033-0.041
Total			1.2-17.5	0.044-0.648

¹ Intake levels of bread, cereal based products and potato based products are based on Wilkinson-Enns et al. (1997). Intake levels of savory ingredients are taken from 21 C.F.R. § 101.12. 90th percentile is 2 times the intake level (DiNovi and Kuznesof, 1995).

² Calculated for a person of 60 kg.

³ Includes 3 categories: 1) Yeast bread and rolls, 2) Quick breads, pancakes and French toast, and 3) Cakes, cookies, pastries and pies.

⁴ Crackers, popcorn, pretzels and corn chips.

⁵ Includes soups and bouillon, sauces and gravy, mixed dishes, processed cheese.

The 90-day oral toxicity study showed a NOAEL of 2300 mg enzyme preparation/kg bw/day, corresponding to 271 mg TOS/kg bw/day. Thus the Margin of Safety lies between $271/0.044=6159$ and $271/0.648=418$.

7.6 Results and Conclusion

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

8. LIST OF ANNEXES

- 2.1.1 Dijck, P.W.M. van, Selten, G.C.M., Hempenius, R.A., On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains, *Regulat. Toxicol. Pharmacol.* 38:27-35 (2003)
- 2.2.1 Figure of the asparaginase expression plasmid, the vector pGBTOPASP-1
- 2.2.2 Nucleotide sequence of the expression unit
- 2.2.3 Map of the selectable marker plasmid
- 2.4.1 Surviving studies of *Aspergillus niger* strains in soil, surface water, and waste water
- 3.2.1 Amino acid sequence of asparaginase from *Aspergillus niger*
- 3.4.1 Biochemical characterization of asparaginase from *Aspergillus niger*
- 4.1.1 Flow diagram of manufacturing process
- 6.2.1 Acrylamide reduction by the use of asparaginase from *Aspergillus niger* in bread doughs – results of laboratory scale experiments
- 6.2.2 Acrylamide reduction by the use of asparaginase from *Aspergillus niger* in doughs for cereal based fine bakery wares – results from laboratory scale experiments
- 6.2.3 Asparagine reduction by the use of asparaginase from *Aspergillus niger* in potato based doughs – results of laboratory scale experiments
- 6.2.4 Asparagine and Acrylamide reduction by the use of asparaginase from *Aspergillus niger* in yeast extracts applications – results of laboratory scale experiments
- 6.4.1 Inactivation of asparaginase – results of laboratory experiments
- 7.1.1 Schuster, E., Dunn-Coleman, N., Frisvad, J.C., Dijck, P.W.M van, *On the Safety of Aspergillus niger – a Review*, *Appl. Microbiol. Biotechnol.* 59:426-435 (2002).
- 7.1.2 JECFA safety evaluation of *A. niger* as a source of enzymes to be used in food, 1987
- 7.1.3 Expert reports submitted to JECFA by Professor J.W. Bennett and Dr. M.O. Moss on the probability of mycotoxins being present in industrial enzyme preparations obtained from fungi (September 1988)
- 7.1.4 JECFA reconsideration of safety of *A. niger* as a source for enzymes to be used in food, 1990
- 7.2 Safety evaluation using the Pariza & Johnson decision tree of asparaginase from *Aspergillus niger* ASP-72
- 7.2.1 Report from the Amfep Working Group on Consumer Allergy: risk from enzyme residues in food (August 1998)
- 7.4.1 Relationship between current production strain and the one used for production of the tox-batch

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2.1.1

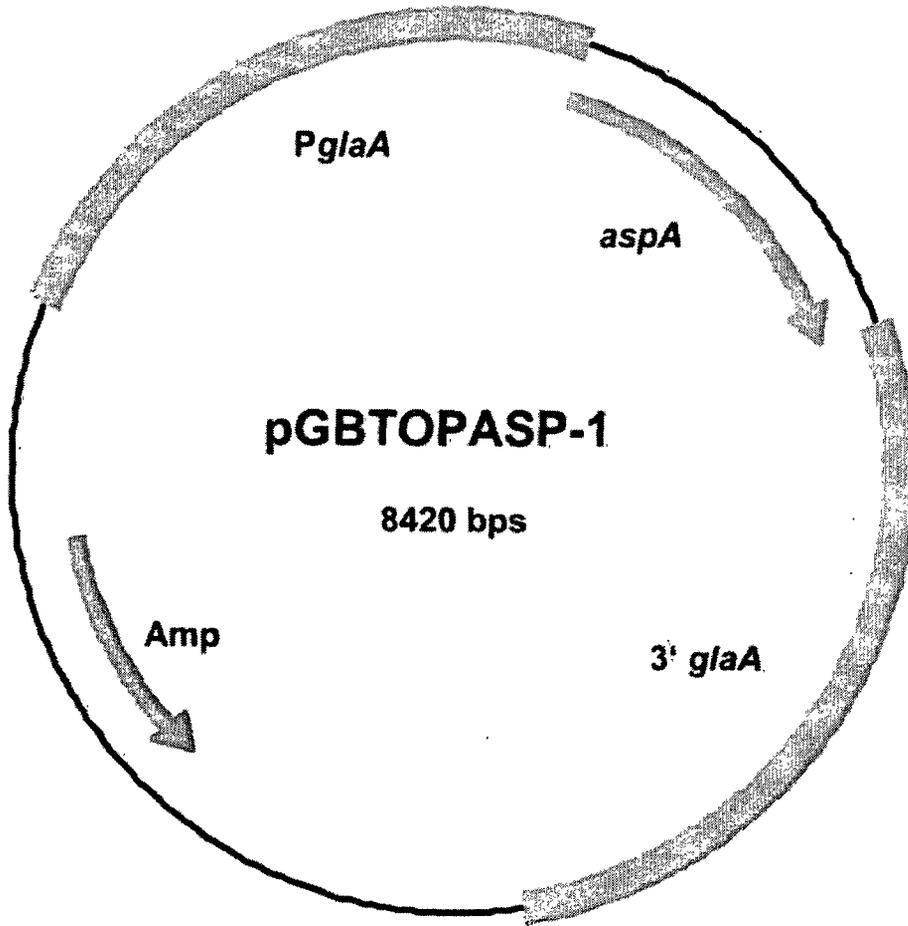
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Pages 000044 - 000052 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

2.2.1

000053

Figure of the asparaginase expression plasmid, the vector pGBTOPASP-1



Physical map of plasmid pGBTOPASP-1. The green arrow represents the *aspA* gene, the expression of which is under control of the *glaA* promoter; the flanking homologies required for targeted integration of the *aspA* expression cassette are shown in blue. The *E. coli* sequences (solid black line) are derived from pTZ19R.

