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GRAS Notice (GRN) No. 345

<http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/default.htm>

ORIGINAL SUBMISSION

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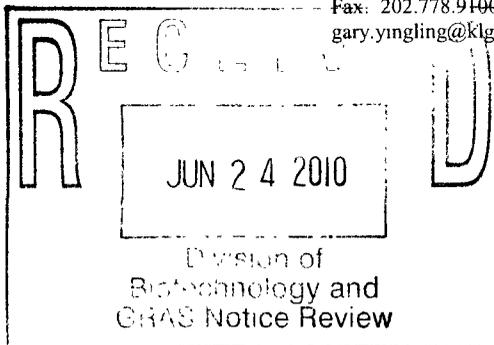
June 15, 2010

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Via Federal Express

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



Re: GRAS Notification for carboxypeptidase from genetically modified *Aspergillus niger*

Dear Sir or Madam:

As counsel for DSM Food Specialties (DSM), we are submitting under cover of this letter three copies of DSM's GRAS notification for a carboxypeptidase preparation from genetically modified *Aspergillus niger*. DSM has determined through scientific procedures that the carboxypeptidase preparation is generally recognized as safe for use in cheese, enzyme modified cheese, and fermented meat as a processing aid to accelerate the development of flavors as well as debittering during the ripening process.

This use of carboxypeptidase derived from genetically modified *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,

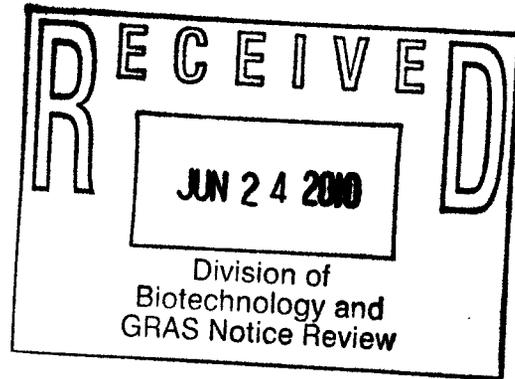
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Gary L. Yingling

cc: DSM Food Specialties

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

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

DSM Food Specialties (“DSM”) manufactures the food enzyme Carboxypeptidase, which is produced by submerged fermentation of a selected, pure culture of *Aspergillus niger*. DSM produces the carboxypeptidase preparations in both a spray-dried and a liquid form. The spray-dried form is standardized with maltodextrin, while the liquid form is stabilized with glycerol. The trade name will be Accelerzyme® CPG.

This carboxypeptidase preparation is intended for use in cheese, enzyme modified cheese or fermented meat as a processing aid to accelerate the development of flavors as well as debittering during the ripening process.

Carboxypeptidase generates C-terminal amino acids from proteins and peptides present in various foods such as milk (casein, whey) and meat, in order to aid in and/or speed up the development of flavors during ripening or to lower the bitter taste of the product.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, DSM has determined that its carboxypeptidase 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 in this Section.

The production organism, *Aspergillus niger*, has a long history of safe use and is discussed in Section 2. FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *Aspergillus niger* strains. See 21 C.F.R. §§ 184.1033 (Citric acid); 184.1685 (Rennet and chymosin). In addition, lipase, asparaginase and phospholipase A2 preparations from genetically modified *Aspergillus niger* strains, derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier, have recently been as the subject of GRAS notifications (GRN 000296, GRN 000214 and GRN000183, respectively), to which FDA had no objections. The FDA recently summarized the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Bier et al., 2006).

Section 2 also describes the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from known harmful sequences, for carboxypeptidase. In Section 3 data showing carboxypeptidase to be substantially equivalent to naturally occurring peptidases¹ are presented.

¹ Peptidases have also been named proteases or proteinases.

The safety of the materials used in manufacturing and the manufacturing process itself are described in Section 4, while Section 5 reviews the composition, specifications as well as the self-limiting levels of use for carboxypeptidase. Section 6 provides information on the mode of action, application, use levels and enzyme residues in final food products in which carboxypeptidase is to be used. Finally, the safety studies outlined in Section 7 indicate that *Aspergillus niger* and carboxypeptidase show no evidence of pathogenicity or toxicity. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

1.1 Name and Address of Notifier

NOTIFIER

DSM Food Specialties

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The Netherlands

MANUFACTURER

DSM Food Specialties

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1.2 Common or Usual Name of Substance

DSM's serine-type carboxypeptidase 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 "carboxypeptidase". It is produced and sold in both liquid and spray-dried form. The liquid formulation is standardized with glycerol, while the spray-dried form is standardized with granulated maltodextrin. The trade name will be Accelerzyme® CPG.

