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March 2, 2012

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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,

(b) (6)

Gary L. Yingling

cc: DSM Food Specialties

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Biotechnology and
GRAS Notice Review

DC-9599644 v1

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GRAS NOTIFICATION FOR AN ASPARAGINASE FROM A GENETICALLY MODIFIED STRAIN OF *ASPERGILLUS NIGER*

INTERNAL DSM REFERENCE: #00058237
IDENTIFIER NUMBEER: DSM1126-001

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

With this document, DSM Food Specialties (DSM) is submitting a GRAS notification for its asparaginase enzyme preparation produced by submerged fermentation of a selected, pure culture of an *Aspergillus niger* strain. The asparaginase enzyme sequence is derived from *Aspergillus niger* and subsequently protein engineered to obtain a protein with a different pH optimum compared to its wild-type equivalent. As the wild-type asparaginase, which was the subject of GRAS notification GRN 000214, the new enzyme is intended to convert asparagine to aspartic acid in order to reduce the formation of acrylamide during the production of cereal and potato based baked products such as bread, cookies, crackers, or potato chips. DSM produces the asparaginase preparations in liquid forms standardized with glycerol. The trade name will be PreventASe ASG.

The active enzyme in PreventASe ASG is an asparaginase (EC 3.5.1.1, CAS 9015-68-3) with identical CAS number, production microorganism and intended use, when compared to DSMs other asparaginase, GRAS Notification (GRN 000214) filed in October 2006. Levels of use differ, depending on the specific application. Naturally, asparaginases can be found in cells of plants, animals, bacteria and fungi (http://www.ncbi.nlm.nih.gov/unigene/?term=L-asparaginase).

PreventASe ASG is a processing aid for use in the food industry to reduce the levels of free L-asparagine, a main precursor in the formation of the food contaminant acrylamide, which is formed during heat treatment. Acrylamide, which is formed during baking or frying of certain foods, raised concern because it is a probable human carcinogen (JECFA, 2005; NTP Technical Report 575, 2011). 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 Progamme, 2006; CAC/CRP-67 2009 Code of Practice for the Reduction of Acrylamide in Foods). The toolbox for acrylamide mitigation published by Food and Drink Europe has recently been updated (Food Drink Europe Acrylamide Toolbox 2011). The use of the enzyme asparaginase is one way to achieve such reduction.

In the presence of water, asparaginase catalyzes the hydrolysis of the amino acid L-asparagine, and forms L-aspartate and ammonia. Most of the acrylamide in food heated above 120°C is a result of 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 and baked or fried potato-based products to reduce the formation of acrylamide.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36 (see 62 Fed. Reg. 18,938 (April 17, 1997)), DSM has determined that its asparaginase enzyme from GMO *Aspergillus niger* is a GRAS substance for the intended food applications and is therefore exempt from the requirement for premarket approval. Information on the enzyme and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme, its applicable conditions for use, DSM's basis for its GRAS determination and the availability of supporting information and reference materials for FDA's review can be found here in Section 1.

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

microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer et al., 2006). In addition, for phospholipase A2 (donor: porcine pancreas, see GRAS notification GRN 000183), asparaginase (donor: *Aspergillus niger*, see GRAS notification GRN 000214), lipase (donor: synthetic gene, see GRAS notification GRN 000296), and a carboxypeptidase (donor: *Aspergillus niger*, see GRAS notification GRN 000345), derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier, have been notified to FDA.

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. Furthermore, this section describes the modifications introduced in the asparaginase gene used to produce the PreventASe ASG enzyme preparation.

In Section 3, data are presented that show the substantial equivalence of the asparaginase in PreventASe ASG to naturally occurring asparaginase as well as the *Aspergillus niger* wild-type enzyme preparation from DSM. The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 4. Section 5 reviews the hygienic measurements, composition and specifications as well as the self-limiting levels of use for asparaginase. Section 6 provides information on the mode of action, applications, use levels of asparaginase and enzyme residues in final food products. The safety studies outlined in Section 7 indicate that asparaginase preparations from *Aspergillus niger* show no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

1.1. Name and Address of Notifier

Notifier:

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Tel: +31 152 79 3592

Manufacturer:

DSM Food Specialties
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France
Tel: 33 320964545
Fax: 33 320964500

Person Responsible for the Dossier:

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Regulatory Affairs
DSM Nutritional Products
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1.2. Common or Usual Name of Substance

DSM's asparaginase enzyme preparation from GMO *Aspergillus niger* is produced by submerged fermentation of a selected, pure culture of *Aspergillus niger*. The common or usual name of the substance is "asparaginase". It is produced and sold in liquid form, stabilized with glycerol. The trade name will be PreventASe ASG.

1.3. Applicable Conditions of Use

The *Aspergillus niger* asparaginase preparation is intended to be used in foods containing asparagine and reducing sugars that are heated above 120°C. The enzyme is added to the food before the heating step. During heating, the enzyme is inactivated by denaturation. The use of asparaginase can thus be regarded as processing aid because it has no function in the final food.

1.3.1. Food Products Used in

The *Aspergillus niger* asparaginase preparation is intended to be used in L-asparagine and carbohydrate-containing food raw materials that are subsequently heated above 120°C, such as:

- bread (e.g. 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 (e.g. corn crackers, cakes, Swiss rolls, Dutch honey cake, breakfast cereals),
- potato-based products (e.g. French fries, potato chips),

1.3.2. Levels of Use

In general, the dosage of the enzyme depends on the type and quality of the ingredients used, their asparagine content, the duration of and temperatures during the production process, and the type of bakery product produced.

<u>Wheat-based products</u>:
In the case of wheat flour, the levels of use may vary between 100 and maximal 1500 NASPU/kg flour, with an average dosage of 600 NASPU/kg flour.

<u>Cereal-based products</u>: In the case of corn masa flour, the levels of use may vary between 275 and 1500 NASPU/kg, with an average dosage of 825 NASPU/kg.

<u>Potato-based products</u>: In the case potato flour is used, the dosage may vary between 825 and 3000 NASPU/kg, with an average dosage of 1500 NASPU/kg.

1.3.3. Purposes

The PreventASe ASG enzyme 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, NTP Technical Report 575, 2011). 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; CAC/CRP-67 2009 Code of Practice for the Reduction of Acrylamide in Foods). The toolbox for acrylamide mitigation published by Food and Drink Europe has recently been updated (Food Drink Europe Acrylamide Toolbox 2011). The use of the enzyme asparaginase is one way to achieve such reduction.
The mechanism by which most of the acrylamide in heat-treated food is formed is a chemical reaction between the amino acid L-asparagine and reducing sugars, which are found naturally in various raw materials for food. 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 foods.

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 (GRN 000214 and GRN 2000201). 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). Many of the naturally occurring sources of asparaginase form part of the human diet. So far, consumption of these asparaginases has not led to any adverse events or allergic reactions. Since the asparaginase produced in *Aspergillus niger* is a normal protein (composed of natural amino acids) it will be digested in the human gastrointestinal tract just as any other food protein/enzyme, too.

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[1] in the final food is expected to be about 2 - 34 mg/kg (= 0.0002 - 0.0034%) for wheat-based products, 2 -35 mg/kg (=0.0002 - 0.0035%) for cereal-based products and 4 - 74 mg/kg (=0.0004 - 0.0074%) for potato-based products.

Since asparaginase is present in the final food products at only very low levels, it is inactivated during baking, and because it is a naturally occurring substance in cells and tissues commonly ingested by humans without any harm, it is unlikely that the consumer population will be affected by the presence of asparaginase in foodstuffs when used as processing aid.

[1] TOS: Total Organic Solids

1.4. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, DSM has determined, through scientific procedures, that its PreventASe ASG enzyme preparation from GMO *Aspergillus 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, or baked or fried potato-based products, 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:

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Washington, DC 20006-1600
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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*). The agnA gene isolated from this donor strain was genetically modified at 85 amino acid positions to generate a protein with an altered pH profile.

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 was 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 Annexes 2.1.1 and 2.1.2). 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 been notified as GRAS (GRN 000183). Another ISO strain is ISO-528, which was used for the construction of wild-type asparaginase (donor: *Aspergillus niger*, see GRAS notification GRN 000214), lipase (donor: synthetic gene, see GRAS notification GRN 000296), and a carboxypeptidase (donor: *Aspergillus niger*, see GRAS notification GRN 000345).
ISO-strains were also used for the construction of production strains for arabinofuranosidase (donor: *Aspergillus niger*), phytase (donor: *Aspergillus niger*), pectin methyl esterase (donor: *Aspergillus niger*), glucoamylase (donor: *Aspergillus niger*), xylanase (donor: *Aspergillus niger*), endo-polygalacturonase (donor: *Aspergillus niger*), proline specific endo-protease (donor: *Aspergillus niger*) and amylase (donor: *Aspergillus niger*).

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

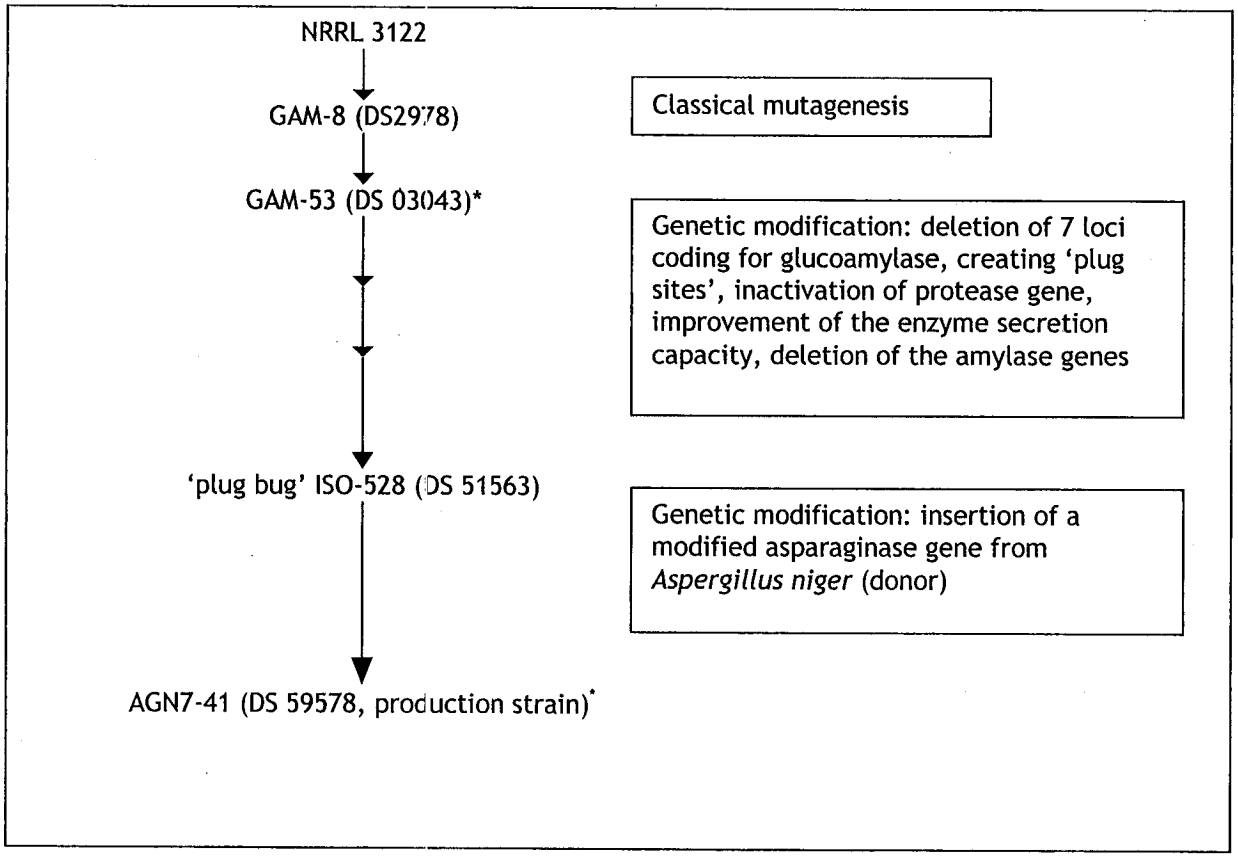
The strain GAM-53 contains 7 loci (i.e. the promoter and coding sequences) for the glucoamylase gene. These 7 loci were removed, creating so-called 'plug-sites' (also called Δ*gla*A loci) into which expression units containing various genes can be integrated ('plugged'). The 7 'plug-sites' were each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

Production Strain:

The asparaginase production strain was obtained by further genetic modification of the *Aspergillus niger* strain ISO-528. The genetic modification techniques used are described in Section 2.2 of this dossier. The production strain was designated AGN7-41 and stored in the DSM Culture Collection as DS 59578.

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



* Strain GAM-53 and AGN7-41 were taxonomically identified as *Aspergillus niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS).

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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 modified asparaginase gene, and the other to derive the cassette containing a selectable marker.

Asparaginase Expression Plasmid

The asparaginase expression plasmid contains strictly defined *Aspergillus niger* chromosomal elements (parts of the *Aspergillus niger* glucoamylase locus, the glucoamylase promoter and part of the glucoamylase *glaA* gene), the *Aspergillus 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 *gla*A promoter from the parental *Aspergillus niger* strain GAM-53.
- The entire 1138 bp sized synthetic sequence encoding the modified asparaginase protein of *Aspergillus niger* GAM-8 from the ATG initiation codon to the TAAA termination signal. The sequence of the agnA gene results in an asparaginase enzyme of 378 amino acids in size, of which 85 differ from those of the wild-type gene.
- 2.3 kb DNA from 3' flanking *gla*A terminator sequence from the parental *Aspergillus niger* strain GAM-53 for efficient termination of *aspA* gene transcription and targeting of the expression unit to the Δ*gla*A loci.
- DNA sequences from the *E. coli* plasmid pTZ19R. 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 *Aspergillus niger* glucoamylase locus as the expression plasmid, the promoter sequence of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*A) gene from the *Aspergillus niger* related fungus *Aspergillus nidulans*, the *A. nidulans amdS* (acetamidase) selectable marker gene and DNA from a well-characterized *Escherichia coli* vector pTZ18R.