2.2.2

000055

Nucleotide sequence of the expression unit

```

1  ATGCCTCTCA AGCCGATTCT CCTGTCTGCC CTGGCCAGTC TCGCCTCGGC CTCTCCGCTG
61 CTCTACTCGC GGACCACCAA TGAAACCTTC GTCTTCACCA ATGCCAATGG CCTCAACTTC
121 ACCCAGATGA ACACCACCCT GCCGAACGTG ACCATTTTCG CAACGGGTAG GTGGACCGAG
181 TATACCTCAG GTAGTGGCAG CGATAGTTAA CCGCAACTCA CAGGTGGTAC CATCGCGGGC
241 TCCGATTCCA GCTCAACCGC CACGACCGGC TACACCTCCG GAGCAGTCGG GGTCTGTCC
301 CTCATCGATG CCGTGCCATC CATGCTGGAT GTGGCCAATG TTGCCGGCGT CCAGGTGCCC
361 AACGTGGGAA GCGAGGATAT CACCTCTGAC ATCCTGATTT CCATGTCCAA GAAGCTGAAC
421 CGCGTTGTAT GTSAGGACCC SACCATGGCC GGTGCTGTCA TCACCCACGG CACCGACACC
481 CTCGAGGAGA CTGCCTTCTT CCTGGACGCC ACTGTCAACT GTGGCAAGCC AATTGTCATC
541 GTGGGTGCCA TCGGCCCATC CACGGCCATC TCAGCTGACG GGCCTTCAA TCTGCTCGAA
601 GCCGTGACCG TGGCTGCCTC CACGTCGGCG CGCGATCGCG GTGCCATGGT GGTCATGAAC
661 GATCCGATTG CCTCGGCCA CTATGTGACC AAGACCAATG CCAACACTAT GGACACCTTC
721 AAGGCCATGG AGATGGGCTA CCTTGGCGAG ATGATCTCCA ACACCCCTTT CTTCTTCTAC
781 CCGCCCGTCA AGCCAACCGG TAAGGTGGCC TTTGACATCA CCAACGTGAC TGAGATCCCC
841 CGTGTGGACA TTCTGTTTTT TTATGAGGAC ATGCACAACG ACACCTCTA CAACGCCATC
901 TCCAGTGGTG CCCAGGGAAT TGTGGTGAGT GTGATTTCTT TGATCTCTCT CTATAAAACT
961 TGGAAATGGAC GCTGATGAGA ATAGATTGCC GGGGCTGGTG CTGGAGGCGT CACAACCTCC
1021 TTCAATGAGG CTATCGAGGA TGTCATCAAC CGTTTGGAGA TCCCTGTCGT GCAGAGTATG
1081 CGCACAGTCA ATGGGGAAGT GCCACTGTCA GACGTGAGCA GCGACACCGC CACCCACATC
1141 GCCAGTGGAT ACCTAAACCC GCAGAAGTCC CGCATTCTGT TGGGATTGCT GCTATCCCAG
1201 GGAAAGAATA TCACCCAAAT CGCTGACGTG TTTGCTCTGG GCACGGATGC GTAG

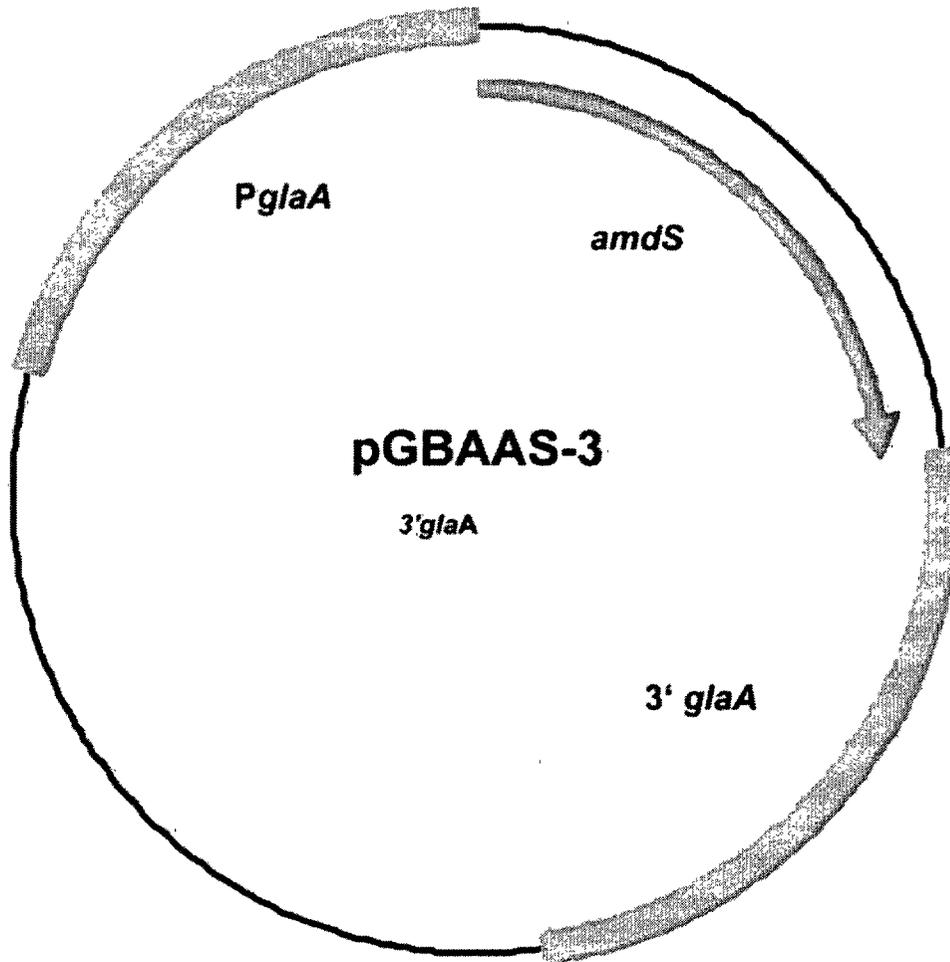
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Nucleotide sequence of the *aspA* gene. The entire open reading frame of the asparaginase-encoding gene from the ATG start codon (underlined at position 1) till the stop codon (TAG underlined at position 1252) is indicated. The introns are shown in blue.

2.2.3

000057

Map of the selectable marker plasmid



Physical map of pGBAAS-3. The *Aspergillus nidulans amdS* gene, shown in red, is functionally expressed in *A. niger* from the *glaA* promoter. The flanking homologies required for targeted integration of the *aspA* expression cassette are shown in blue. The *E. coli* sequences (solid black line) are derived from pTZ18R.

2.4.1

000059

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.

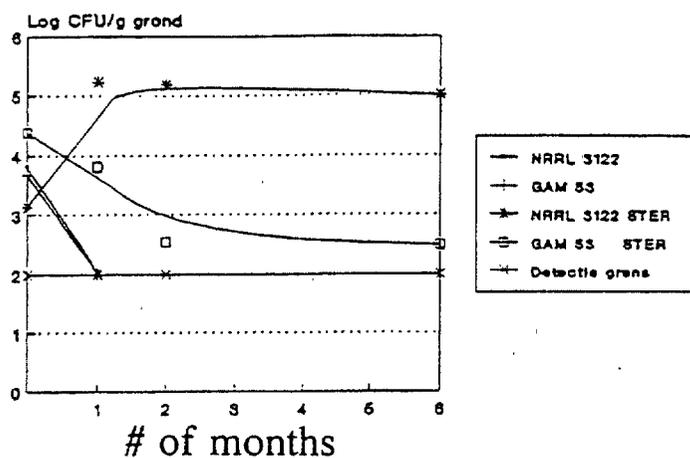
In all sterile environments at 25°C inoculated with *A. niger* spores, having a titre between 10^4 - 10^5 , colony forming units/gram sample were found. At 8°C the wild-type strain was surviving well in soil as well as in surface water, whereas in waste water it was decreased beneath the detection level.

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.

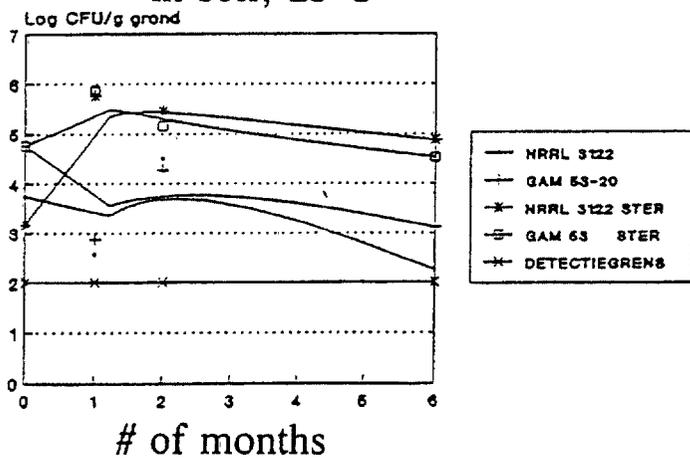
In natural environments and in the presence of competitive micro-organisms surviving *Aspergillus niger* strains were found after 6 month only in soil samples at 25°C, inoculated with at least 10^3 spores/g. In both aqueous environments at 8°C and 25°C and in soil of 8°C a fast decline of surviving strains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

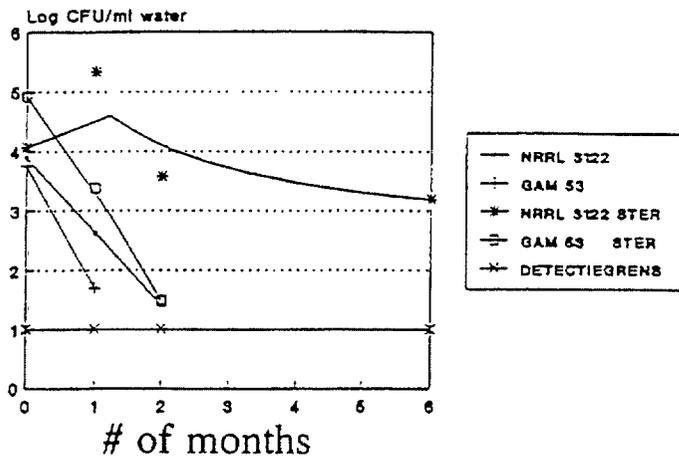


Incubation of *A. niger* mycelium
in soil, 25°C

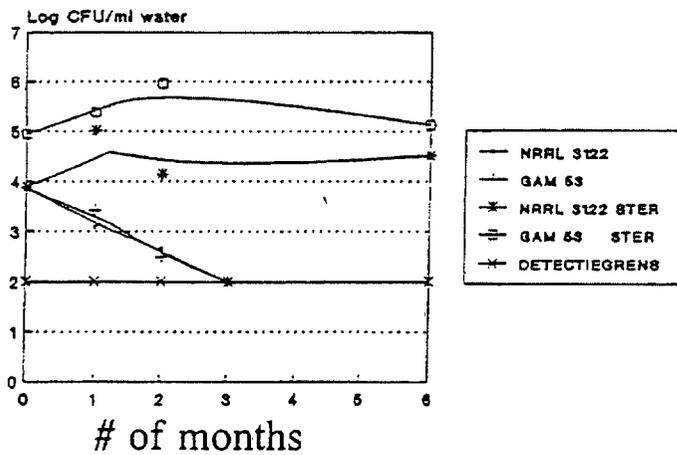


Surviving of *Aspergillus niger* I

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

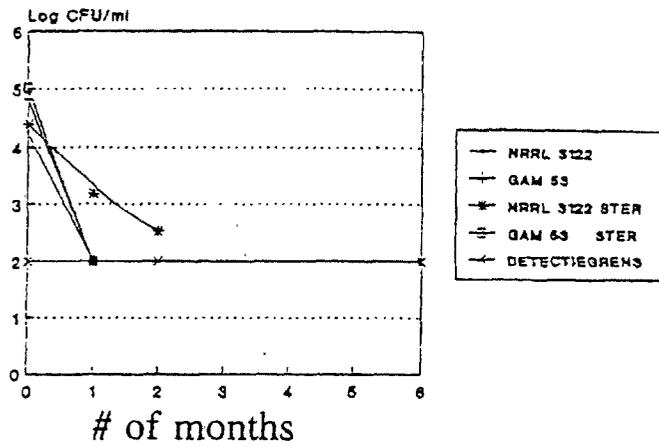


Incubation of *A. niger* mycelium
in surface water, 25°C

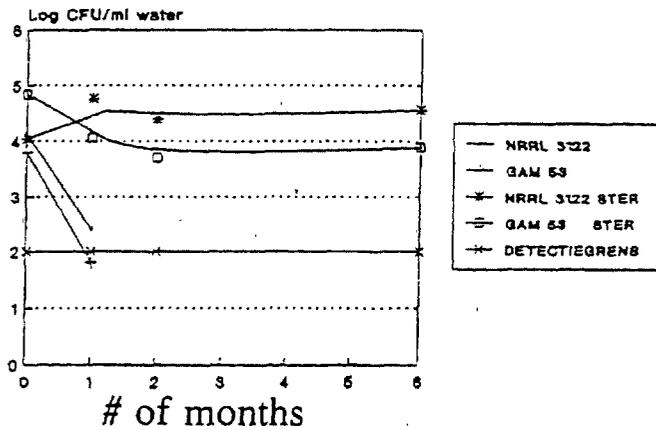


Surviving of *Aspergillus niger* II

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



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



Surviving of *Aspergillus niger* III

000063

3.2.1

000064

Amino acid sequence of asparaginase of *Aspergillus niger*

Met-Pro-Leu-Lys-Pro-Ile-Leu-Leu-Ser-Ala-Leu-Ala-Ser-Leu-Ala-Ser-Ala-Ser-Pro-Leu-
Leu-Tyr-Ser-Arg-Thr-Thr-Asn-Glu-Thr-Phe-Val-Phe-Thr-Asn-Ala-Asn-Gly-Leu-Asn-Phe-
Thr-Gln-Met-Asn-Thr-Thr-Leu-Pro-Asn-Val-Thr-Ile-Phe-Ala-Thr-Gly-Gly-Thr-Ile-Ala-Gly-
Ser-Asp-Ser-Ser-Ser-Thr-Ala-Thr-Thr-Gly-Tyr-Thr-Ser-Gly-Ala-Val-Gly-Val-Leu-Ser-Leu-
Ile-Asp-Ala-Val-Pro-Ser-Met-Leu-Asp-Val-Ala-Asn-Val-Ala-Gly-Val-Gln-Val-Ala-Asn-Val-
Gly-Ser-Glu-Asp-Ile-Thr-Ser-Asp-Ile-Leu-Ile-Ser-Met-Ser-Lys-Lys-Leu-Asn-Arg-Val-Val-
Cys-Glu-Asp-Pro-Thr-Met-Ala-Gly-Ala-Val-Ile-Thr-His-Gly-Thr-Asp-Thr-Leu-Glu-Glu-Thr-
Ala-Phe-Phe-Leu-Asp-Ala-Thr-Val-Asn-Cys-Gly-Lys-Pro-Ile-Val-Ile-Val-Gly-Ala-Met-Arg-
Pro-Ser-Thr-Ala-Ile-Ser-Ala-Asp-Gly-Pro-Phe-Asn-Leu-Leu-Glu-Ala-Val-Thr-Val-Ala-Ala-
Ser-Thr-Ser-Ala-Arg-Asp-Arg-Gly-Ala-Met-Val-Val-Met-Asn-Asp-Arg-Ile-Ala-Ser-Ala-Tyr-
Tyr-Val-Thr-Lys-Thr-Asn-Ala-Asn-Thr-Met-Asp-Thr-Phe-Lys-Ala-Met-Glu-Met-Gly-Tyr-
Leu-Gly-Glu-Met-Ile-Ser-Asn-Thr-Pro-Phe-Phe-Phe-Tyr-Pro-Pro-Val-Lys-Pro-Thr-Gly-
Lys-Val-Ala-Phe-Asp-Ile-Thr-Asn-Val-Thr-Glu-Ile-Pro-Arg-Val-Asp-Ile-Leu-Phe-Ser-Tyr-
Glu-Asp-Met-His-Asn-Asp-Thr-Leu-Tyr-Asn-Ala-Ile-Ser-Ser-Gly-Ala-Gln-Gly-Ile-Val-Ile-
Ala-Gly-Ala-Gly-Ala-Gly-Gly-Val-Thr-Thr-Ser-Phe-Asn-Glu-Ala-Ile-Glu-Asp-Val-Ile-Asn-
Arg-Leu-Glu-Ile-Pro-Val-Val-Gln-Ile-Ser-Met-Arg-Thr-Val-Asn-Gly-Glu-Val-Pro-Leu-Ser-
Asp-Val-Ser-Ser-Asp-Thr-Ala-Thr-His-Ile-Ala-Ser-Gly-Tyr-Leu-Asn-Pro-Gln-Lys-Ser-Arg-
Ile-Leu-Leu-Gly-Leu-Leu-Leu-Ser-Gln-Gly-Lys-Asn-Ile-Thr-Glu-Ile-Ala-Asp-Val-Phe-Ala-
Leu-Gly-Thr-Asp-Ala

3.4.1

000066

Biochemical characterization of asparaginase from *Aspergillus niger*

1. Summary

The following biochemical characteristics of asparaginase from *Aspergillus niger* were investigated:

- pH-curve
- temperature-curve
- salt tolerance
- Molecular weight and purity of the UF concentrate, based on SDS-PAGE
- Iso-electric Point (IEP), based on Iso-electro Focusing (IEF)

The results are given below.

2. Methods