1.3 Applicable Conditions of Use

The carboxypeptidase preparation is to be used in cheese, enzyme modified cheese (EMC) and fermented meat. Regarding cheese, the enzyme is added to milk together with the lactic acid bacteria, resulting in an accelerated ripening of the cheese. Regarding meat, the enzyme is added together with lactic acid bacteria before the fresh pieces of meat are dried. Due to the physical state of both end products, the enzyme is not active at the end of the process in these applications.

Regarding EMC, carboxypeptidase is replacing other peptidases normally present in enzyme mixtures used to release flavor components. This leads to a less bitter taste of the EMC. The final processing step of the EMC involves pasteurization, which inactivates the enzymes (including carboxypeptidase).

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

1.3.1 Substances Used In

The carboxypeptidase preparation is to be used in cheese, enzyme modified cheese and fermented meat.

1.3.2 Levels of Use

Enzyme preparations are generally used in *quantum satis*. The dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions.

Cheese

The carboxypeptidase preparation will be used in an average dosage of 18-90 CPGU/liter milk. The maximal dosage will be 225 CPGU/liter milk. 1 liter milk will result in 100 g cheese and 900 ml whey.

EMC

The carboxypeptidase preparation will be used in an average dosage of 400-1600 CPGU/kg EMC. The maximal dosage will be 4000 CPGU/kg EMC.

Fermented meat

The carboxypeptidase preparation will be used in an average dose of 80-180 CPGU/kg meat. The maximal dosage will be 400 CPGU/kg meat.

1.3.3 Purposes

The technological function of carboxypeptidase is to release C-terminal amino acids from proteins and peptides present in various foods such as milk (casein, whey) and meat, in order to aid in and/or speed up the development of flavors during ripening.

Cheese

In cheese, the use of carboxypeptidase results in accelerated development of flavors as well as debittering during the ripening process.

During cheese production, the lactic acid bacteria that is added to the milk forms lactic acid and flavor components. The formation of these flavor components is a slow process and takes several months. This period is referred to as “ripening of the cheese”. Cheese producers like to shorten this ripening period to increase the flexibility of the supply chain and to reduce the storage costs of the cheese.

When added to milk together with lactic acid bacteria, carboxypeptidase will be evenly distributed in the cheese during cheese making. This enables the enzyme to act on the proteins present throughout the cheese and to release the amino acids that act as precursors for the flavor components during the ripening period. In this way, the same types of flavor components are developed as during regular ripening, but the speed of the flavor development is accelerated resulting in a shorter ripening period of the cheese. Due to the physical solid state of the cheese, the carboxypeptidase enzyme is no longer functional in the final food.

Enzyme modified cheese (EMC)

In EMC, carboxypeptidase is mainly used as a debittering aid.

EMC is produced by hydrolyzing components (i.e., lipids and proteins) of young cheese (such as Cheddar) with a mixture of enzymes such as peptidases and lipases. During an incubation time of several hours at 30-40°C, the enzymes release several flavor components, giving the mixture the flavor of ripened cheese. After incubation, the mixture is pasteurized to inactivate the enzymes and often dried to an EMC powder.

The EMC-powder is used to create a cheesy-taste to several foods like soups and sauces. However, the regular enzymes used in EMC production often also release bitter-tasting peptides, which are undesired in most applications. When carboxypeptidase is added to the regular enzyme mixture, the C-terminal amino-acids are split off from the bitter-tasting peptides, resulting in a less bitter taste.

Fermented meat

In fermented meat, carboxypeptidase can be used to speed up the development of flavor during ripening.

Fermented dry meat is produced by cutting and mixing fresh pieces of meat and bacon together with lactic acid bacteria. The mixture is filled in foil or natural pig-intestine, and left to dry and ferment in a temperature- and moisture-controlled ripening cell. During the ripening period of three weeks, the moisture decreases by evaporation, the pH decreases because the lactic acid bacteria form lactic acid, and the taste develops because of flavor components formed by the lactic acid bacteria.