The different elements of the plasmid are:

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

The plasmid map is shown in Annex 2.2.3.

Transformation and selection of the final production strain
The asparaginase expression cassettes containing the genetically modified asparaginase gene of *Aspergillus niger* 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 *gla*A promoter and 3'-*gla*A terminator parts of the two expression units, they are targeted to one of the seven ΔglaA 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*gla*A loci of the recipient strain. The selection of these transformants was done by PCR analyses, applying *asp*A and *gla*A specific primers.

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

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

From the available recombinants a strain was chosen that contained sufficient gene copies to allow for commercial attractive expression levels of the modified asparaginase enzyme: this strain was designated AGN7-41.

The nucleotide sequence of the final asparaginase expression unit as present in the AGN7-41 production organism has been determined.

Translation of this *agn*A expression unit and subsequent secretion by the microbial cell into the fermentation broth results in a fully functional, biologically active, modified asparaginase protein of 378 amino acids. The sequence of the expressed protein shows 77.5% homology to the wild-type protein from *Aspergillus niger*.

2.3. Stability of the Transformed Genetic Sequence

The strains belonging to the *Aspergillus niger* GAM-lineage - from which the host ISO-528 and the recombinant modified asparaginase production strain are 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 modified 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 *Aspergillus niger* production organism to another, non-related, organism.

2.4. Good Industrial Large Scale Practice (GILSP)

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

The host organism is non-pathogenic, does not produce adventitious agents under the fermentation conditions employed and has an extended history of safe industrial use (see Section 7.1). 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). 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 (wild-type) asparaginase, albeit in less efficiently than the asparaginase production strain.

Therefore, the modified 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 the 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 *Aspergillus niger*.

2.7. Absence of Antibiotic Genes

As noted above, no antibiotic resistance markers or other heterologous markers are present in the strain. The enzyme preparations are tested to ensure the absence of antibiotic activity in accordance with the recommendation from the Joint Expert Committee of Food Additives of the FAO/WHO ("JECFA"). As is shown in Section 4.6 of this dossier, quality control testing of the finished 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 nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species."*

The test showed that mycotoxins, as reported in the literature to be produced by *Aspergillus niger* strains, are not produced by the AGN7-41 strain under the standard industrial submerged fermentation conditions.

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

PreventASe ASG asparaginase from *Aspergillus niger* is a glycoprotein with a primary sequence of 378 amino acids and a calculated molecular weight of 39,583 Dalton. This sequence shows 77.5% homology to the wild-type protein from *Aspergillus niger*. The predicted signal peptide (amino acids 1-17), directs

transport of the protein into the endoplasmic reticulum of *Aspergillus niger* and, ultimately, into the extracellular medium; the signal peptide cleavage site is located after position 17. The mature AgnA protein is 361 amino acids in size, has a molecular mass of approx. 50 kD (in its glycosylated form) and an iso-electic point (pI) of 4.4. Annex 3.2 shows the amino acid sequence of the PreventASe ASG asparaginase from *Aspergillus niger* and a comparison with the wild-type sequence.

3.3. Sequence Comparison to Other Asparaginase Enzymes

In order to compare the PreventASe ASG asparaginase from *Aspergillus niger* with other presently known asparaginases, an extensive sequence comparison has been carried out. The amino acid sequence of the PreventASe ASG asparaginase was used as a query to search sequence databases using 'BLAST' software for related sequences. About 200 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. The PreventASe ASG asparaginase belongs to the fungal cluster that also contains asparaginase from *Aspergillus niger, 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

Main Enzymatic Activity

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

$$\text{L-asparagine} + H_2O \rightarrow \text{L-aspartate} + NH_3$$

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 PreventASe ASG asparaginase is expressed in so-called NASPU (Neutral ASParaginase Units). One NASPU 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 (pH 7.0, 37°C).

The biochemical properties of PreventASe ASG asparaginase from *Aspergillus niger* have been investigated extensively (see also Annex 3.4). The enzyme has shown to exhibit activity over a pH range between 5 and 8 with an optimum around pH 7. The temperature optimum for asparaginase activity is between 50 and 60°C. The enzyme is inactivated (denatured) at temperatures above 70 °C, thus the enzyme is inactivated during baking.

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

PreventASe ASG 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.

4.2. Raw Materials

All raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the fermentation and recovery of the product are suited for the intended use; this leads to the required safety status of the product. The safety is confirmed by the toxicological studies (see Section 7.4 of this dossier). The antifoam and flocculant are listed in the FDA September 11, 2003 letter to ETA as acceptable for use in enzyme manufacturing (Annex 4.2). The raw materials used for the formulation are of food grade quality and meet FCC specifications.

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.

- Glucose
- Yeast extract
- Inorganic salts (potassium chloride, manganese sulfate, zinc sulfate, copper sulfate, magnesium sulfate, iron sulfate)
- Phosphoric acid, sodium hydroxide, ammonium hydroxide
- Antifoam Clerol FBA 3107 (polypropylene glycol ether oleate (CAS 78041-14-2)[2])
- 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 or Sodium acetate (killing-off agent)
- Flocculant C577 (Dimethylamine-epichlorohydrin copolymer (CAS 25988-97-0)[2])
- Filter aid Dicalite BF (DBF)
- Calcium chloride ($CaCl_2$)

[2] The antifoam and flocculant are included on the ETA list (see also Annex 4.2)

000019

- Phosphoric acid
- Ammonia
- Water
- Glycerol

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 24-36 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 38 hours at a constant temperature of 30°C and a fixed pH. 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, until a few hours before the end of fermentation (duration approximately 72-84 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 5 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 or sodium acetate 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 again germ filtrated and then standardized with approx. 50% glycerol to an average enzyme concentration of 2500 NASPU/g at a pH of 3.5 - 5.0.

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 (7th edition), the final PreventASe ASG asparaginase preparations from *Aspergillus niger* meet the following specifications:

Parameter	Specification limit
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 monocytes	0/25g
Yeasts	<100/g
Moulds	<100/g
Total viable count	$< 5*10^4/g$

In addition, the following specifications were set:

Parameter	Specification
Appearance	Brown, clear liquid
Asparaginase activity	2500 NASPU/g
pH	3.5 - 5.0

5. COMPOSITION AND SPECIFICATIONS

5.1. Formulation

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

Item	Value	Unit
Enzyme activity	2500-5000	NASPU/g
Dry matter	6-13	%
Ash	0.7-1.5	%

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

The Total Organic Solids (TOS) of the asparaginase preparations and the asparaginase activities were determined for three different batches of the UF concentrate:

Calculation of the TOS					
Batch number	Water (%)	Ash (%)	TOS (%)	Activity (NASPU/g)	NASPU/g TOS
APE.GRZ.1122	87.8	0.91	11.3	4100	36283
611018401	93.4	0.72	5.9	2830	47966
GRK1147b	87.4	1.26	11.3	4650	41150
MEAN	89.5	0.96	9.5	3860	41799

The TOS values of the final, formulated enzyme preparations can be calculated on basis of the above values. For example, a standardized enzyme preparation containing 100 mg TOS/g will contain 4180 NASPU/g.

5.2. General Production Controls and Specifications

Quality standards require a strictly controlled fermentation process. The DSM enzyme fermentation factory at Seclin, France, which has fermentation experience since 1922, produces the enzyme under 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. Prior to inoculation, the fermentor is cleaned, rinsed and sterilized. Membrane valves, air filters and seals are regularly checked, cleaned and replaced if necessary. Only sterilized air is used in the fermentation. 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 are strictly followed.

Microbial contamination is prevented during downstream processing by several germ reduction filtrations. The filters are thoroughly cleaned before 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, 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 taken and checked for the level of microbial contamination. If these checks show that significant contamination has occurred, the downstream processing is discontinued.

The finished product is subjected to extensive controls and complies with JECFA and FCC specifications: see Section 4.6: Quality Control of Finished Product.

6. APPLICATION

6.1. Mode of Action

The enzyme in PreventASe ASG is an asparaginase that hydrolyses the amino acid L-asparagine to aspartic acid and ammonia by hydrolyzing the amide in asparagine to the corresponding acid. Apart from asparagine, asparaginase only acts on glutamine (although with a lower preference) and it has no activity on other amino acids. It has also no activity on asparagine residues in peptides or proteins.
PreventASe ASG is to be used for acrylamide reduction in various food applications. Acrylamide is formed as a reaction product from asparagine and reducing sugars when food products are baked or fried at temperatures above 120°C. Both asparagine and reducing sugars are commonly found in many food raw materials. By using asparaginase, the asparagine content will be reduced and hereby also the acrylamide in the final product. Typical dough based applications include: biscuits, crackers, crisp bread, tortilla chips,

fabricated potato chips, pretzels, bread, etc. The enzyme can also be used for direct treatment of cut potatoes for production of French fries, sliced potato chips, potato flakes and potato granules.

During baking or frying, the enzyme will be inactivated. The use of asparaginase can thus be regarded as a processing aid, having no function anymore in the final food.

6.2. Application

Many baked products contain flour from grains, corn or potato. 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).

6.2.1. Wheat-based products

Bread, cookies and cakes are usually prepared with wheat flour. During baking acrylamide can be formed. The results of laboratory scale bakery trials show that, depending on the type of bread/bakery product and amount of enzyme added, the acrylamide formation in the final product (cracker/cookie) can be reduced from 40% to 90% by the addition of asparaginase to the dough. The results of these trials are given in Annex 6.2.1.1 and 6.2.1.2.

6.2.2. Other cereal-based products

Apart from wheat, also other cereals like e.g. corn flour are used for many other baked products, such as tortilla chips and crackers. 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 addition of PreventASe ASG to dough made from corn flour can reduce the asparagine content by up to 90% when using 0.1 units asparaginase per gram masa flour during a 30 minutes incubation time at room temperature. 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 dough has shown to remove free L-asparagine (almost) completely when using test dough at pH 7-8 treated with 5-6 NASPU/g potato flour for 30 minutes at room temperature. The results of these trials are given in Annex 6.2.3.

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.

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
Wheat-based products[1]	100-1500 NASPU/kg flour	67-91%	67 - 1365 NASPU/kg	1.6 - 33.6 mg/kg
Cereal-based products[2]	275-1500 NASPU/kg flour	25-95%	69 - 1425 NASPU/kg	1.7 - 35.1 mg/kg
Potato-based products	825-3000 NASPU/kg flour	20-100%	165 - 3000 NASPU/kg	4.1 - 73.8 mg/kg

[1] Includes 3 categories: i. Yeast bread and rolls, ii. Quick breads, pancakes and French toast and iii. 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

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, animals and humans. 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 human nutrition or health.

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).

Aspergillus niger is not a human pathogen and does not produce toxins under the fermentation conditions used. 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, *Aspergillus niger* has been safely used in the commercial production of organic acids and various food enzymes, such as glucose oxidase, pectinase, alpha-amylase and glucoamylase. Industrial production of citric acid by *Aspergillus niger* has taken place since 1919 (Schuster et al., 2002, attached as Annex 7.1.1).

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

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

Enzymes from *Aspergillus niger* have been used in food for many decades and there is no evidence that the industrial strains used produce toxins under the routine conditions of industrial submerged fermentations. 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 *Aspergillus 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 *Aspergillus niger*, based on the concern that some strains may produce unknown toxins. Two expert reports submitted to JECFA in 1988 concluded that the production of toxins was highly unlikely (see Annex 7.1.3). The long history of use as an enzyme source, the numerous toxicological studies and the two expert reports caused JECFA to review its decision in 1990 and change the ADI for enzyme preparations derived from *Aspergillus niger* into "not specified."(See Annex 7.1.4 to this notification for JECFA's 1990 review)

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

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

The *Aspergillus 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 modified asparaginase production strain AGN7-41 (DS59578) 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.

Based on the genetic modification performed (see Section 2.2), there is no reason 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 *Aspergillus 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).

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 modified asparaginase production strain is not able to produce any toxins under standard submerged fermentation conditions. The results of these tests showed that the production strain does not produce such 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, the FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *Aspergillus niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132), and lipase (GRN 000111 and GRN 000158). They also recently summarized the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer et al., 2006). More recently, several enzyme preparations, from genetically modified *Aspergillus niger* strains, derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier, such as phospholipase A_2 (donor: pig, see GRAS notification GRN 000183), wild-type asparaginase (donor: *Aspergillus niger*, see GRAS notification GRN 000214), lipase (donor: synthetic gene, see GRAS notification GRN 000296), and a carboxypeptidase (donor: *Aspergillus niger*, see GRAS notification GRN 000345) have been notified to FDA as GRAS.

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 accepted 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 consumed as 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 AGN7-41, over-expressing the modified 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 1983, which was extended by the IFBC into the decision tree format and published in 1990. In 2001, Pariza and Johnson published an update. Furthermore, the safety of protein engineered enzymes has also been discussed in literature (Pariza and Cook, 2010) where the authors conclude that protein engineered enzymes, when derived from non-toxic proteins and hosts can be considered safe and toxicity studies are not needed. There is no evidence that evolution and natural variation within enzyme families has not resulted in the generation of toxins active via the oral route. DSM's decision tree analysis, based on the most recent update of the decision tree, is described in Annex 7.2.

Furthermore, enzyme preparations of the wild-type asparaginase from *Aspergillus niger* have been evaluated in a number of toxicity studies in which no adverse effects or mutagenic activity were discovered. The results of theses studies as well as studies performed with the PreventASe ASG enzyme are summarized in Section 7.4.1.

7.2.1. Allergenicity

Virtually all food allergens are proteins, although only a small percentage of proteins are allergens (Taylor, 1987). Any food containing protein has the potential to cause allergic reactions, however a few food groups are known to cause allergies more frequently than others. These major allergenic food groups are: milk, egg, fish, crustacea (shrimp, lobster, crab), soybeans, peanuts, tree nuts and wheat. Allergens from these food groups account for more than 90% of food allergic reactions. The prevalence of allergic sensitivities to specific foods obviously varies between countries, depending on the frequency with which

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

Based on the potentially life threatening consequences of allergic reactions to food and the possibility to introduce new antigens by genetic engineering, hazard identification concerning the allergenicity of newly engineered foods/food components is essential. The risk evaluation is done by a weight-of-evidence approach and is performed in several steps: First, it is investigated whether or not the source of the newly expressed protein has a history of allergenicity. Then, a search for sequence homologies and/or structural similarities between the new protein and known allergens is performed. Furthermore, resistance to degradation by the proteolytic enzyme pepsin can be assessed *in silico*. Where necessary, other, additional tests may be used.