pH and temperature profiles were made using an adaptation of the standard protocol for measuring asparaginase activity. In order to cover a pH range from 4 to 9, the standard citric acid buffer was replaced by a 50 mM solution of citric acid, di-sodium hydrogen phosphate and boric acid. 0.1M asparagine was added as substrate, similar to the standard assay. For the pH range the activities were measured at 37 °C at pH 4, 5, 6, 7, 8 and 9. For the temperature profile the activities were measured at pH 5 at temperatures of 20, 37, 50 and 65 °C. Incubation time was 10 minutes. Activities were measured by measuring formed ammonia on the Cobas Mira analyzer.

Salt tolerance of asparaginase was tested by measuring the activity under standard conditions but in the presence of an increasing concentration of NaCl. For this, salt was added to the standard substrate solution resulting in final assay concentrations of 0, 1.9, 3.8, 5.7, 7.6 and 9.5% NaCl.

In order to determine the molecular weight and the iso-electric point of asparaginase, an SDS-PAGE and iso-electric focusing was performed with enzyme containing UF concentrate. The SDS-PAGE also gives an indication of the purity of the UF concentrate.

3. Results and Conclusions

3.1 pH profile

As can be seen in Figure 1, asparaginase has an apparent pH optimum between 4 and 5. At and above pH 9 no activity can be measured anymore. In a separate experiment, in which the activity of asparaginase under stomach conditions was tested, it was shown that the enzyme is instantly and irreversibly inactivated at pH 1 and 2.

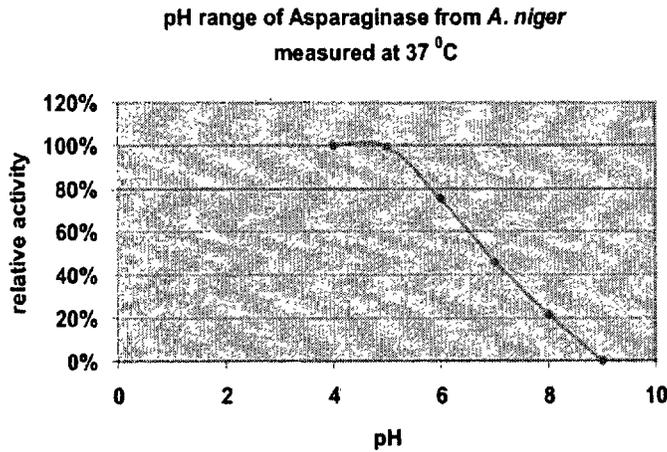


Figure 1. Relative activity of asparaginase between pH 4-9, at 37°C.

3.2 Temperature profile

As is shown in Figure 2, asparaginase is active between 20 and 65 °C and has a temperature optimum around 50 °C. In a separate experiment, in which asparaginase was inactivated for use as a control in a toxicity study, it was observed that the enzyme was quickly inactivated at a temperature of 70 °C.

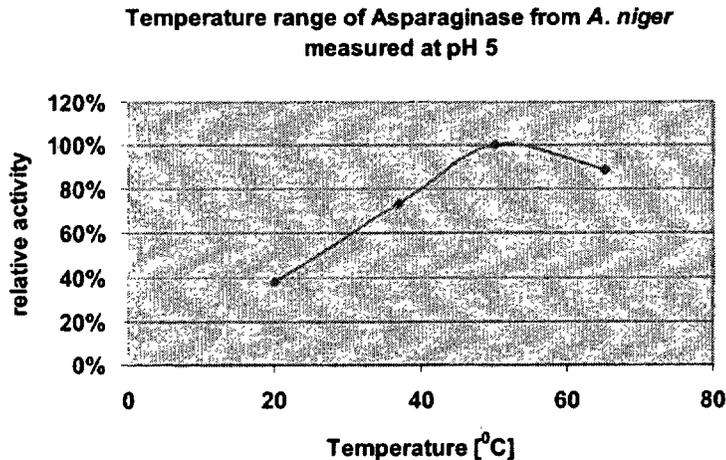


Figure 2. Relative activity of asparaginase at temperatures between 20-65 °C at pH 5.

3.3 Asparaginase tolerance for salt

Because the salt content in some applications is relatively high, the stability of asparaginase in the presence of salt was investigated. As is shown in Figure 3, almost 80% of the activity is still retained at the highest salt concentration. It may therefore be concluded that asparaginase can be used for 'salty' applications.

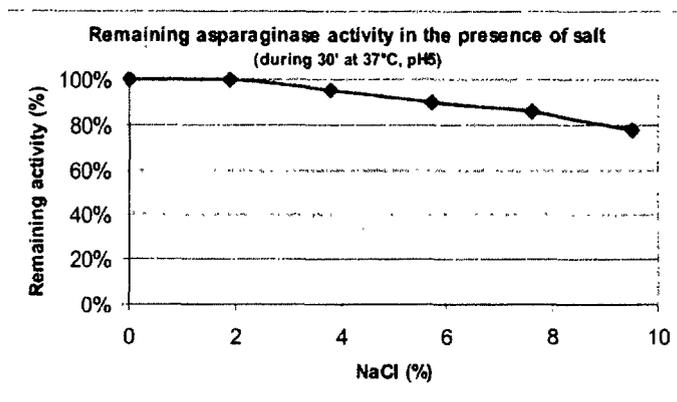


Figure 3. Asparaginase activity measured in the presence of salt (NaCl).

3.4 Molecular weight and iso-electric point of the asparaginase, and purity of the UF concentrate

Figure 4 shows the results of a SDS-PAGE and IEF performed on the asparaginase containing UF concentrate. From this Figure, it can be concluded that the apparent molecular weight of the enzyme is about 50 kD and the iso-electric point is 3.6. Based on its amino acid sequence, however, asparaginase has a theoretical molecular weight of 39584 Da and iso-electric point of 4.48. The difference in molecular weight can be explained by the fact that the enzyme is glycosylated. In fact, from the amino acid sequence it can be concluded that the enzyme contains 7 glycosylation sites. To verify this, the enzyme was treated with endoglycosidase H. This treatment removed much of the glycosylation and the molecular weight dropped to values around 40-42kD.

As can be concluded from the SDS-PAGE, the UF concentrate is rather pure, with only about 5 other minor visible protein bands.

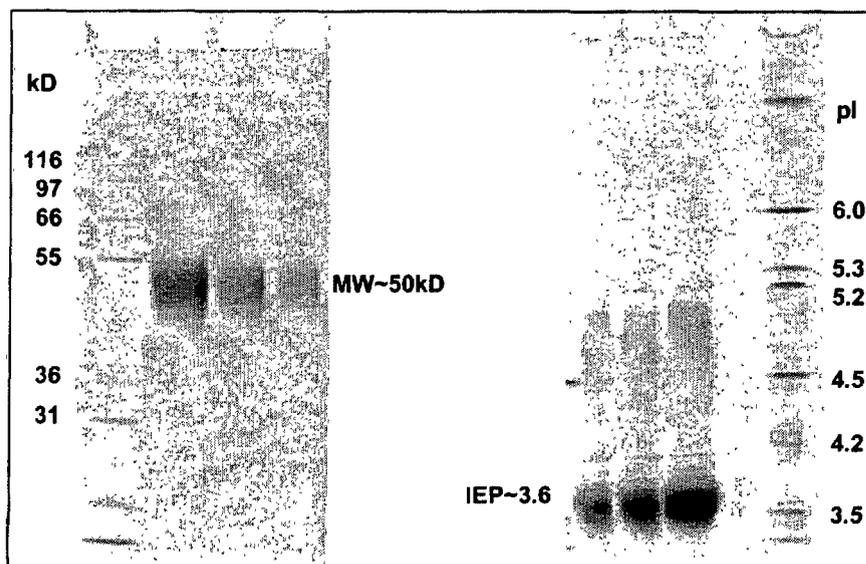
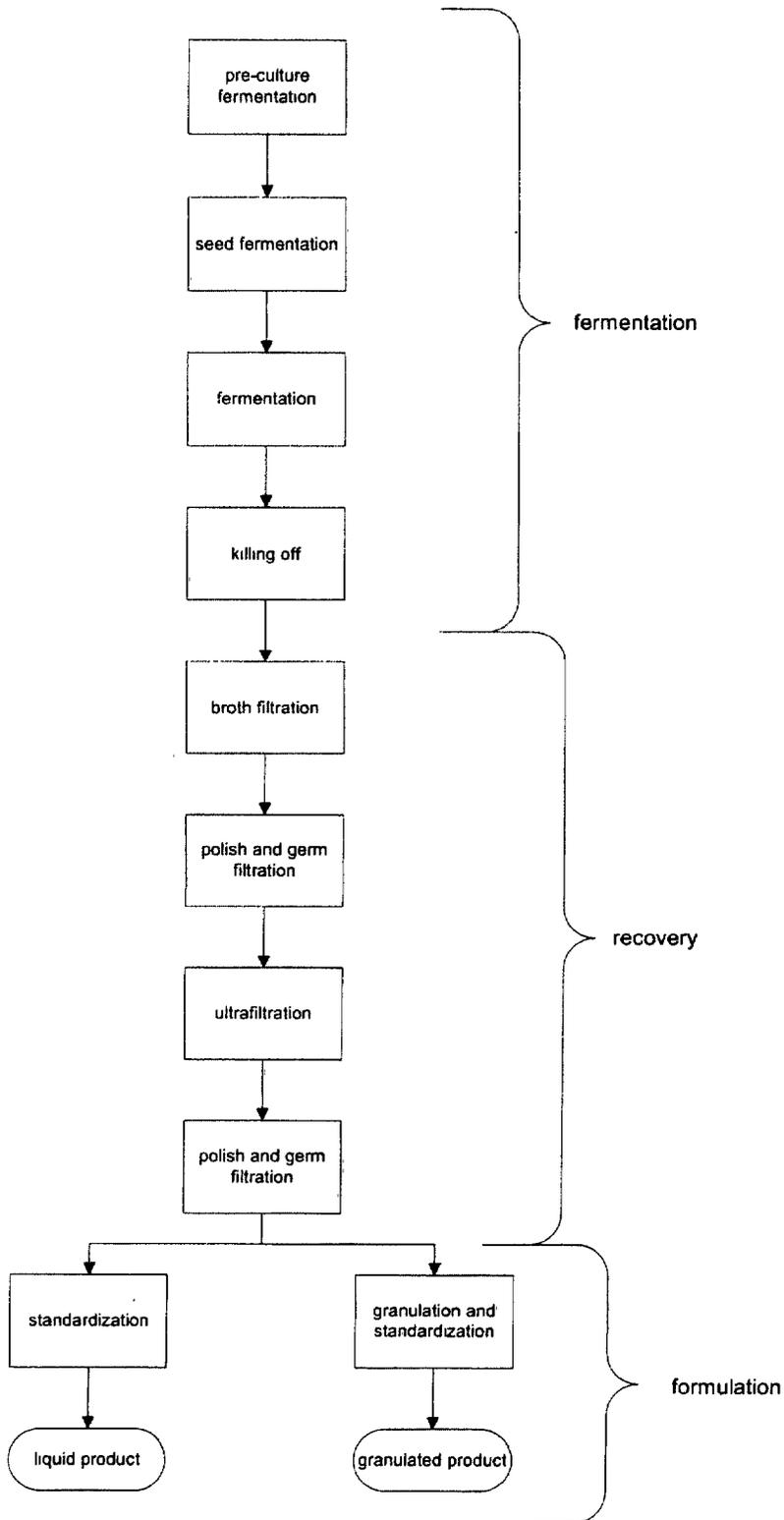


Figure 4. SDS-PAGE (left) and IEF-gel (right) of asparaginase UF concentrate, stained with Sypro Ruby. From left to right (in the SDS-gel) a molecular weight marker, 20, 10 and 5µl of UF are loaded. On the IEF-gel this is the opposite (with an IEF-marker instead of MW marker). Molecular weights and IEP's are indicated.

4.1.1

000070

Flow diagram of manufacturing process



6.2.1

000072

Acrylamide reduction by the use of asparaginase from *Aspergillus niger* in bread doughs – results of laboratory scale experiments

Summary

Various bread doughs were treated with asparaginase from *Aspergillus niger* to reduce the content of the free amino acid L-asparagine. The results show that asparaginase treatment of the dough results in bread products containing much lower levels of acrylamide than bread products obtained from untreated doughs (up to 75% reduction of acrylamide).

Methods

Various doughs were prepared according standard recipes (based on flour and water) and accordingly treated with different amounts of asparaginase from *Aspergillus niger*. Treatment took place at room temperature using several asparaginase levels ranging from 2-5 ppm. After treatment, the acrylamide was extracted and measured by gas chromatography. Untreated doughs were taken as control.

Results

As is shown in the Table below, treatment of different doughs with asparaginase resulted in a reduction of acrylamide in bread crusts up to 75%.

Bread Product	Enzyme use level in dough (ppm*)	Acrylamide in bread crusts (ppb**)	% Reduction of acrylamide
French batard	0	75	
	2	40	47
Potato bread (batard type)	0	800	
	5	200	75
Corn bread (batard type)	0	44	
	5	28	36
Dutch tin bread	0	74	
	5	47	36

*: parts per million (mg/kg dry weight)

** : parts per billion (µg/kg dry weight)

6.2.2

000074

Acrylamide reduction by the use of asparaginase from *Aspergillus niger* in doughs for cereal based fine bakery wares – results from laboratory scale experiments

Summary

Different cereal based doughs were treated with asparaginase from *Aspergillus niger* to reduce the content of the free amino acid L-asparagine. The results show that asparaginase treatment of the doughs results in fine bakery wares containing much lower levels of acrylamide than fine bakery wares obtained from untreated doughs (86-92% reduction of acrylamide).