For DSMs asparaginases, the risk evaluation is described below:
The source of the DSM asparaginases (wild-type and PreventASe ASG) is *Aspergillus niger*. *Aspergillus niger* does not belong to the eight major groups of food allergens. Furthermore, *Aspergillus niger* is not considered an allergen producer. *In silico* sequence comparison with known allergens did not result in any match with known food allergens for neither the wild-type asparaginase nor PreventASe ASG. For this screening, the databases Allermatch (www.*Allermatch.org*) and SDAP (http://fermi.utmb.edu/SDAP/sdap_who.html) were used. The databases are (mainly) based on WHO-IUIS, SwissProt, TrEMBL and UniProt allergen data. The search was performed following the guidelines from the FAO/WHO (FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 2001), who consider a protein a potential allergen when there is more than 35% homology between the amino acid sequence of the tested protein and a known allergen using a sliding window of 80 amino acids and a suitable gap penalty. Since DSM PreventASe ASG asparaginase did not show any relevant match with known food allergens during this screening, it is considered unlikely that the enzyme will produce an allergenic or sensitization response upon oral consumption.

In addition, *in silico* analysis of pepsin digestibility of the protein sequence using the program "PeptideCutter" (ExPASy) revealed easy degradability. For DSM's *Aspergillus niger* wild-type asparaginase preparations no allergic reactions in consumers have been reported so far.

Furthermore, exposure of consumers to enzymes used as processing aids in food is usually very low, which renders the likelihood of allergic sensitization to these proteins to virtually zero. The absence of food allergenicity has been confirmed by an extensive literature search and survey of producers' files, in which no cases have been reported describing sensitization or allergic reactions after 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.

Taken together, the PreventASe ASG enzyme is therefore considered not to be allergenic when ingested with food.

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

The safety of the production organism is the main point of attention when assessing the safety of enzymes used in food processing. In this case, the production organism, *Aspergillus niger*, has been demonstrated to

be nonpathogenic and nontoxigenic under the fermentation conditions used 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, *Aspergillus niger* is included in the authors' listing of the organisms being commonly used in the industry.

The FDA has also accepted the Enzyme Technical Association's and its members' GRAS Notifications stating that lipase (GRN. 000111 and GRN 000158), asparaginase (GRN 000214) and carboxypeptidase (GRN 000345) enzyme preparations from *Aspergillus niger* are generally recognized as safe. *Aspergillus niger* is listed as a production organism for enzymes (Pariza and Johnson, 2001) and has a long history of safe use (see Annex 7.1.1).

As is clear from the information provided in this notification, there have been genetic modifications to the *Aspergillus niger* used by DSM, but these genetic modifications are well characterized and specific in that the DNA encoded is host-own and does not express any harmful or toxic substances. 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 publication of Pariza and Foster (1983). 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. For the DSM host strain ISO 528 this information is found in Section 2.

The activity of asparaginase is very comparable with the one of glutaminase (see Section 7.2.3), 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).

Asparaginase from *Aspergillus niger* (GRN000214) and asparaginase from *Aspergillus oryzae* (GRN 000201) have been notified as GRAS to the FDA, and the FDA had no questions.

Because of its medical use, for which the enzyme is administered intravenously, asparaginase has been evaluated in a number of toxicological studies. However, the studies were performed in the 1960's and 1970's and their quality is unknown. LD_{50} values are available for asparaginases produced in *Escherichia coli* or *Erwinia carotovora*, showing oral toxicity of at least a factor 10 lower than after parenteral (intravenous) administration ($LD_{50\ oral}$ >5000-7500 kU/kg bw vs. $LD_{50\ i.v.}$ 50-750 kU/kg bw) (Ohguro et al., 1969; Lorke and Tettenborn, 1970). The rabbit seems to be exceptionally sensitive with a LD_{50} (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 hypersensibility, hematological effects and changes in some blood chemical parameters. In addition, fatty deposition in liver cells was observed (Ohguro et al., 1969). Furthermore, developmental toxicity studies have been performed (Ohguro et al., 1969; Lorke and Tettenborn, 1970; Adamson and Fabro, 1968; Adamson et al., 1970). In these tests, rats and mice were administered intravenously or intraperitoneally with asparaginase at different periods during pregnancy. Observed effects included 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.Furthermore, developmental effects were not observed when the DSM asparaginase was given via the oral route.
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 cancer therapy, while DSM's intended application is in food. In general, toxic effects are less severe after oral administration compared to parenteral injection due to lower bioavailability after oral exposure and chemical interactions (deactivation/degradation) in the stomach and gastrointestinal tract.

Second, the enzyme is inactivated in the intended food applications during baking, resulting in a denatured protein (see 6.4.4), whereas for medical use the enzyme is active. The therapeutic 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 growth and viability. The high requirement for asparagine in fast growing tissues like an embryo is probably also the reason for the embyrotoxic properties of intravenously administered asparaginase. Again, consumers of asparaginase treated food products are only exposed to inactive enzyme via the oral route. Thus, the effects that are observed in studies with asparaginase of *Escherichia coli* and *Erwinia carotovora* administered intravenously are not expected after oral consumption of processed food treated with asparaginase from *Aspergillus niger*.

These arguments show that the use of the PreventASe ASG asparaginase in food applications is unlikely to produce any adverse effects in humans.

7.2.3. Substantial Equivalence

Several expert groups have discussed the concept of substantial equivalence in relation to food safety assessment of enzymes. 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 tests other than those for the existing ingredient are necessary. This concept is also accepted by the FDA, which in addition has evaluated the safety aspects of differences in glycosylation but concluded that chemical modification or site-directed mutagenesis would not raise safety concerns and the proteins are still considered substantially equivalent.

The following arguments describe why DSM considers its PreventASe ASG asparaginase as substantially equivalent to known enzymes:

a) asparaginase (EC 3.5.1.1) and glutaminase (EC 3.5.1.2) are substantially equivalent:
   - Both enzymes catalyze a comparable chemical conversion; the enzymes hydrolyze an amino acid and release ammonia and an acid amino acid.
   - Both enzymes have similar substrates: the two amino acids asparagine and glutamine differ only by one carbon atom in the side chain
   - glutaminases and asparaginases have also hydrolyzing activity towards the substrate of the other enzyme, i.e. asparaginase hydrolyzes glutamine and glutaminase asparagine.
     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, low Km values for both substrates.
   - 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 generally available metabolites. They are, however, quite abundant in the human diet. Humans can readily convert one into the other amino acid. Hence, there is no basis to believe that conversion of asparagine to aspartate will have significant effects on the human body as is the conversion of glutamine to glutamate without any effect.

b) The PreventASe ASG asparaginase is substantially equivalent to the *Aspergillus niger* wild-type enzyme:
   - Both enzymes catalyze the same reaction, i.e. hydrolysis of asparagine to aspartic acid and ammonia
   - Both enzymes are based on the asparaginase sequence of *Aspergillus niger* and have 77.5 % homology based on amino acid sequence.
   - Both enzymes are expressed in *Aspergillus niger* under GMP, using food grade ingredients.

7.3. Safety of the Manufacturing Process

PreventASe ASG 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 and is subject to testing to assure the enzyme product meets the stated specification. 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 DSM's asparaginase preparations. It includes studies performed with both, the *Aspergillus niger* wild-type enzyme and with the PreventASe ASG enzyme. All safety studies were performed according to internationally accepted guidelines (OECD or FDA) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

7.4.1. Summary of Safety Studies

The following studies were performed for the *Aspergillus niger* wild-type asparaginase enzyme preparation:
- Ames test
- Chromosome aberration test
- Mouse lymphoma assay
- 90-day oral toxicity study in rats
- Developmental toxicity study in rats

The following studies were performed with the PreventASe ASG asparaginase enzyme preparation:
- Ames test

The safety studies for the *Aspergillus niger* wild-type asparaginase were performed on batch APE.0501 containing 4160 ASPU/g and 11.8 %TOS (liquid batch) and batch APE.0604 containing 34552 ASPU/g and 89.7% TOS (spray dried). Batch APE.0604 is derived from strain ASP-72 which contains 16 copies of the

wild-type *Aspergillus niger* AspA gene integrated by site-directed recombination. Batch APE.0501 is produced by a predecessor strain of ASP-72, i.e. the gene coding for asparaginase was integrated in a random way in the genome. The Ames test for the PreventASe ASG asparaginase was performed with batch APE.GRZ.1122 containing 4100 NASPU/g and 11.3% TOS derived from AGN7-41 containing 5 copies of the protein engineered asparaginase as described in the previous sections. In all tests, the active, non-denatured enzyme was applied.

Results of the Safety Studies

The *Aspergillus niger* **wild-type asparaginase** enzyme preparation of batch APE.0501 was not mutagenic in the Ames test neither in the presence nor the absence of S9 metabolic activation up to the highest dose tested. In the chromosome aberration test, the enzyme preparation was found to be cytotoxic, but did not lead to an increase in the number of cells with chromosome aberrations at any of the concentrations or time points analyzed. It was therefore concluded that the wild-type asparaginase enzyme preparation is not clastogenic for cultured human lymphocytes. In the mouse lymphoma assay, the wild-type asparaginase preparation again showed cytotoxicity, but no mutagenic activity at the TK-locus of the mouse lymphoma L5178 cells. Comparable results were obtained with batch APE.0604, which was not mutagenic in the Ames test and showed cytotoxicity but no clastogenicity in the cultured human lymphocyte chromosome aberration test.

Based on these results, the *Aspergillus niger* wild-type asparaginase preparation was considered non-mutagenic and non-clastogenic.

In the sub-chronic 90-day oral gavage toxicity study in rats with batch APE.0501, a NOAEL of 2300 mg/kg bw was established at the highest dose tested. This corresponds to 271 mg TOS/kg bw/day or 9569 ASPU/kg bw/day. The 90-day oral feed study in rats with batch APE.0604 revealed a NOAEL of 1157 mg/kg bw/day for males and 1331 mg/kg bw/day for females which was the highest dose. This corresponds to 1038 mg TOS/kg bw/day or 40000 ASPU/kg bw/day for males and 1194 mg TOS/kg bw/day or 46000 ASPU/kg bw/day for females.

In the developmental toxicity study performed with batch APE.0501, in which rats were treated with asparaginase preparations by oral gavage from gestation day 0-19, a NOAEL of 8500 mg/kg bw was observed (corresponding to 1001 mg/kg bw/day TOS or 35364 ASPU/kg bw/day). This study showed no effects on the 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 at the highest dose tested. In a second developmental study, rats were administered the asparaginase enzyme of batch APE.0604 via feed from gestation day 0 to 21. The NOAEL of this study was found to be at the highest dose tested, corresponding to 1205 mg/kg bw/day (or 1081 mg/kg bw/day TOS or 41600 ASPU/kg bw/day).

The **PreventASe ASG asparaginase** enzyme preparation was tested in the Ames test and was not mutagenic in any of the strains tested when incubated in the absence or presence of S9 metabolic activation.

Taking together the results of all safety studies and the published literature (Pariza and Foster (1983), Pariza and Johnson (2001), Olempska-Beer (2006) and van Dijck (2003)), asparaginase expressed in *Aspergillus niger* is considered safe for human consumption.

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 for adults:

Final food	Residual amount of (inactive) enzyme in final food (NASPU/kg)	90th percentile intake level (g food/per person/day)[1]	Estimated daily intake of (inactive) enzyme (NASPU/kg bw/day)[2]	Estimated daily intake of TOS (mg/kg bw/day)
Wheat-based products[3]	67 - 1365	260	0.3 - 5.9	0.007 - 0.146
Cereal-based products[4]	69 - 1425	60	0.07 - 1.43	0.002 - 0.035
Potato-based products	165- 3000	58	0.16 - 2.9	0.004 - 0.07
Total			**0.52 - 10.2**	**0.01-0.25**

[1] Intake levels of bread, cereal based products and potato based products are based on Wilkinson-Enns et al. (1997). 90th percentile is 2-times the intake level (FDA Guidance 2006).
[2] Calculated for a person of 60 kg.
[3] Includes 3 categories: i. Yeast bread and rolls, ii. Quick breads, pancakes and French toast and iii. Cakes, cookies, pastries and pies.
[4] Crackers, popcorn, pretzels and corn chips and ready-to-eat cereals.

The 90-day oral toxicity study of the wild-type enzyme (batch APE.0501) showed a NOAEL of 2300 mg enzyme preparation/kg bw/day, corresponding to 271 mg TOS/kg bw/day. This study shows the lowest NOAEL in terms of TOS/kg bw/day among the two batches of asparaginase tested in the 90-day study. Assuming similar safe levels for the PreventASe ASG enzyme, safe amounts of 2935 mg/kg bw/day (or 11328 NASPU/kg bw/day) can be calculated. This results in a safety margin of 1111 to 21785.

8. Conclusion

Based on the results of the safety evaluation and review of the published literature it can be concluded that the PreventASe ASG asparaginase is safe for its intended use aiding in the reduction of acrylamide levels in food. Toxicity evaluation did not raise any concern and the estimated safety margins are high. This is further supported by the long history of safe use of *Aspergillus niger* as production organism for food enzymes and the natural occurrence of asparaginase in plants and animals consumed as foods. Furthermore, the conclusions are supported by many studies reported in literature. Based upon these factors, as well as upon the limited and well-characterized genetic modifications and the standardized production of the enzyme preparations, it is DSM's conclusion that PreventASe ASG asparaginase preparation from *Aspergillus niger* is GRAS for its intended use.

9. LIST OF ANNEXES

2.1.1	Selten, G.C.M., Gorcom, R.F.M. van, Swinkels, B.W., *Selection Marker Gene Free Recombinant Strains: A Method for Obtaining Them and the Use of These Strains*, European Patent Application EPO0635574A1 (1995)
2.1.2	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 pGBTOPAGN-7
2.2.2	Nucleotide sequence of the expression unit
2.2.3	Map of the selectable marker plasmid
2.4	Surviving studies of *Aspergillus niger* strains in soil, surface water, and waste water
3.2	Amino acid sequence of modified asparaginase from *Aspergillus niger*
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6.2.1.1	Asparagine and acrylamide reduction by the use of PreventASe ASG asparaginase from *Aspergillus niger* in wheat-based dough - results of laboratory scale experiments
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6.2.3	Asparagine reduction by the use of PreventASe ASG asparaginase from *Aspergillus niger* in potato dough - results of laboratory scale 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. Micobiol. Biotechnol. 59:426-435 (2002).
7.1.2	JECFA safety evaluation of *Aspergillus 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 *Aspergillus niger* as a source for enzymes to be used in food, 1990
7.2	Safety evaluation using the Pariza & Johnson decision tree of modified asparaginase from *Aspergillus niger* AGN7-41
7.2.1	Report from the Amfep Working Group on Consumer Allergy: risk from enzyme residues in food (August 1998)

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11. Annexes

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Annex 2.1.1

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Pages 000041-000093 have been removed in accordance with copyright laws. The removed reference is:
Selten, G.C.M., Gorcom, R.F.M. van, Swinkels, B.W., Selection Marker Gene Free Recombinant Strains: A Method for Obtaining Them and the Use of These Strains, European Patent Application EP00635574A1 (1995).

Annex 2.1.2

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Pages 000095-000103 have been removed in accordance with copyright laws. The removed reference is:
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. Toxic& Pharmacol. 38:27-35 (2003).

Annex 2.2.1

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Annex 2.2.2

Annex 2.2.2 Nucleotide sequence of the expression unit of the PreventASe ASG asparaginase

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Molecule:       pGBTOPAGN-7,  8303 bps DNA Circular
File Name:      pGBTOPAGN-7.cm5,  dated 06 Dec 2011
Description:    Ligation of inverted aspV7 into pGBTOP-12*EcoRI*SnaBI
Printed:        1 to 8303 bps (Full)

   1  tggcctggga tgcaatttct ttcaattccc ggggattcgc cgtccccggt cgtgcgtggt
  61  gacttgattg gctggtataa cgatggtact ataactaacg ggaagctata acgcaggtcc
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Annex 2.2.3

(b) (4)

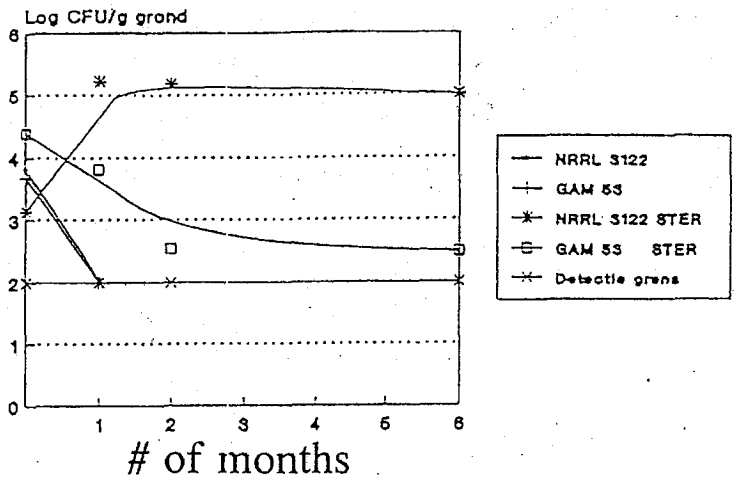
Annex 2.4

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

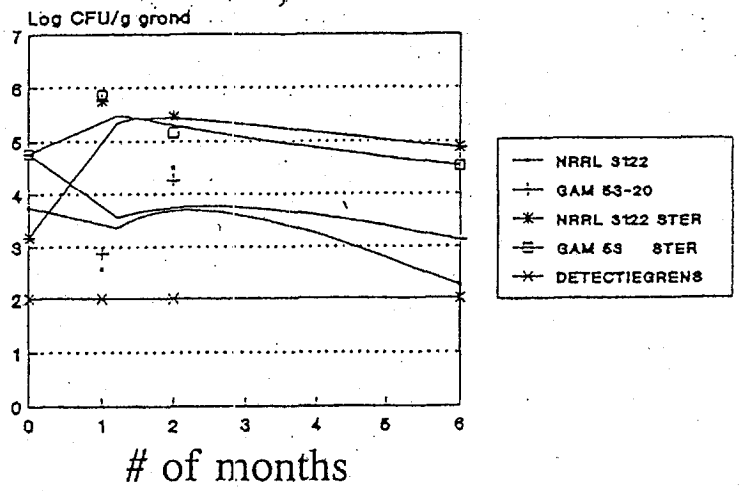
Aspergillus niger was inoculated in three different environments, namely: soil, surface water and sewage (waste water of Gist-brocades production plant in Delft (The Netherlands)).
As positive control, that is to say to judge whether the environment contained sufficient substrates for the organisms to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

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

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

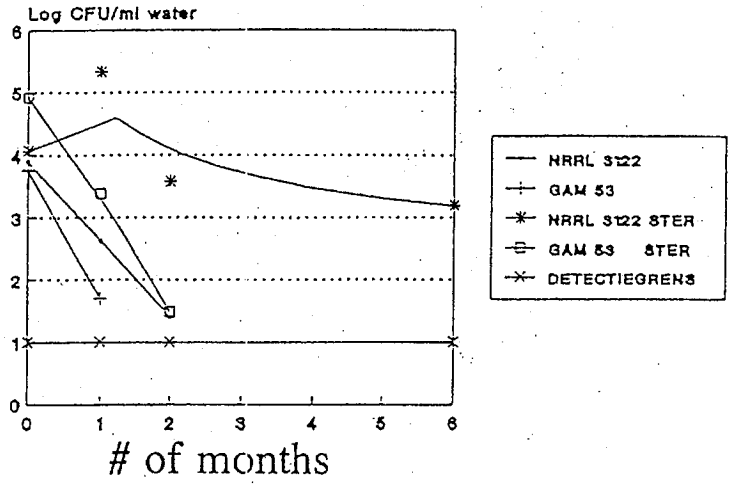


Incubation of *A. niger* mycelium in soil, 25^0C

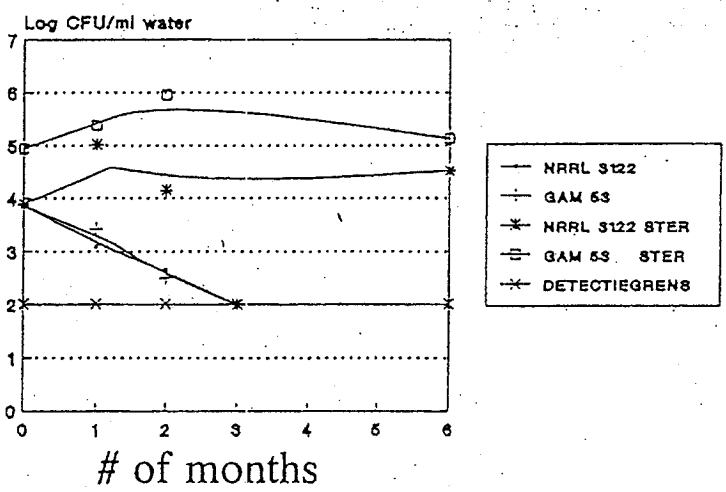


Surviving of *Aspergillus niger* I

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

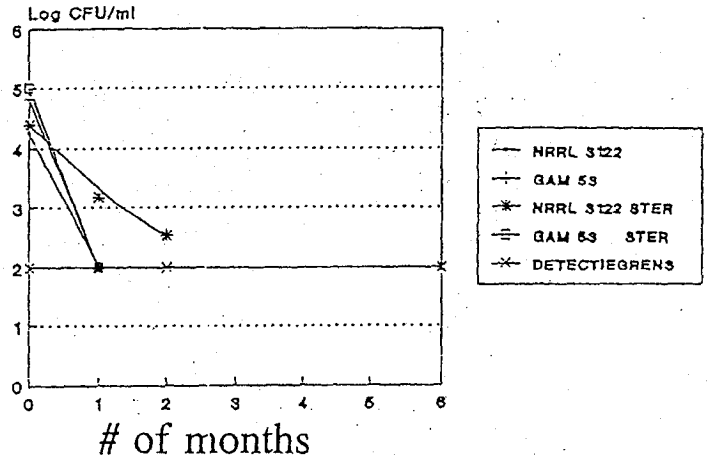


Incubation of *A. niger* mycelium in surface water, 25^0C

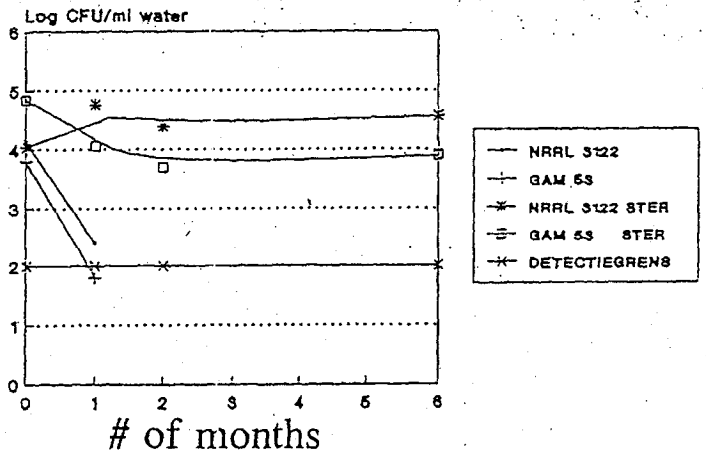


Surviving of *Aspergillus niger* II

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



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



Surviving of *Aspergillus niger* III

Annex 3.2

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Annex 3.2 Amino Acid Sequence of Asparaginase PreventASe ASG

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SYQDMQNDTLYSAIDNGAKGIVIAGSGAGSVSTGFSDAIDDIASKHSIPIVLSTRTGNGEVPTSDVSSDTATHIASGFLNPQKARILLG
LLLAEGKGFKEIREVFAKVTVA

The predicted signal peptide, directing transport of the protein into the endoplasmic reticulum of *Aspergillus niger* and, ultimately, into the extracellular medium is underlined.

Comparison of the amino acid sequences of the wild type *Aspergillus niger* asparaginase and the PreventASe ASG asparaginase

```
WT AS    MPLKPILLSA LASLASASPL LYSRTTNETF VFTNANGLNF TQMNTTLPNV TIFATGGTIA GSDSSSTATT GYTSGAVGVL
PrevASG  .......... .......... ....A..T.Y ....S..... .......... ..L....... ..SADN.... ...A..I.IQ

WT AS    SLIDAVPSML DVANVAGVQV ANVGSEDITS DILISMSKKL NRVVCEDPTM AGAVITHGTD TLEETAFFLD ATVNCGKPIV
PrevASG  T......E.. .......... .....P.V.. S..L..A.T. .E...D.... S......... .......... ..........

WT AS    IVGAMRPSTA ISADGPFNLL EAVTVAASTS ARDRGAMVVM NDRIASAYYV TKTNANTMDT FKAMEMGYLG EMISNTPFFF
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WT AS    MRTVNGEVPL SDVSSDTATH IASGYLNPQK SRILLGLLLS QGKNITEIAD VFALGTDA
PrevASG  T..G.....T .......... ....F..... A........A E..GFK..RE ...KV.V.
```

Annex 3.4

000119

Biochemical Properties of PreventASe ASG

1. Summary

The following biochemical characteristics of the asparaginase enzyme preparation PreventASe ASG from *Aspergillus niger* were investigated:

- activity at different pH and pH optimum
- activity at different temperatures and temperature optimum
- 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 measured using an adaptation of the standard protocol for determination of asparaginase activity. In order to cover a pH range from 3 to 9.5, the standard citric acid buffer was replaced by a 50 mM solution of citric acid and potassium pyrophosphate. 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 3 to 9.5 with 0.5 pH unit increments. For the temperature profile the activities were measured at pH 5 - 9 at temperatures of 37, 50, 60, 70 and 80°C. Incubation time was 30 minutes. Activities were determined by measuring formed ammonia on the Konelab Arena 60 analyzer based on the Berthelot reaction, which makes use of alkaline hypochlorite and phenol nitroprusside are reagents.
In order to determine the molecular weight and the iso-electric point of the asparaginase, an SDS-PAGE and iso-electric focusing was performed with enzyme-containing concentrated ultra-filtrate (UF). The SDS-PAGE also gives an indication of the purity of the UF concentrate.

3. Results

3.1. pH profile

Figure 1 shows the pH profile of PreventASe ASG. The enzyme has a very broad pH spectrum, which is on or near maximum between pH 5.5 and 7. Below pH 3.5 no activity can be measured anymore.

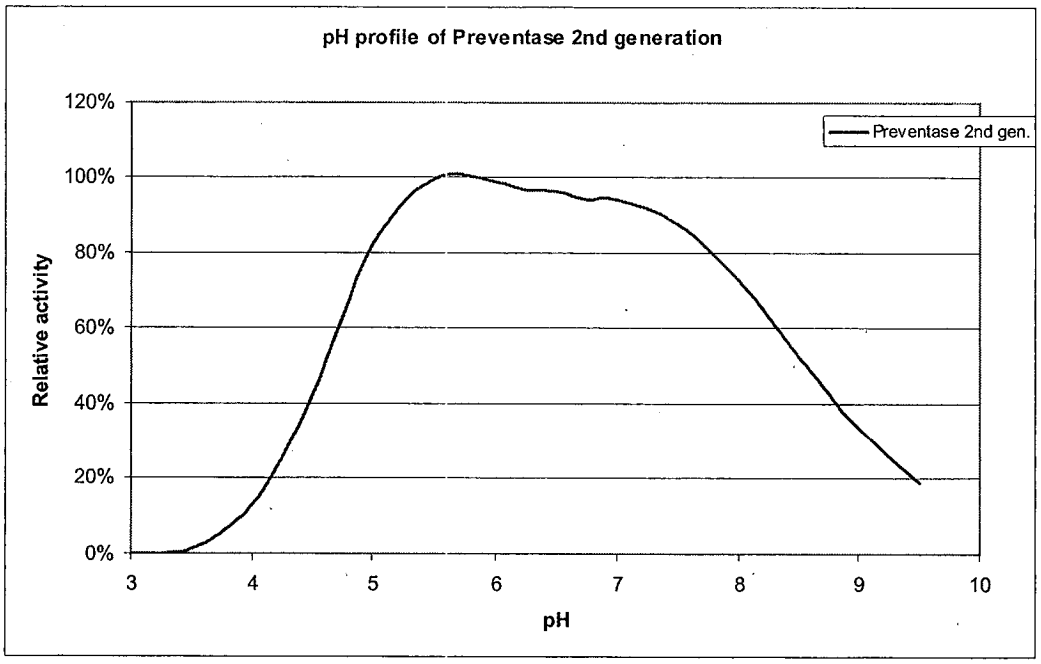


Figure 1 **Relative activity of PreventASe ASG between pH 3 and 9.5 at 37°C.**

3.2. Temperature profile

Figure 2 shows the temperature profiles of PreventASe ASG at different pH. The temperature optimum lies between 50 and 60 °C at all pH conditions. The enzyme is inactivated at temperatures above 70 °C.