Methods

Different cereal based doughs were prepared according standard recipes for the different applications and accordingly treated with different amounts of asparaginase from *Aspergillus niger*. Treatment took place at room temperature using several asparaginase levels ranging from 1-6 ppm. After treatment, the acrylamide was extracted and measured by gas chromatography. Untreated doughs were taken as control.

Results

As is shown in the Table below, treatment of different cereal based doughs with asparaginase resulted in a reduction of acrylamide in the fine bakery wares up to 92%.

Cereal based product	Asparaginase level in dough (ppm*)	Acrylamide content (ppb)	% Reduction of acrylamide
Crackers	0	45	
	6	6	87
Dutch honey cake (crumb)	0	2600	
	1	200	92
Fritters (crust)	0	57	
	4	8	86
Donuts (crust)	0	94	
	4	12	87

* ppm is calculated on the amount of flour in the dough

6. 2. 3

000076

Asparagine reduction by the use of asparaginase from *Aspergillus niger* in potato based doughs – results of laboratory scale experiments

Summary

Potato based doughs were treated with asparaginase from *Aspergillus niger* to reduce the content of the free amino acid L-asparagine. The reduction was measured using untreated potato dough as control. It appeared that the L-asparagine reduction was time and dose dependent.

Methods

Potato dough prepared from dry potato flakes and water was treated with different amounts of asparaginase from *Aspergillus niger*. Treatment took place at room temperature using several incubation times ranging from 5 up to 35 minutes. After treatment, the L-asparagine level was measured by HPLC. Untreated potato dough was taken as control.

Results

As shown in the Table below, the treatment of potato dough with asparaginase resulted in a time and dose dependent reduction of L-asparagine in the dough.

Incubation time (minutes)	Enzyme level (U/g)	Asparagine concentration (mg/g dry matter)	Asparagine reduction (%)
Test 1. (30-11-2004)			
30	0	12.8	
12	5	7.0	45
30	5	0.7	95
Test 2. (10-12-2004)			
30	0	12.6	
10	10	1.7	87
20	10	0.1	99
30	15	0.02	100
Test 3. (05-04-2005)			
0	0	12.2	
10	7.5	3.4	72
15	7.5	1.5	88
35	7.5	0.7	94

Based on the fact that L-asparagine is one of the main precursors in the formation of acrylamide, it is scientifically sound to assume that removal of more than 90% of the asparagine present in potato dough, will result in a strong reduction of acrylamide formation in potato-based products.

6.2.4

000078

Asparagine and Acrylamide reduction by the use of asparaginase from *Aspergillus niger* yeast extracts applications – results of laboratory scale experiments

Summary

Yeast extracts were treated with asparaginase from *Aspergillus niger* to reduce the content of the free amino acid L-asparagine. The reduction was measured using untreated yeast extracts as control. It appeared that the L-asparagine reduction was very effective (no Asn could be measured).

It was also proven that the reaction flavors produced with this low Asn YE contained much lower levels of acrylamide (73-80% reduction of acrylamide).

Methods

200 l of a 20 % w/w solution of autolytic yeast extract (Gistex® LS, DSM Food Specialties-The Netherlands) was made in water. The pH of this solution was adjusted to 5.1 using 4N KOH. 20 ml of Asparaginase solution (4602 ASP units/ml) was added to the yeast extract solution and the mixture was incubated for 4 hours at 51 °C. Subsequently, the yeast suspension was subjected to heat treatment and the pH was adjusted to 6.5. The resulting solution was spray dried. The final product contained less than 3.5% w/w water. The free asparagine content was measured in the yeast extract powder before and after the described enzyme treatment.

Acrylamide formation was measured in a reaction flavor (roasted beef flavor) made with untreated yeast extract (formulation 1) as well as a reaction flavor made with asparaginase treated yeast extract (formulation 2). Table 1 shows the composition of the formulations:

Table 1 Composition of formulations for production of reaction flavors

Ingredients	Formulation 1	Formulation2
Gistex® LS powder (g)	81.3	
Gistex® LS powder treated with asparaginase (g)		81.3
Maxarome® Plus HS powder (g)	16.0	16.0
Dextrose monohydrate (g)	24.3	24.3
Sunflower oil (g)	3.4	3.4

Results

The result of the treatment of yeast extract with asparaginase is shown in Table 2.

The results clearly demonstrate that asparaginase treatment results in a complete reduction of L-asparagine in yeast extract.

Table 2 - Reduction of L-asparagine in yeast extract by asparaginase treatment

	Standard Gistex LS powder	Gistex LS powder treated with asparaginase
Asparagine content (mg/g dm)	7.2	0.0

The results of the determination of acrylamide in reaction flavors made with untreated (formulation 1) and with asparaginase treated yeast extract (formulation 2) are shown in Table 2. As can be concluded from this Table, a more than 70% reduction of the acrylamide content in the reaction flavor prepared with asparaginase-treated yeast extract was obtained when compared to the reaction flavor prepared with untreated yeast extract.

Table 3 - Reduction of acrylamide in a reaction flavor prepared with asparaginase treated yeast extract

Experiment conditions	Acrylamide (µg/kg)	% Reduction of acrylamide
Formulation 1, 165°C, 40 minutes	935	
Formulation 1, 180°C, 40 minutes	1497	
Formulation 2, 165°C, 40 minutes	255	73
Formulation 2, 180°C, 40 minutes	296	80

000080

G.4.1

000081

Inactivation of asparaginase – results of laboratory experiments

Summary

Doughs and yeast extract were treated with asparaginase and the loss of enzyme activity was measured after processing of these food ingredients according to standard manufacturing methods. In the case of bread the activity was measured in the dough (before baking) and in the baked bread. In the case of yeast extract the activity was measured after the heating step that normally takes place in the process, and after the production of a yeast extract-based reaction flavor. The results of these experiments are given below.

Methods

Doughs were treated with 3 different doses of asparaginase (37.2 ASPU, 372 ASPU and 3720 ASPU/kg flour). The enzyme was extracted from the doughs as well as the baked bread and the asparaginase activity was measured. To increase the sensitivity of the enzyme analysis the incubation time in the assay was extended from 30 minutes (standard) to 5 hours.

Yeast extracts were treated with 2 different doses of asparaginase (1000 ASPU and 37000 ASPU/kg). The enzyme activity was measured after the standard heat treatment (15 min at 115°C) applied in the yeast extract process. The enzyme- and heat treated yeast extracts were subsequently used in the production of a reaction flavor, where again a heating step is applied. To judge the inactivation of the enzyme during this second heating step, asparaginase was added at a level of 1100 ASPU/g before the heat treatment. The enzyme activity was measured after the production of the reaction flavor.

Results

Bread production

The results of the measurements of asparaginase in dough and in bread are given in Table 1. The high recovery of the enzyme in dough proves that most of the added enzyme could be retrieved with the extraction method and enzyme analysis applied. After baking, however, no residual asparaginase activities could be detected in the bread crumb anymore. These results confirm that the enzyme is completely inactivated during baking.

Table 1 - Asparaginase activity in dough and bread samples

Sample	Dosage	Activity (ASPU/g) 30' incubation	Recovery (%)	Activity (ASPU/g) 5h incubation	Recovery (%)
Dough 1	0 (blank)	n.d.		n.d.	
Dough 2	0 (blank)	n.d.		n.d.	
Dough 3	Normal	n.d.		n.d.	
Dough 4	Normal	n.d.		n.d.	
Dough 5	100x normal	2.9	123	2.7	115
Dough 6	100x normal	2.8	118	2.7	113
Dough 7	1000x normal	27.3	115	19.8	84
Dough 8	1000x normal	27.2	115	19.0	80
Bread 1		n.d.		n.d.	
Bread 2		n.d.		n.d.	
Bread 3		n.d.		n.d.	
Bread 4		n.d.		n.d.	
Bread 5		n.d.		n.d.	
Bread 6		n.d.		n.d.	
Bread 7		n.d.		n.d.	
Bread 8		n.d.		n.d.	

n.d.: not detectable.

bread 1 corresponds with dough 1.

Production of yeast extract and yeast extract-based reaction flavor

The results of the measurements of asparaginase in yeast extracts are given in Table 2. As is shown in this Table, the enzyme activity is lost already after the normal heat treatment of yeast extract, irrespective of the dose. Therefore, inactivation during the production of the yeast extract-based reaction flavor was measured by an additional dose of the enzyme just before the heating step. As is shown in the Table, also in this case the enzyme is completely inactivated. The results show that even if yeast extract would not have been heat treated itself, the heat treatment during the production of yeast extract-based reaction flavors is sufficient to completely inactivate the activity of asparaginase.

Table 2 - Asparaginase activity in yeast extract and reaction flavor samples

Asparaginase addition	activity before heating step [ASPU/kg]	activity after heating step [ASPU/g]
Normal dose in yeast extract process	1000	n.d.
High dose in yeast extract process	37000	n.d.
Additional dose in extrusion process ¹	1100	n.d.

¹ only applied for inactivation purposes; normally no enzyme applied at this stage

7.1.1

000084

Pages 000085 - 000094 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

7.1.2

000095

ENZYMES DERIVED FROM ASPERGILLIS NIGER¹

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.

AMYLOGLUCOSIDASES (E.C. 3.2.1.3)

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies

Special studies on aflatoxin-related effects

Ducklings

¹ Extract from: Joint FAO/WHO Expert Committee on Food Additives, Toxicological Evaluation of Certain Additives, WHO Food Additives Series 22 (1988)

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 toxicity¹

Species	Route	LD ₅₀ (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

000097

¹ 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.

Observations in man

000098

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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β -GLUCANASE (E.C. 3.2.1.6)

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).

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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 ₅₀ (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

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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 10 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

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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.

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

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Biochemical aspects

No information available.

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

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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 ₅₀ (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

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analyses, organ weight analyses, and gross and microscopic pathology showed no compound-related effects (Garvin et al., 1972).

Long-term studies

No information available.