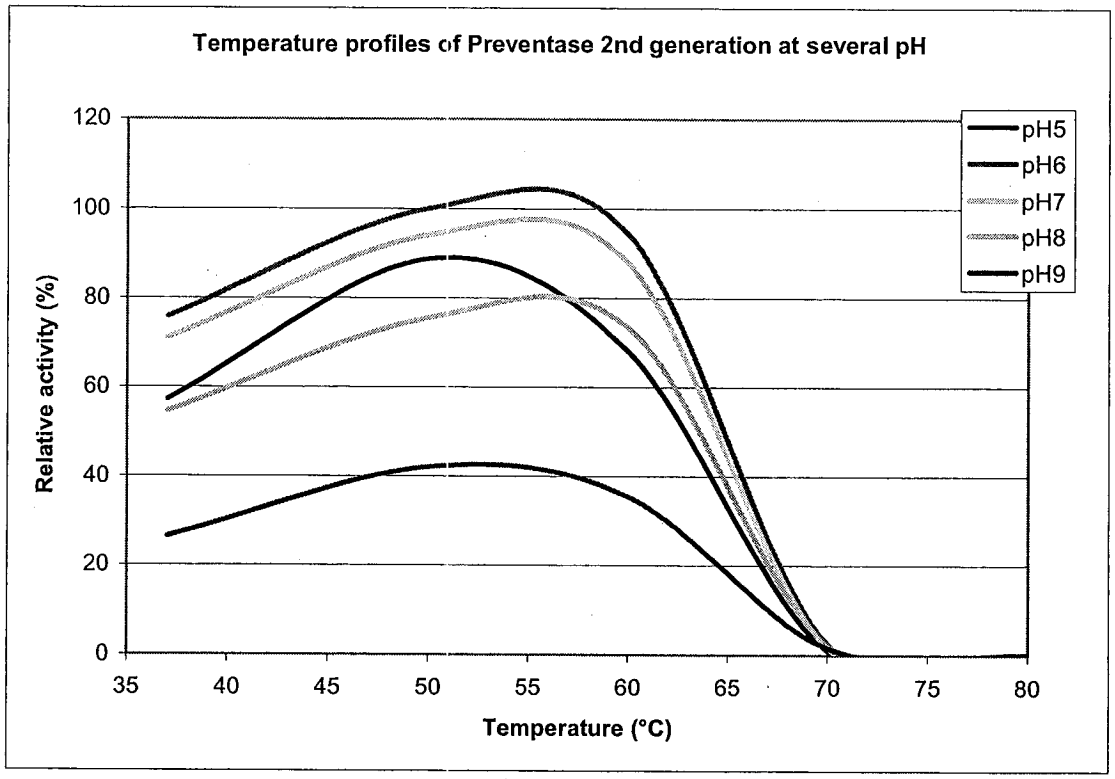


Figure 2 **Temperature profiles of PreventASe ASG at different pH (pH range 5-9) relative to the measurement at 50°C, pH6, which was set at 100%.**

3.3. Molecular weight and iso-electric point of the asparaginases

Figure 3 and 4 show the results of an IEF-gel and SDS-PAGE performed on the asparaginase containing UF concentrates. PreventASe ASG has a molecular weight of approx. 50 kD (due to glycosylation; deglycosylated samples show molecular weights of ~40 kD; not shown) and an iso-electric point of ~4.4.

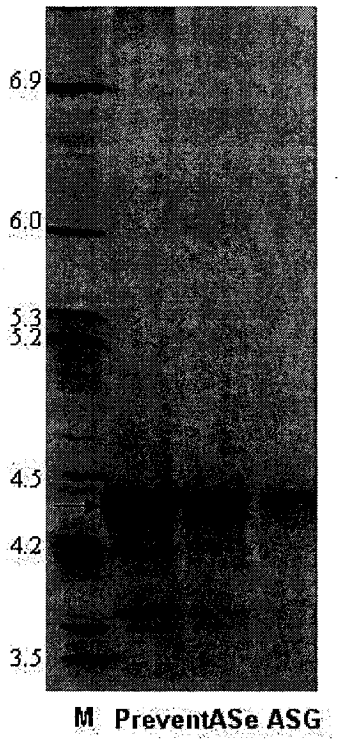


Figure 3 Image of the IEF gel loaded with three concentrations of PreventASe ASG (~3, 1 and 0.3 mg/mL protein). The arrow indicates the PreventASe main band. M represents IEF marker with iso-electric points as indicated.

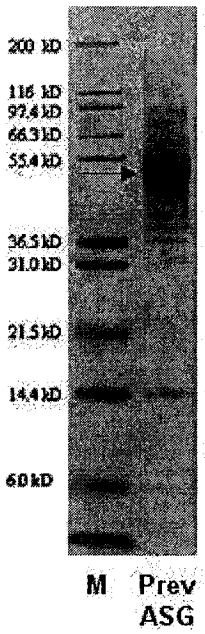
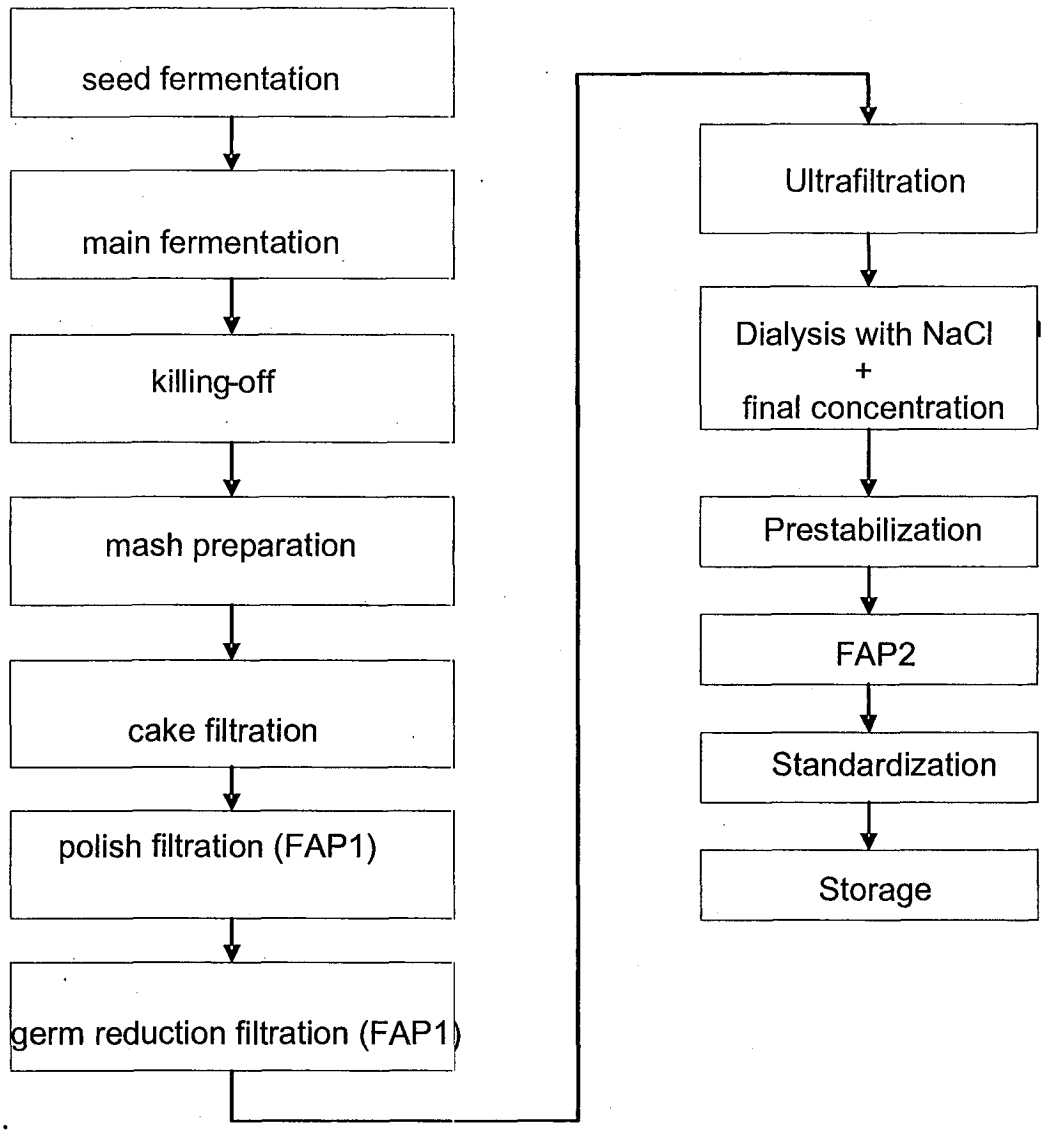


Figure 4 Image of an SDS-PAGE gel loaded with PreventASe ASG. The arrow indicates the PreventASe ASG main band. M represents the molecular weight marker with sizes as indicated.

Annex 4.1

Annex 4.1 Flow Diagram Manufacturing Process



Annex 4.2

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

September 11, 2003

Mr. Gary Yingling
Kirkpatrick & Lockhart LLP
1800 Massachusetts Avenue, NW
Second Floor
Washington, DC 20036-1221

Dear Mr. Yingling:

You requested, on behalf of the Enzyme Technical Association, that OFAS review the use of certain defoaming and flocculating agents in the manufacture of enzyme preparations used in food. You provided information related to these compounds in your letters of December 20, 1996 (to Dr. Alan Rulis), 4-24-1998 (to Dr. Zofia Olempska-Beer), and 11-30-99 (to Dr. Zofia Olempska-Beer). You also arranged for a teleconference between ETA members and OFAS representatives, facilitated telephone contacts with technical experts from ETA member companies, and responded to numerous requests for clarification. We appreciate your and ETA's cooperation.

We reviewed the information on defoaming and flocculating agents that you submitted as well as the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. The enclosed attachment provides a brief overview of our evaluation and itemizes the evaluated defoamers (Table 1) and flocculants (Table 2). We conclude that these compounds are used by enzyme manufacturers in accordance with the principles of good manufacturing practice (GMP).

Sincerely yours,

(b) (6)

Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition

Defoaming and Flocculating Agents Used in the Manufacture of Enzyme Preparations Used in Food

Enzyme Preparations

Most enzymes currently used in food are derived from microorganisms. The manufacturing process of such enzymes includes three major steps: fermentation, enzyme recovery, and enzyme formulation. The formulated products are generally referred to as enzyme preparations. In addition to the enzymes of interest, enzyme preparations contain added substances such as diluents, preservatives, and stabilizers. They may also contain metabolites derived from the production microorganism and the residues of substances used in the manufacturing process, such as components of the fermentation medium or defoaming and flocculating agents used during fermentation and recovery. When FDA reviews safety data on enzyme preparations, it considers all components of the preparation.

Defoaming Agents

Defoaming agents (defoamers) are used by enzyme manufacturers to reduce or prevent foaming during fermentation and recovery. They are formulated with ancillary ingredients such as surface-active agents or carriers. Defoamers currently used in the manufacture of food enzymes are listed in Table 1. The Table includes five major defoamers that are identified by a double asterisk and several compounds that are used either as secondary defoamers or ancillary ingredients in defoamer formulations.

The major defoamers are added to the fermentation broth at levels within the range of 0.05-1% on a weight basis. Some of these defoamers, for example, polyoxyethylene-polyoxypropylene block copolymer, may contain trace levels of ethylene oxide, propylene oxide, and 1,4-dioxane which are known to cause cancer in laboratory animals. The Office of Food Additive Safety (OFAS) has evaluated the use of defoamers listed in Table 1 and determined that human exposure to the residues of these defoamers in enzyme preparations does not present human safety concern.

Flocculating Agents

Flocculating agents (flocculants) are used in the enzyme recovery step to separate microbial cells and cell debris from the fermentation broth containing the dissolved enzyme. The flocculation typically consists of two steps - primary flocculation and secondary flocculation. In the primary flocculation, inorganic salts (such as calcium chloride or aluminum sulfate) or "low molecular weight" polymers (such as polyamines) are used to agglomerate the cellular debris. The primary flocculation is usually followed by the secondary flocculation in which "high molecular weight" polymers are used to aid the formation of larger agglomerates that are subsequently removed by centrifugation or filtration. The polymers used as flocculants can be either cationic or anionic. The cationic polymers are added to the fermentation broth at levels not higher than 1% on a

weight basis. The anionic polymers are used at levels at or below 0.025%.

The flocculants used in the manufacture of food enzymes are listed in Table 2. They include inorganic salts, polyamines, and polyacrylamides. Several of these compounds are regulated in 21 CFR either as food additives or GRAS substances. Certain polyamines may contain traces of epichlorohydrin and 1,3-dichloro-2-propanol. Polyacrylamides usually contain very low levels of acrylamide. These contaminants of polyamines and polyacrylamides are known to cause cancer in laboratory animals. OFAS has evaluated all polymers included in Table 2 and determined that human exposure to the residues of these flocculants in enzyme preparations does not present human safety concern.

Sources of Information on Defoamers and Flocculants

OFAS compiled data on defoamers and flocculants listed in Tables 1 and 2 using information voluntarily submitted by the Enzyme Technical Association. OFAS also relied on the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. Other sources of information included published articles, computer searches, and Material Safety Data Sheets issued by manufacturers of defoamers and flocculants.

Table 1. Defoamers Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Polypropylene glycol**	25322-69-4	Average MW: 2000
Polyglycerol polyethylene-polypropylene glycol ether oleate**	78041-14-2	
Polyoxyethylene-polyoxypropylene block copolymer**	9003-11-6	Average MW: 2000
Polypropylene glycol monobutyl ether**	9003-13-8	
Polydimethylsiloxane**	63148-62-9 68083-18-1	
Silica	7631-86-9 63231-67-4	
Stearic acid	57-11-4	
Sorbitan sesquioleate	8007-43-0	
Glycerol monostearate	123-94-4	
Polysorbates (polyoxyethylene sorbitan fatty acid esters)		Polysorbate 60 (CAS No. 9005-67-8), Polysorbate 65 (CAS No. 9005-71-4), and polysorbate 80 (CAS No. 9005-65-6) are regulated as food additives and components of defoamer formulations
Rape oil mono- and diglycerides	93763-31-6	
White mineral oil	64742-47-8	

Table 2. Flocculants Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Dimethylamine-epichlorohydrin copolymer	25988-97-0	Cationic polyamine
Methylamine-epichlorohydrin copolymer	31568-35-1	Cationic polyamine
Dimethylamine-epichlorohydrin-ethylenediamine terpolymer	42751-79-1	Cationic polyamine
Polyacrylamide modified by condensation with formaldehyde and dimethylamine	67953-80-4	Cationic polyacrylamide
Acrylamide-acryloxyethyl-trimethyl-ammonium chloride copolymer	69418-26-4	Cationic polyacrylamide
Acrylamide-acrylic acid copolymer	25987-30-3 9003-06-9	Anionic polyacrylamide
Aluminum sulfate	10043-01-3	
Calcium chloride	10035-04-3 10043-52-4	

Annex 6.2.1.1

Annex 6.2.1.1 Asparagine and acrylamide reduction by the use of PreventASe ASG asparaginase from *Aspergillus niger* in wheat-based dough - results of laboratory scale experiments

Asparagine and Acrylamide Reduction by the Use of PreventASe ASG produced in *Aspergillus niger* in savoury crackers

Results of laboratory scale experiments

Summary

Wheat-based dough for savoury crackers (pH 8) were treated with different amounts of the PreventASe ASG asparaginase to evaluate the reduction of the free amino acid L-asparagine in the dough and the acrylamide contents in the crackers after baking. The results show that treatment of the dough with 1000 NASPU/kg flour of PreventASe ASG results in almost complete hydrolysis of free asparagine and a subsequent reduction of cracker acrylamide content by almost 90%. The data support the finding that there is a direct correlation between asparagine content in the dough and acrylamide formation in the baked product.

Materials and Methods

Dough preparation

Cracker dough was prepared according to the following recipe:

Ingredient	Unit	Amount	Percentage of Ingredient
Shortening	g	60	7.1 %
Lecithin	g	1.7	0.2 %
Sugar	g	11.7	1.4%
Egg	g	24.2	2.9%
Skim milk powder	g	20	2.4%
Invert sugar	g	18.3	2.2%
Glucose syrup (42 DE)	g	21.7	2.6%
Water (45°)	g	146.06 to 146.7	17.3- 17.4%-
Salt	g	6.7	0.8%
Wheat flour (Ysvogel)	g	500	59.4%
Ammonium bicarb.	g	3.6	0.4%
Sodium bicarb	g	7.8	0.09%
Bakezyme B500 (200ppm) (2,5gr/0,5L)	g	20	2.4%

All ingredients, except for the enzyme, were mixed. Subsequently, the diluted enzyme solution was added. The final enzyme concentrations were 130, 260, 520, 780 and 1000 NASPU/kg flour. The dough was kept to rest for 3 hours before moulding.

After the resting period, just before baking, 10 g of each dough was taken for asparagine measurement.

For this, the enzyme reaction was stopped by mixing the 10 g of the dough with 50 mL 0.1 M HCl. Subsequently, the mixture was analyzed for asparagine contents by UPLC-MSMS. Untreated dough was taken as control.

Asparagine analysis
Asparagine contents in the dough were measured using a UPLC-MSMS method. The separation is based on reversed phase chromatography using UPLC; MSMS detection is based on positive electrospray ionization. Isotope-labeled asparagine was used as internal standard. For measurement of the asparagine contents in the potato dough, the dough/HCl mixture was centrifuged and diluted again before labeling via a derivatization reaction using Waters AccQ-Fluor Reagent (6-aminoquinoyl-N-hydroxysuccinimdyl carbamate or AQC). The AccQ-Fluor reagent reacts rapidly with primary and secondary amino acids to yield highly stable carbamide derivatives.

Acrylamide analysis
Acrylamide contents were analyzed in crackers from 2 control dough and the dough treated with 260, 520 and 1000 NASPU PreventASe ASG, respectively, using a GC/MS method. The separation is based on gas chromatography; detection is based on mass spectrometry. ^{13}C-labeled acrylamide was used as internal standard.

Results

As shown in the graphs and table below, treatment with PreventASe ASG resulted in a clear and dose-dependent reduction of L-asparagine and acrylamide in the tested cracker dough (see Table 1). Using 260 NASPU/kg flour, reduced free asparagine and acrylamide by approx. 60 % compared to the untreated dough. A further reduction of both, asparagine and acrylamide, was observed with higher asparaginase doses (up to 1000 NASPU/kg flour), showing reductions by 70 - 90 % of the control dough (see Figure 1and Figure 2). A linear correlation is observed between asparagine content in the dough and acrylamide concentrations in the baked savoury crackers (see Figure 3).

Table 1 Asparagine and Acrylamide contents in dough and crackers treated with different amounts of PreventASe ASG

PreventASe ASG Units	Asparagine content in dough (mg/kg)	% Reduction compared to untreated control	Acrylamide content in baked cracker (µg/kg)	% Reduction compared to untreated control
control	62		184	
130	43	32%	nm	
260	27	57%	74	60%
520	19	71%	54	71%
780	5	92%	nm	
1000	8	88%	24	87%

nm: not measured

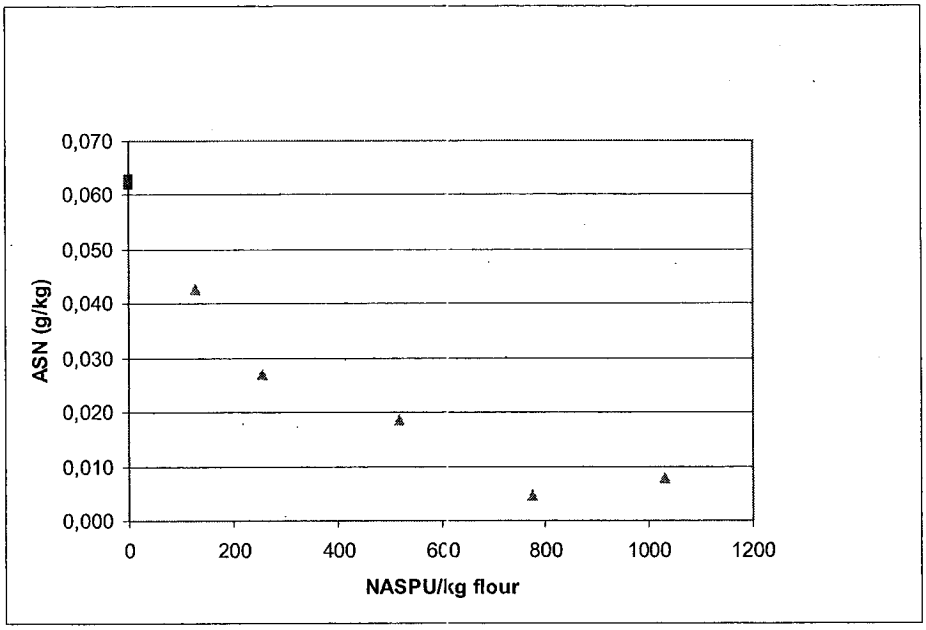


Figure 1 Asparagine content of cracker dough (pH 8) after incubation with different amounts of PreventASe ASG

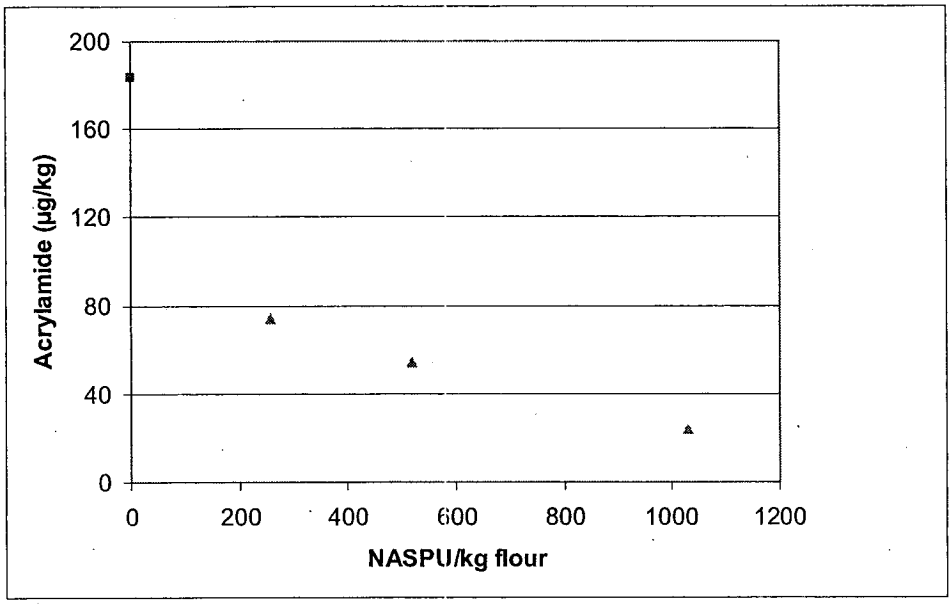


Figure 2 Acrylamide contents in savory crackers after incubation with different amounts of PreventASe ASG

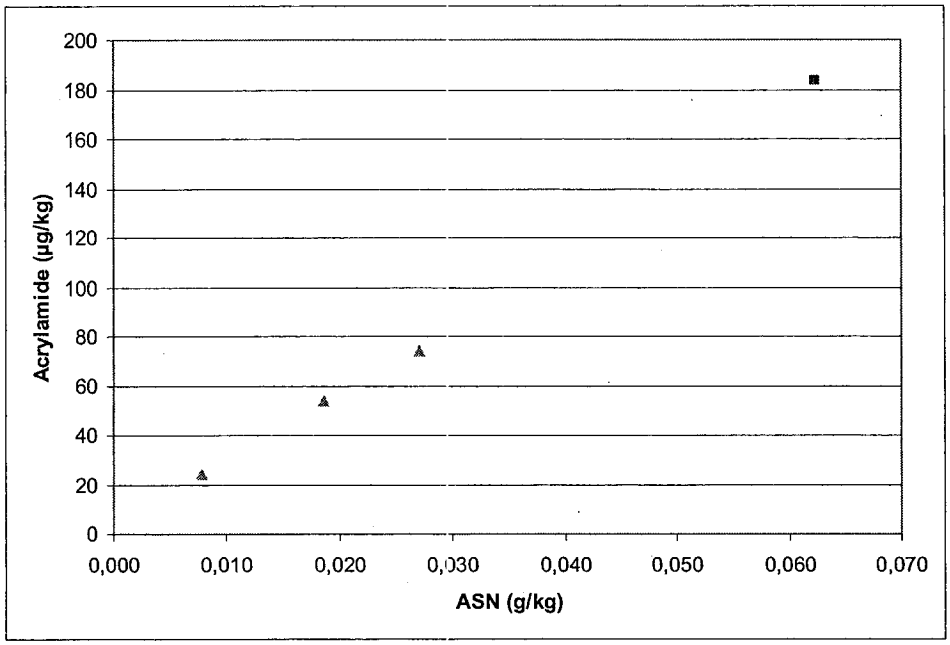


Figure 3 Correlation between asparagine content in cracker dough and acrylamide content in baked crackers

Annex 6.2.1.2

000138

Annex 6.2.1.2 Asparagine and acrylamide reduction by the use of PreventASe ASG asparaginase from *Aspergillus niger* in cookie dough - results of laboratory scale experiments

Asparagine and Acrylamide Reduction by the Use of PreventASe ASG produced in *Aspergillus niger* in cookies

Results of laboratory scale experiments

Summary

Wheat-based dough for cookies (pH 7) was treated with PreventASe ASG asparaginase to evaluate the reduction of the free amino acid L-asparagine in the dough and the acrylamide contents in the cookies after baking. The results show that treatment of the dough with PreventASe ASG results in a reduction of free asparagine and a subsequent reduction of cookie acrylamide content by almost 40-50%. The data support the finding that there is a direct correlation between asparagine content in the dough and acrylamide formation in the baked product.

Materials and Methods

Dough preparation

Cookie dough was prepared according to the following recipe:

Ingredient	Unit	Amount	Percentage of Ingredient
Frying Fat (conditioned at 26°C)	g	230	13.6%
Powdered sugar	g	294.5	17.4%
Skimmed milk Powder	g	17.5	1.0%
Salt	g	10.5	0.6%
Sodium Bicarbonate	g	3.5	0.2%
Ammonium Bicarbonate	g	2	0.1%
Water	g	130	7.7%
Flour	g	1000	59.2%
Total		1688	
PreventASe ASG	NASPU	180	
Water (% in dough)		7.7	
Fat (% in dough)		13.6	

All ingredients, except for the enzyme, were mixed. Subsequently, the diluted enzyme solution was added. The final enzyme concentration was 180 NASPU/kg flour. The dough was kept to rest for 20 minutes before moulding and another 20 minutes after moulding.