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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Garvin, P.J., Ganore, C.E., Merubia, J., Delahany, E., Bowers, S., Varnado, A., Jordan, L., Harley, G., DeSmet, C., & Porth, J. (1972). Carbohydrase from Aspergillus niger (pectinase, cellulase and lactase). Unpublished report from Travenol Laboratories, Inc., Horton Grove, IL, USA. Submitted to WHO by Gist-brocades NV, Delft, Holland.

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PROTEASE

No information available.

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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.

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DATE: September 14, 1988

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

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

I have read the report of the Joint FAO/WHO Expert Committee of Food Additives 31st meeting, Geneva, February 16-25, 1987

This report reiterates a conclusion reached at an earlier meeting of JECFA that an acceptable daily intake (ADI) should be established for certain enzyme preparations derived from microorganisms not normally used as food, or for enzyme preparations not removed from the food products to which they are added. This conclusion is based, in part, on the notion "that source organisms may produce toxins under certain conditions of growth"

Neither the name of the putative toxins, nor the name of the organisms implicated as toxin producers, was given in the report.

I would assume that the "offending" species are Aspergillus niger, Trichoderma harzianum, Trichoderma reesei, Penicillium funiculosum, and Aspergillus alliaceus since these are the producing organisms for enzymes for which the Joint FAO/WHO Expert Committee seeks to establish ADI's. Since none of these

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

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

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

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

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

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

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

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

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

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

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

The fact that mycotoxins are secondary metabolites is very relevant. Unlike enzymes, which are direct gene products synthesized directly from a structural gene via a series of RNA and amino acid intermediates, secondary metabolites are the result of numerous biosynthetic steps, each step enzymatically catalyzed. In most cases we do not know the exact number of steps in a biosynthetic pathway for a given secondary metabolite. Therefore, we do not know the number of genes required to encode for the enzymes of the pathway. However, 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 B1, 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 B₁, 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 B₁, sterigmatocystin, T-2 toxin, or zearalenone.

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Sep. 1988

THE OCCURRENCE AND SIGNIFICANCE
OF MYCOTOXINS

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THE OCCURENCE AND SIGNIFICANCE OF MYCOTOXINS

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1. Description of mycotoxins

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

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

2. Mycotoxins as natural contaminants in food

Of the several hundred known toxic mould metabolites (see Moreau, 1974; Wyllie & Morehouse, 1977; Watson, 1985) only about three dozen have been shown to occur as natural contaminants in food (Krogh, 1987). Table I lists the majority of these with the species of mould known to produce them. A further selection of mycotoxins, such as the satratoxins, verrucarins, sporidesmins and slaframine, have been identified in animal feeds and fodders.

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

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

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

4. Species specific mycotoxins

Table 2 lists some of the secondary metabolites associated with species of mould used for the production of enzymes. Only *Aspergillus alliaceus* is known to produce one of the mycotoxins (ochratoxin) included in those routinely tested for using the method of Patterson and Roberts (i.e. aflatoxin B₁, 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>
ZEARALENONE	<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	5328	MYCOTOXIN
S2	16258	AFLATOXIN
S3	195	DIHYDROXYFLAV?
S4	736	DIACETOXYSCIRPENOL
S5	2352	OCHRATOXIN
S6	238	LUTEOSKYRIN
S7	0	EPOXY(W)TRICOTHECENE
S8	1226	STERIGMATOCYSTIN
S9	172721	TOXIN? ?
S10	2352	T(2W)2(2W)TOXIN
S11	2094	ZEARALENONE
S12	36	TRICOTHECENE
S13	394	RUBRATOXIN
S14	1506	PATULIN
S15	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
Chemical 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

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7.1.4

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Pages 000129 - 000133 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

7.2

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SAFETY EVALUATION using the PARIZA & JOHNSON DECISION TREE of asparaginase from *Aspergillus niger* ASP-72

Introduction

The "Decision Tree for evaluation of the relative safety of food ingredients derived from genetically modified organisms" was published in 1991. This publication from the International Food Biotechnology Council (IFBC) was an extension, based on an earlier publication by Pariza and Foster in 1983¹. The 1991 IFBC Decision Tree was updated by Pariza and Johnson in 2001².

The enzyme asparaginase derived from *Aspergillus niger* ASP-72 was evaluated according to the P&J Decision Tree. The result is described below.