After the resting period, just before baking, 10 g of each dough was taken for asparagine measurement.

For this, the enzyme reaction was stopped by mixing the 10 g of the dough with 50 mL 0.1 M HCl. Subsequently, the mixture was analyzed for asparagine contents by UPLC-MSMS. Untreated dough was taken as control.

Asparagine analysis
Asparagine contents in the dough were measured using a UPLC-MSMS method. The separation is based on reversed phase chromatography using UPLC; MSMS detection is based on positive electrospray ionization. Isotope-labeled asparagine was used as internal standard. For measurement of the asparagine contents in the potato dough, the dough/HCl mixture was centrifuged and diluted again before labeling via a derivatization reaction using Waters AccQ-Fluor Reagent (6-aminoquinoyl-N-hydroxysuccinimdyl carbamate or AQC). The AccQ-Fluor reagent reacts rapidly with primary and secondary amino acids to yield highly stable carbamide derivatives.

Acrylamide analysis
Acrylamide contents were analyzed in cookies from control dough and the dough treated with 180 NASPU PreventASe ASG, respectively, using a GC/MS method. The separation is based on gas chromatography; detection is based on mass spectrometry. ^{13}C-labeled acrylamide was used as internal standard.

Results
As shown in the table below, treatment with PreventASe ASG resulted in a clear reduction of L-asparagine and acrylamide in the tested cookie dough (see Table 1). Pre-treatment of the dough with PreventASe ASG reduced free asparagine and acrylamide by 40-50 % compared to the untreated dough. A good correlation is observed between asparagine content in the dough and acrylamide concentrations in the baked cookies.

Table 1 Asparagine and Acrylamide contents in dough and baked cookies treated with PreventASe ASG

PreventASe ASG Units	Asparagine content (mg/kg)	% Reduction Asparagine compared to untreated control	Acrylamide content in baked cookie (µg/kg)	% Reduction acrylamide compared to untreated control
control	41		93	
180	26	37	43	54

Annex 6.2.2

Annex 6.2.2 Asparagine reduction by the use of PreventASe ASG asparaginase from *Aspergillus niger* in corn flour dough - results from laboratory scale experiments

Asparagine Reduction by the Use of PreventASe ASG Produced in *Aspergillus niger* in Corn-Based Products

Results of laboratory scale experiments

Summary

Corn-based dough of different pHs was treated with the PreventASe ASG asparaginase for different time intervals to evaluate the reduction of the free amino acid L-asparagine. The results show that treatment of the dough with PreventASe ASG for 10 minutes results in a 50-60% reduction, treatment for 20 minutes results in 70-87% reduction and incubation for 30 minutes leads to 85-91% removal of free asparagine in the dough.

Materials and Methods

Dough preparation

Masa dough was prepared from 46 % masa flour, 50% water and 4 % olive oil; the pH was adjusted with NaOH to achieve a pH of 7 and 8, respectively. The dough were treated with 0.08 - 0.1 U PreventASe ASG per gram masa flour and incubated for 10, 20 and 30 minutes at room temperature. The enzyme dosage was chosen to achieve (almost) complete hydrolysis of the asparagine in about 30 minutes. The enzyme reaction was stopped by mixing 5 g of the dough with 100 mL 0.1 M HCl. Subsequently, the mixture was analyzed for asparagine contents by UPLC-MSMS. Untreated potato dough was taken as control.

Asparagine analysis

Asparagine contents in the dough were measured using a UPLC-MSMS method. The separation is based on reversed phase chromatography using UPLC; MSMS detection is based on positive electrospray ionization. Isotope-labeled asparagine was used as internal standard. For measurement of the asparagine contents in the potato dough, the dough/HCl mixture was centrifuged and diluted again before labeling via a derivatization reaction using Waters AccQ-Fluor Reagent (6-aminoquinoyl-N-hydroxysuccinimdyl carbamate or AQC). The AccQ-Fluor reagent reacts rapidly with primary and secondary amino acids to yield highly stable carbamide derivatives.

Results

As shown in the graphs below, treatment with PreventASe ASG resulted in clear reduction of L-asparagine in the tested masa dough at both, pH 7 and pH 8. Using 0.08 - 0.1 NASPU/g corn flour, a 50-60 % reduction of free asparagine was observed after 10 minutes incubation at room temperature. A further reduction was observed after longer incubation times showing reductions by 70-87 % of the initial asparagine level after 20 minutes and 85-91 % after 30 minutes at room temperature.

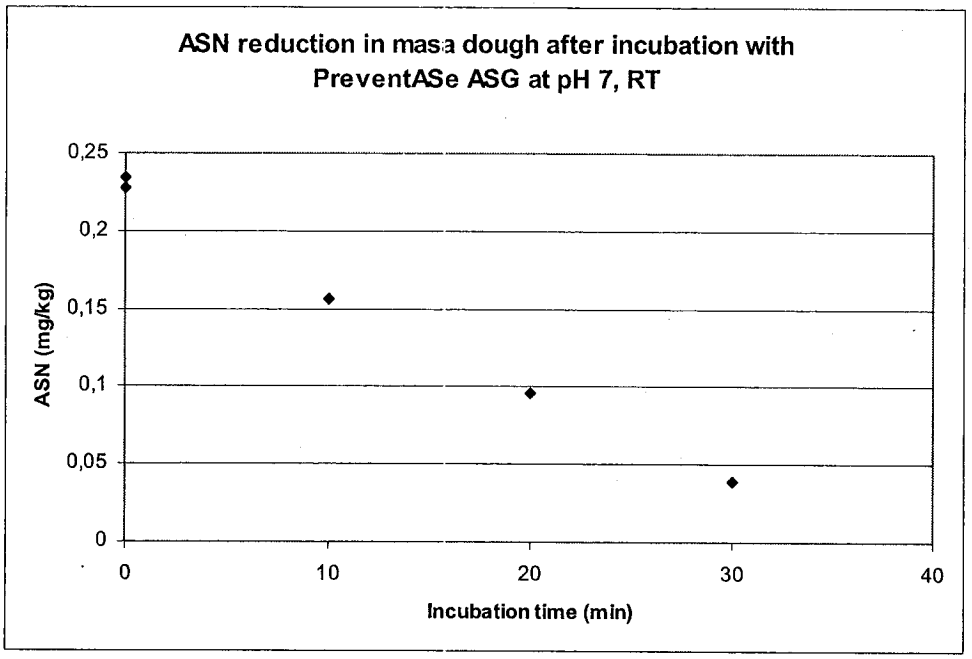


Figure 1 Asparagine contents in masa dough (pH 7) after incubation with PreventASe ASG (0.08 NASPU/g flour) for different time intervals

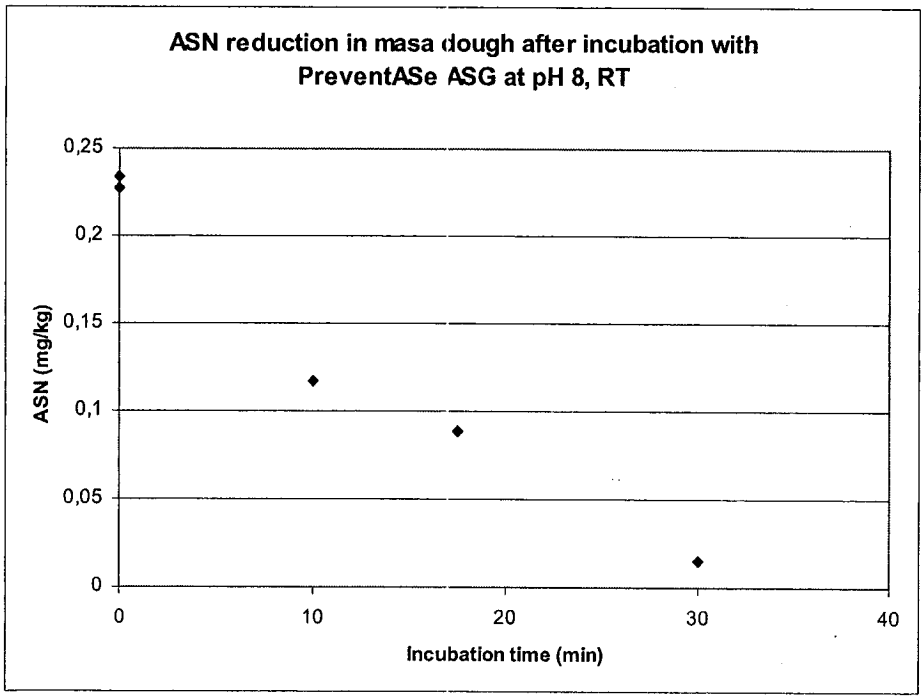


Figure 2 Asparagine contents in masa dough (pH 8) after incubation with PreventASe ASG (0.1 NASPU/g flour) for different time intervals

Annex 6.2.3

Annex 6.2.3 Asparagine reduction by the use of PreventASe ASG asparaginase from *Aspergillus niger* in potato dough - results of laboratory scale experiments

Asparagine Reduction by the Use of PreventASe ASG Produced in *Aspergillus niger* in Potato-Based Products

Results of laboratory scale experiments

Summary

Potato-based dough of different pHs was treated with the PreventASe ASG asparaginase for different time intervals to evaluate the reduction of the free amino acid L-asparagine. The results show that treatment of the dough with PreventASe ASG for 10 minutes results in a 50 % reduction, treatment for 20 minutes results in up to 87 % reduction and incubation for 30 minutes leads to (almost) complete removal of free asparagine in the dough.

Materials and Methods

Dough preparation

Potato dough was prepared from 38.4 % dry potato flakes, 57.5% water and 4.1% olive oil; the pH was adjusted with NaOH to achieve a pH of 7 and 8, respectively. The dough were treated with 5-6 U PreventASe ASG per gram dry potato flakes and incubated for 10, 20 and 30 minutes at room temperature. The enzyme dosage was chosen to achieve (almost) complete hydrolysis of the asparagine in about 30 minutes. The enzyme reaction was stopped by mixing 5 g of the dough with 100 mL 0.1 M HCl. Subsequently, the mixture was analyzed for asparagine contents by UPLC-MSMS. Untreated potato dough was taken as control.

Asparagine analysis

Asparagine contents in the dough were measured using a UPLC-MSMS method. The separation is based on reversed phase chromatography using UPLC; MSMS detection is based on positive electrospray ionization. Isotope-labeled asparagine was used as internal standard. For measurement of the asparagine contents in the potato dough, the dough/HCl mixture was centrifuged and diluted again before labeling via a derivatization reaction using Waters AccQ-Fluor Reagent (6-aminoquinoyl-N-hydroxysuccinimdyl carbamate or AQC). The AccQ-Fluor reagent reacts rapidly with primary and secondary amino acids to yield highly stable carbamide derivatives.

Results

As shown in the graphs below, treatment with PreventASe ASG resulted in clear reduction of L-asparagine in the tested potato dough at both, pH 7 and pH 8. Using 5-6 NASPU/g potato flour, a 50 % reduction of free asparagine was observed after 10 minutes incubation at room temperature. A further reduction was observed after longer incubation times showing reductions by 80 - 87 % of the initial asparagine level after 20 minutes and (almost) complete removal after 30 minutes at room temperature.

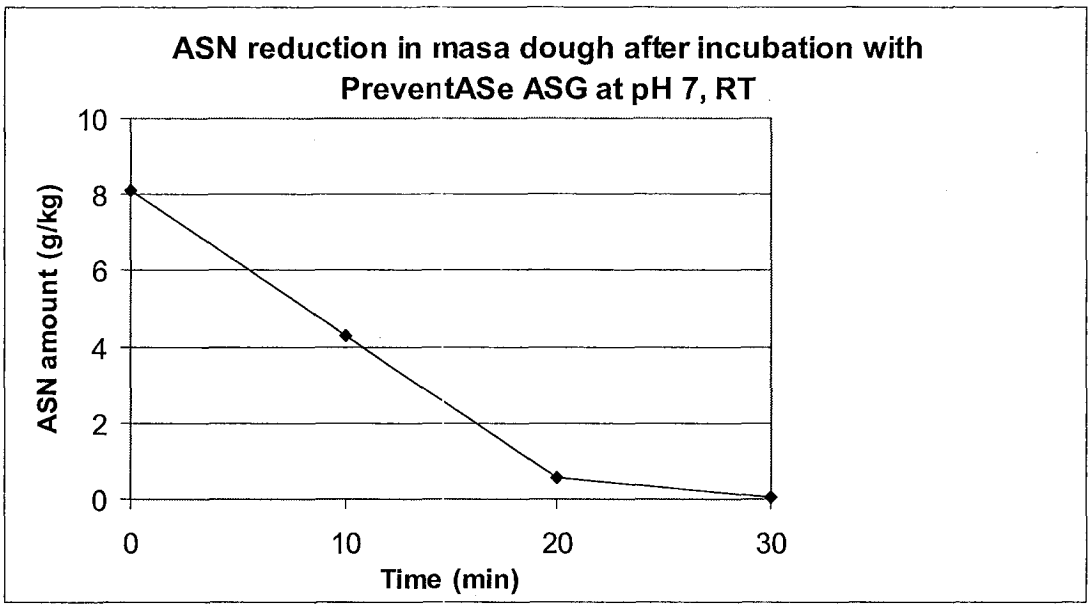


Figure 1 Asparagine contents in potato dough (pH 7) after incubation with PreventASe ASG (5 NASPU/g flour) for different time intervals

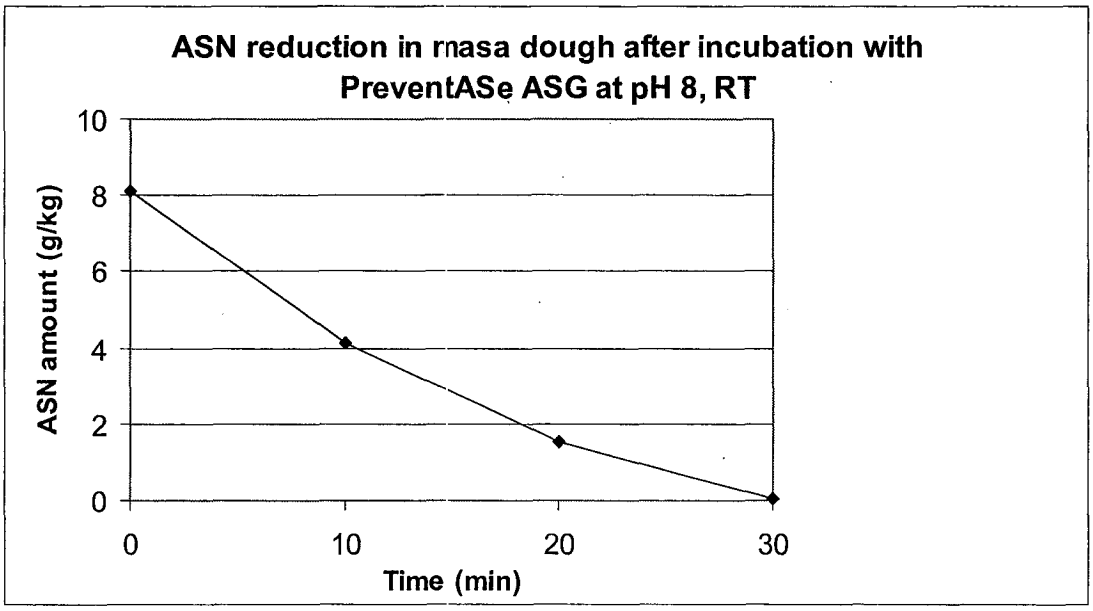


Figure 2 Asparagine contents in potato dough (pH 8) after incubation with PreventASe ASG (6.5 NASPU/g flour) for different time intervals

Annex 7.1.1

Pages 000148-000157 have been removed in accordance with copyright laws. The removed reference is:
Schuster, E., Dunn-Coleman, N., Frisvad, J.C., Dijck, P.W.M. van, On the Safety of Aspergillus niger - a Review, Appl. Micobiol. Biotechnol. 59A26-435 (2002).

Annex 7.1.2

Pages 000159-000160 have been removed in accordance with copyright laws. The removed reference is:
Joint FAO/WHO Expert Committee on Food Additives, Toxicological Evaluation of Certain Additives, WHO Food Additives Series 22 (1988), available at: http://www.inchem.org/documents/jecfa/jecmono/v22je04.htm.

Annex 7.1.3

000161

DATE: September 14, 1988

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

multistep pathways with many genes and many enzymes involved.

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

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

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

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

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

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

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

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

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

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

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

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

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Sep. 1988

THE OCCURRENCE AND SIGNIFICANCE OF MYCOTOXINS

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THE OCCURENCE AND SIGNIFICANCE OF MYCOTOXINS

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1. Description of mycotoxins

A conservative estimate suggests that there are at least 100,000 species of fungi (Hawksworth, Sutton & Ainsworth, 1983) and many of these are able to produce one or more low molecular weight organic compounds known as secondary metabolites. These metabolites are a structurally diverse group of molecules (Turner & Aldridge, 1983) some of which have biological activity as antibiotics, phytotoxins and mycotoxins. The term mycotoxin is generally confined to those toxic metabolites produced by moulds growing on foods, animal feeds, or the raw materials and additives used in their manufacture.
The biological activity of mycotoxins is characterized by a toxic response when consumed by man or animals. Depending on the type of mycotoxin and animal species, even low concentrations of mycotoxins can create an acutely toxic, carcinogenic, oestrogenic or immuno-suppressive effect. A number of fungi producing macroscopic fruiting bodies (mushrooms and toadstools) also produce toxic metabolites and these are a hazard when such fruiting bodies are eaten. It is convenient to deal with these compounds separately and not include them as mycotixons.

2. Mycotoxins as natural contaminants in food

Of the several hundred known toxic mould metabolites (see Moreau, 1974; Wyllie & Morehouse, 1977; Watson, 1985) only about three dozen have been shown to occur as natural contaminants in food (Krogh, 1987). Table 1 lists the majority of these with the species of mould known to produce them.
A further selection of mycotoxins, such as the satratoxins, verrucarins, sporidesmins and slaframine, have been identified in animal feeds and fodders.

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

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

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

4. Species specific mycotoxins

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

5. Effect of mutations on mycotoxin production

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

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

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

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

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

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

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

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

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

* recognised as mycotoxins

APPENDIX 1

Search Strategy Used

Set	Items	Description
S1	5328	MYCOTOXIN
S2	16258	AFLATOXIN
S3	195	DIHYDROXYFLAV?
S4	736	DIACETOXYSCIRPENOL
S5	2352	OCHRATOXIN
S6	238	LUTEOSKYRIN
S7	0	EPOXY(W)TRICOTHECENE
S8	1226	STERIGMATOCYSTIN
S9	172721	TOXIN? ?
S10	2352	T(2W)2(2W)TOXIN
S11	2094	ZEARALENONE
S12	36	TRICOTHECENE
S13	394	RUBRATOXIN
S14	1506	PATULIN
S15	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 Biotechnology Current Advances in Microbiol. Current Advances in Molecular Biol. Current Advances in Cell + Dev. Biol. Current Advances in Toxicology and many more
Biosis Previews	Dialog	Biological Abstracts
EMBASE	Dialog	Abstracts & Citations from 4000 worldwide Biomedical Journals
International Pharmaceutical Abstracts	Dialog	500 Pharmaceutical, medical + related Journals
Life Sciences Collection	Dialog	Industrial + Applied Microbiology, Microbiological abstracts
Chemcial Exposure	Dialog	Databank
Martindale on line	Dialog	Databank
Medline	Dialog	Index Medicus (3000 International Journals)
Occupational Safety & Health	Dialog	400 Journals 70,000 monographs
Chemical Regulations & Guideline system	Dialog	US Federal Databank on controlled substances
Drug information full text	Dialog	
Agrochemicals Handbook	Dialog	
CA Search	Dialog	Chemical abstracts
Merck index on line	Dialog	Merck index

Annex 7.1.4

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Pages 000180-000185 have been removed in accordance with copyright laws. The removed reference is:
Joint FAO/WHO Expert Committee on Food Additives, Evaluation of Certain Additives and Contaminants, 35th report of JECFA, WHO Technical Report Series 789 (1990), available at: http://whqlibdoc.who.int/trs/WHO_TRS_789.pdf

Annex 7.2

SAFETY EVALUATION OF PREVENTASE ASG ASPARAGINASE DERIVED FROM *Aspergillus niger* AGN7-41 USING THE PARIZA & JOHNSON DECISION TREE

Introduction

A first "Decision Tree for evaluation of the relative safety of food ingredients derived from genetically modified organisms" was published in 1990 by the International Food Biotechnology Council (IFBC)[1]. This publication was an extension, based on an earlier publication by Pariza and Foster in 1983[2]. In 2001, the IFBC Decision Tree was updated by Pariza and Johnson[3].
The enzyme preparation PreventASe ASG derived from *Aspergillus niger* AGN7-41 was evaluated according to the decision tree published by Pariza and Johnson in 2001. The result is described below.

Decision Tree

1. Is the production strain genetically modified?
   YES
   The strain *Aspergillus niger* AGN7-41 is derived from host ISO-528, which is a genetically modified strain (GMO self-clone) derived from the DSM GAM lineage of *Aspergillus 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~~
   Asparaginases are present in many food sources and have been notified as GRAS (GRN 000201, GRN 000214). The asparaginase produced by strain AGN7-41 is an asparaginase modified by protein engineering. This was done to make the enzyme more effective under the temperature and pH conditions of some applications. The modified gene enzyme was analyzed for its allergenic properties (*in silico*) but no indications were found that the modified enzyme is allergenic upon consumption. Furthermore, the enzyme consists of natural occurring amino acids

[1] International Food Biotechnology Council (IFBC). Reg. Toxicol. Pharmacol. 12 (1990), S1-S196 (page 119)
[2] Pariza M.W. and Foster E.M. J. Food Protection **46**. (1983), 453-468
[3] Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. **33** (2001) 173-186)

which are denatured in the application. The protein is easily digestible by gastric enzymes. The safety of protein engineered enzymes has also been evaluated by Pariza and Cook[4], where the authors conclude that protein engineered enzymes, when derived from non-toxic proteins and hosts can be considered safe and toxicity studies are not needed. There is no evidence that evolution and natural variation within enzyme families has resulted in the generation of toxins active via the oral route.

[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 *Aspergillus niger* production strains, for which the safety has been covered by safety studies on previous test articles from the same safe strain lineage[5].]

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 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?
YES
If yes, go to 4. ~~If no, go to 12.~~
The final production strain is complete marker gene free and devoid of any uncharacterized heterologous DNA (see 2c).

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

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[4] Pariza and Cook. Regulat. Toxicol. Pharmacol. **56** (2010) 332-342.
[5] P.W.M. van Dijck et al. Reg. Toxicol. Pharmacol. **38** (2003) 27-35.

Nevertheless, the strain has been analyzed for its production of secondary metabolites, including mycotoxins. The strain AGN7-41 did not produce any mycotoxins under the conditions of the fermentation procedure. This was verified by analyses on broth and concentrated UF samples.
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]

Annex 7.2.1

WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

AMFEP	*Members*
Thierry Dauvrin	**Frimond**
Gert Groot	**Gist-brocades**
Karl-Heinz Maurer	**HenkelCognis**
David de Rijke	**Quest International**
Henning Ryssov- Nielsen	**Danisco Ingredients**
Merete Simonsen	**Novo Nordisk**
Torben B. Sorensen (chairman)	**TBS Safety Consulting ApS**

Copenhagen, August 1998

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3.6. The consumption of enzymes for medical purposes and as digestive aids
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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'ies, and particularly since 1992 it has been repeatedly claimed that enzyme residues in foods may represent a hazard to consumers in the form of allergies, and that a certain percentage of the population are at risk of having allergicy reactions to enzymes in bread and other foods.

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

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

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

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

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

2.0 Background

2.1 General

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

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

2.2 *Occupational respiratory allergy*

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

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

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

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

3.0. *Food allergy*

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

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

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

Symptoms

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

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

Types of food allergens

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

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

Properties of food allergens

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

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

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

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

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

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

Doses at which food allergy occurs

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

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

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

Food produced by GMO's

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

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

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

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

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

3.2. Epidemiology of Food Allergy

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

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

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

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

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

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

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

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

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

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

3.3. Enzymes in food

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

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

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

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

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

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

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

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

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

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

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

3.4. The Theory of cross reactions

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

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

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

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

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

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

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

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

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

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

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

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

3.5. Food related reactions in occupationally sensitised people

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.6.1. Medical uses:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4.0. Conclusion

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

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

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

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

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

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

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GRAS Notice Review

Ramos-Valle, Moraima

From: Yingling, Gary L. [Gary.Yingling@klgates.com]
Sent: Tuesday, March 27, 2012 9:27 AM
To: Srinivasan, Jannavi
Cc: West-Barnette, Shayla; Ramos-Valle, Moraima
Subject: FW: Attached Files
Attachments: DC-#9610884-v1-DSM_supplement_submission_to_GRN_214_asparaginase.DOC; DC-#9610898-v1-DSM_comparison_table_asparaginase_GRN_214_and_new_product.DOC

Dear Jannavi: Here is a proposed letter and attachment to explain the small differences between the GRN 0000214 and the current product. I will be calling you to discuss the next step. gary

K&L GATES

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March 26, 2012

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Division of Biotechnology and GRAS Notice
Review
Office of Food Additive Safety
Center for Food Safety & Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Supplemental Submission to GRAS Notice No. GRN 000214 - Asparaginase derived from *Aspergillus niger*

Dear :

This letter is to provide supplemental information for a slightly different asparaginase being produced by my client, DSM Food Specialties ("DSM"). In October of 2006, DSM filed GRAS Notice No. GRN 000214 for an asparaginase derived from a wild-type *Aspergillus niger*, which resulted in a letter from FDA dated March 12, 2007 noting that the agency had no questions. DSM has developed another asaparginase from an enzyme modified wild-type *Aspergillus niger* with the trade name Preventase ASG. There are some minor differences and attached is a table noting them. However, the basic issues of safety and use were covered in the 2006 Notice and for that reason, DSM is submitting this supplemental information and also stating that DSM is of the opinion that the new asparaginase is generally recognized as safe based on the data submitted in 2006.

In sending this letter, it is my understanding that FDA will be sending a response.

If FDA has any questions or needs any documents, please do not hesitate to contact me.

Very truly yours,

Gary L. Yingling

GLY:
Enclosure

cc: DSM Food Specialties

Asparaginase comparison WT (GRN 000214) and new product.

The following table notes some of the differences in the GRAS notification of asparaginase wild type (GRN 000214) and the second generation asparaginase which is the subject of a supplement to the GRN 00214 notice.

Description	**GRN 000214 Asparaginase**	**Second Generation Asparaginase**
Name of product	Preventase L, M or W	Preventase ASG
Formulation	L (liquid, glycerol), M (maltodextrin, powder) or W (wheat, powder)	L (liquid, glycerol)
Application	Bread, other cereal based products, potato based products, reaction flavors	Bread, other cereal based products, potato based products
Enzyme unit	ASPU	NASPU
Amount of TOS in final food	Bread 2-13 mg/kg Other cereals 0.2-30 mg/kg Potato 4-562 mg/kg	Bread 2-34 mg/kg Other cereals 2-35 mg/kg Potato 4-74 mg/kg
Gene encoding asparaginase	Wild type *Aspergillus niger*	Modified wild type *Aspergillus niger* variant
pH range application	4-8 (optimum 4-5)	5-8 (optimum 7)
Analytical method	Measured at pH 5.0	Measured at pH 7.0
Application data	Comparable acrylamide reduction levels	
Pariza-Johnson decision tree	No questions	No questions
Allergenicity	No questions	No questions
Leading publications	Same	Same and reference to GRNs 214 and 201 added
Safety studies	Same references	Same references and Ames test on variant and 90-day study on WT product added
Estimated daily intake of TOS (mg/kg bw/day)	0.044-0.648	0.01-0.25
Margin of safety	418-6159	1111-21785

There is no significant safety issue requiring review that was not addressed in GRAS Notice No. GRN 000214.

SUBMISSION END