Decision Tree

1. Is the production strain genetically modified?
YES
The strain *Aspergillus niger* ASP-72 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?
YES
If yes, go to 3c. ~~If no, go to 3b~~
Asparaginase is present in many food sources and is also produced by the GRAS organism *Saccharomyces cerevisiae* (bakers yeast).
- 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 lineage³.
- 3c. Is the test article free of transferable antibiotic resistance gene DNA?
YES
If yes, go to 3e. ~~If no, go to 3d.~~
In the transformation of this lineage of DSM production strains the *E. coli* part of the plasmid is removed using the appropriate restriction enzymes and purification of the asparaginase

¹ Pariza M.W. and Foster E.M. J. Food Protection **46**. (1983), 453-468

² Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. **33** (2001) 173-186

³ P.W.M. van Dijck et al. Reg. Toxicol. Pharmacol. **38** (2003) 27-35.

expression cassette by gel-electrophoresis. Absence of any E. coli sequences in the transformant is verified by genetic tests.

- 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?

NA

~~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 (see 3c).

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 targetedly integrated.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects, which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?

NA

Nevertheless, the strain has been analyzed with respect to its potential to produce secondary metabolites, including mycotoxins. The strain ASP-72 showed no potential to produce secondary metabolites or mycotoxins of importance in food.

~~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

7.2.1

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WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

AMFEP *Members*

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Copenhagen, August 1998

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 - 3.6. The consumption of enzymes for medical purposes and as digestive aids
 - 3.6.1. *Medical uses*
 - 3.6.2. *Digestive aids*

- 4.0. **Conclusion**

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

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

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

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

1.0. Introduction

Since the late 80'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 α -amylase. The public was somewhat alarmed and there have been complaints, questions and other reactions of concern to bakers and other suppliers.

The media's interest was based on results from a study by Schata¹, published only as a 1/2-page abstract which does not allow for scientific evaluation.

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

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

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

2.0 Background

2.1 General

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

- The frequency of common allergy (all allergies included) is 20 - 30%, in most populations around the world. The figure is increasing. Part of the increase may be due to higher awareness and improved diagnostic methods, however, a true increase cannot be ruled out.
- The frequency of occupational allergy in bakers is 8 - 27%. About 30 - 35%, of the bakers with occupational allergy to flour have an additional respiratory allergy to α -amylase and/or other baking enzymes.
- There is a reasonably good documentation of the frequency of food allergy in the general population at 1 - 2%. However, the frequency of perceived food allergy 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^{2,4}, Trypsin⁵, protease's from the skin yeast *Candida albicans*⁶, from bacteria/ subtilisins^{7,8}, fungal amylases^{9,10}, bacterial amylases¹¹, fungal hemicellulases¹², lipases¹³, xylanases and cellulases^{14,15} are all examples of industrial enzymes known to induce allergic sensitisation and respiratory occupational allergy. This is a feature characterised by highly purified enzyme protein products rather than the origin or the methods of production.

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

The classical food allergens are also capable of inducing respiratory allergy when they are brought into a dust- or aerosol form and inhaled. Soya¹⁶, eggs^{17,18}, milk¹⁹ and fish²⁰ 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 allergen²¹.

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 food²². 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 population²²⁻²⁵.

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

Symptoms

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

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

Types of food allergens

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

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

Properties of food allergens

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

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

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

The TNO Institute performed a study⁵⁸ on native α -amylase from *Aspergillus oryzae* in a gastrointestinal model simulating the physiological events in the stomach.

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

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

Doses at which food allergy occurs

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

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

Steinman²⁹ 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 technology¹⁰⁻³³ and allergy seem to agree that gene technology and the results thereof expressed in foods should not cause concern with regard to allergy risk. However, gene technology does bring about new proteins, and it is important to be aware that some of these new proteins may be allergenic.

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

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

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

3.2. Epidemiology of Food Allergy

In a survey of 5000 households in the USA carried out in 1989, 1992 and again in 1993²⁵ 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 Holland²³ 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 allergy²⁴. 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 additives³⁴.

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)^{35,36}, is the method of choice to confirm or reject indications of food allergy that may derive from the patient's perception and in many cases also from skin prick testing.

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

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

3.3. Enzymes in food

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

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

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

In 1983 Mansfield and co-workers³⁷ published a case story of a person who had allergic symptoms after ingestion of papain used as a meat tenderiser. - Later, in 1985 they reported a study of 475 patients³⁸ 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 Binkley³⁹, 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 Wüthrich⁴⁰ of enzymes in food concluded that orally ingested enzymes are not potent allergens and that sensitisation to ingested enzymes is rare as is also the case of reactions to bread in bakers with occupational allergy to enzymes.

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

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

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

3.4. The Theory of cross reactions

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

The theory that people with allergy to common moulds which are related to those used for the fermentation of enzymes might react to enzyme residues in food was one of Schata's¹ 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 Cullinan⁴¹ 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,⁴² and Baur & Czuppon⁴³ 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 al⁴⁴ investigated occupational allergy to α -amylase in a pharmaceutical plant and found a number of employees sensitised to α -amylase. None reported reactions related to ingestion of bread. Five patients, all positive to α -amylase were given oral doses of native α -amylase in doses up to 10 mg.

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

Baur et al⁴⁵ described the possible background for consumer sensitisation to α -amylases in bread. 138 subjects, of which 98 were allergic, and 11 bakers with occupational allergy were tested. The bakers reacted to α -amylase as may be expected. None of the atopics and none of the control persons reacted to skin prick test with α -amylase. Two atopics had weak RAST to native α -amylase and one reacted also to heated ce-amylase. Reactions to other related compounds, for example *Aspergillus* was not tested.

Tarlo and co-workers⁴⁶ 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 al⁴⁷ and describes a man with occupational asthma after exposure to flours and other grain dusts. He was sensitised to barley, and experienced gastrointestinal reaction upon ingestion of foods and beverages made from barley.

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

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

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

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

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

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

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

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

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

3.6.1. Medical uses:

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

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

Proteolytic enzymes and mixtures of different enzymes are commonly used for treatment of a number of physical lesions and also for a number of more special conditions⁴⁸⁻⁵⁰.

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

We have not been able to find any evidence of sensitisation or allergy symptoms caused by the ingestion of enzymes from these enzyme preparations. One example is the use of enzymes given as tablets for the treatment of non-articular rheumatism. Uffelmann⁵¹ 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 preparations⁵²⁻⁵⁴. This of course might also happen to the Cystic fibrosis patients when they handle the enzyme preparations themselves. However no cases of enzyme allergy in Cystic Fibrosis patients have been described, but there are reports of allergy to common food allergens⁵⁵.

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 RAST⁵⁶ did not confirm sensitisation to any of the enzymes. Challenge tests have not been performed⁵⁷.

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.

Binkley³⁹, 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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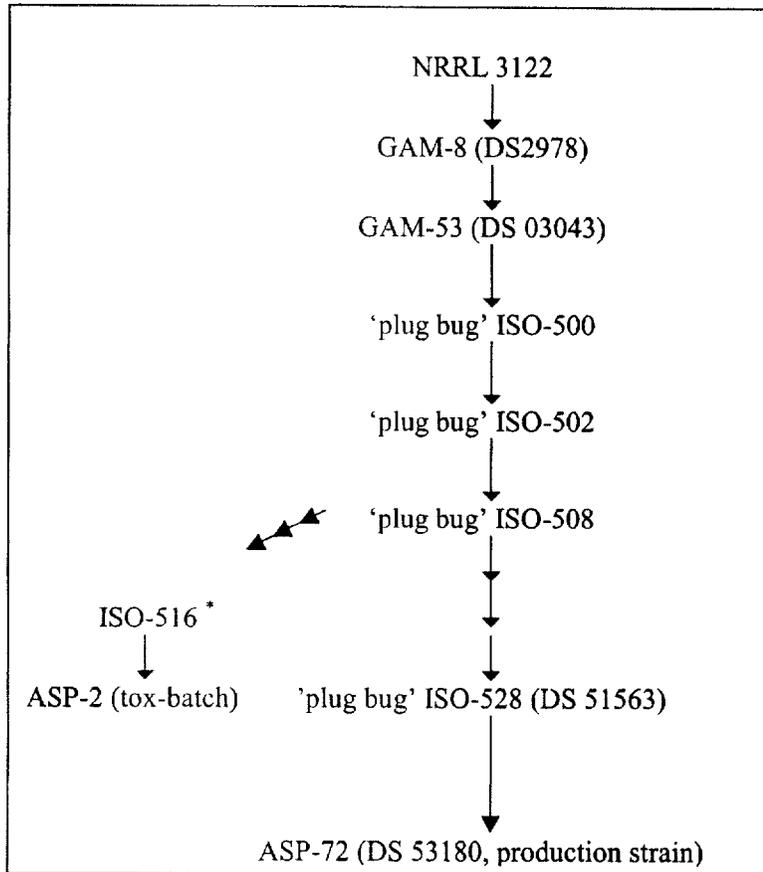
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7.4.1

Relationship between the production strain ASP-72 and the strain used for producing the tox-batch



* Strain ISO-516 is not considered to be a 'plug bug'; in the strain ASP-2 the asparaginase gene copies were integrated at random and not as in the case of ASP-72 at predefined loci in the genome of the 'plug bug' host ISO-528.

SUBMISSION END

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Reference List for Industry Submission, GRN 000214

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NA- Not applicable