By adding carboxypeptidase together with the lactic acid bacteria during cutting and mixing of the meat and bacon, amino acids are released that stimulate the lactic acids bacteria in their formation of flavor components. As a result, the meat reaches the desired taste after 22 instead of 28 days. Due to the physical solid state of the dried meat, the enzyme is no longer functional in the final food.

1.3.4 Consumer Population

Serine carboxypeptidases are abundantly present in nature as has been described by Breddam in 1986. They are found in the vacuoles of higher plants and fungi and in the lysosomes of animal cells. One of the micro organisms producing carboxypeptidases is *Saccharomyces cerevisiae*, i.e., baker's yeast (Félix and Labouesse-Mercoureff, 1956, Hayashi et al., 1970). In addition, many fungi, including *Aspergilli* species, excrete serine carboxypeptidases (Dal Degan, 1992). The addition of the carboxypeptidase preparation will therefore not lead to reaction products not considered normal constituents of the human diet. Because carboxypeptidase is an enzyme protein naturally occurring in microorganisms, animals and plants, DSM expects it will be digested, as would any other protein occurring in food.

Carboxypeptidase hydrolyzes C-terminal amino acids from proteins and peptides. Amino acids, small and large peptides all play an important role in a number of metabolic processes in all organisms, from bacteria to mammals. As a result, they are abundant in the human diet. Hence, there is no basis to believe that the extra addition of carboxypeptidase in any of the applications will have a significant effect, if any, on the human body.

As is shown in Section 6.4 of this dossier, the total amount of enzyme TOS in the final food is expected to be about 9.9-100.1 mg/kg (= 0.00099 – 0.01001%).

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

1.4 Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, DSM has determined, through scientific procedures, that its carboxypeptidase enzyme preparation from GMO *Aspergillus niger* is GRAS for use as accelerator in cheese and meat ripening and in their debittering, in levels not to exceed good manufacturing practices.

1.5 Availability of Information for FDA Review

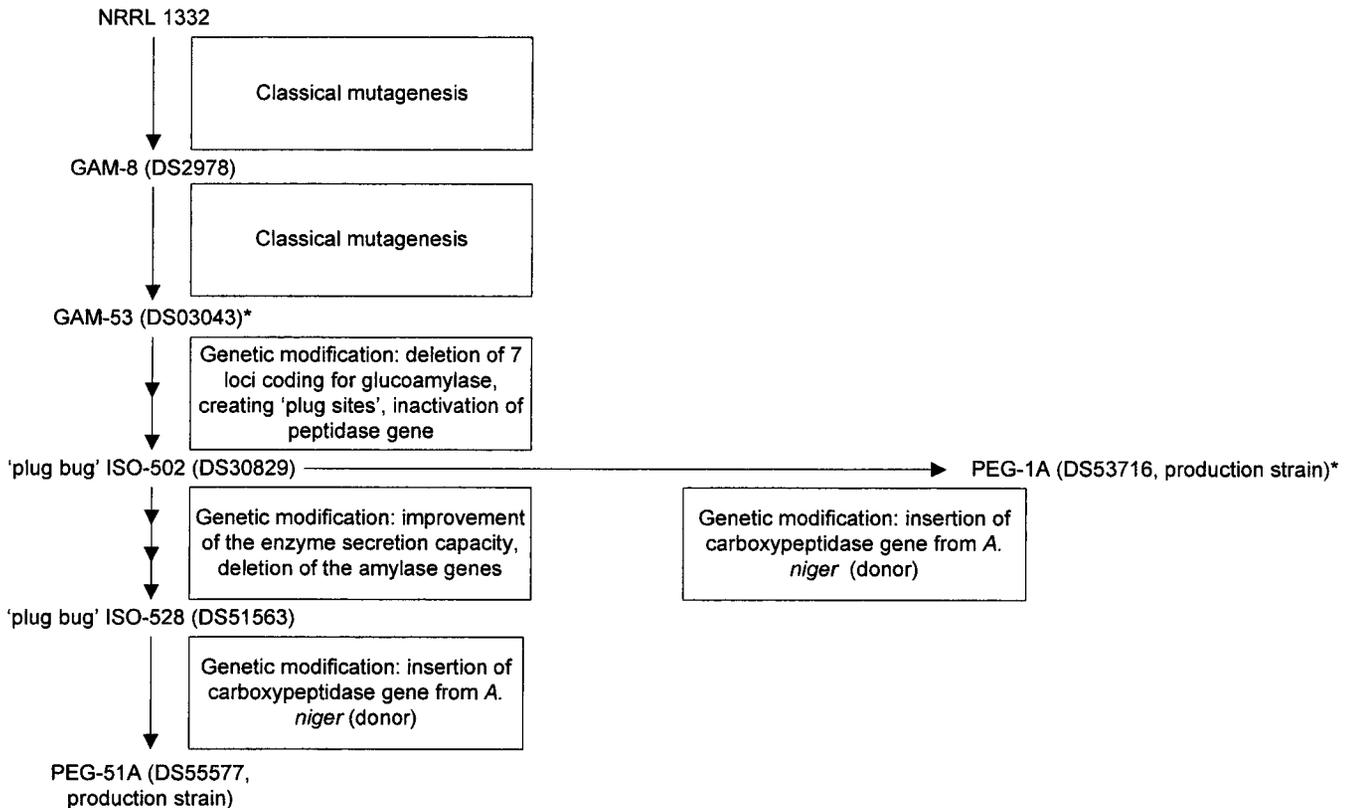
The data and information that are the basis for DSM's GRAS determination are available for the FDA's review, and copies will be sent to FDA upon request. Requests for copies and arrangements for review of materials cited herein may be directed to:

Gary L. Yingling, Esq.
K&L Gates LLP
1601 K Street, NW
Washington, DC 20006-1600

2. PRODUCTION MICROORGANISM

2.1 Donor, Recipient Organism and Production Strain

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



* Strain GAM-53 and PEG-1A were taxonomically identified as *Aspergillus niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS).

Donor:

The gene coding for carboxypeptidase (*pepG* gene) was derived from *Aspergillus niger* strain N400, which was obtained from Wageningen University.

Recipient organism

The recipient organism used in the construction of the carboxypeptidase 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 51563. The strain ISO-528 was declared as suitable host strain for the construction of genetically modified organisms belonging to Group I safe microorganisms by the Dutch authorities.

The strain ISO-528 is derived from the fully characterized DSM *Aspergillus niger* strain GAM-53 (DS 03043) by genetic modification. The strain GAM-53 was derived by several classical mutagenesis steps from *Aspergillus niger* strain NRRL 3122, a strain purchased from the Culture Collection Unit of the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, USA.

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

The strain GAM-53 was taxonomically identified as *Aspergillus niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS). This is an independent, internationally recognized laboratory. The strain GAM-53 is being used to construct a new generation of strains according to the 'design and build' concept, in which introduced genes are targeted ('plugged') to a predetermined region of the genome. The exact technique used to construct such 'plug bugs' (designated as 'ISO-strains') and its advantages are described in literature (Selten et al., 1995 and van Dijck et al., 2003, included as Annex 2.1.1).

One of these ISO-strains, ISO-502, was used for the construction of the production strain for phospholipase A2 (donor: porcine pancreas), which is subject to a GRAS notification (GRN 000183), to which FDA had no questions. The ISO-502 strain has been used for the construction of a previous production strain for carboxypeptidase, PEG-1A (see genealogy).

The recipient organism ISO-528 used in the construction of the carboxypeptidase production strain was derived from GAM-53 as follows: The strain GAM-53 contains 7 loci (i.e., the promoter and coding sequences) for the glucoamylase gene. These 7 loci were removed, creating so-called 'plug-sites' (also called $\Delta glaA$ loci) into which expression units containing various genes can be integrated ('plugged'). The 7 'plug-sites' were each provided with unique restriction sites (also called 'DNA-flags'), marking the location of the 'plug-sites' on the genome. In addition, the gene coding for the major protease (*pepA*) was inactivated and the major amylases (*amyA* and *amyB*) were deleted both by established rDNA techniques and the strain's capacity to secrete proteins was improved by classical mutation and selection.

The resulting 'plug bug', ISO-528, was classified as a self-cloned GMO by the Dutch competent authorities.

The ISO-528 strain has been used for the construction of the production strains for asparaginase (donor: *A. niger*) and lipase (synthetic gene). Both enzymes are subject to GRAS Notifications (GRN 000214 and GRN 000296). FDA had no questions with these GRAS notifications.

Production strain

The carboxypeptidase 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 PEG-51A and stored in the DSM Culture Collection as DS 55577.

2.2 Genetic modification

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

Donor DNA

Based on the published sequence of the carboxypeptidase *pepG* gene (Svendsen and Dal Degan, 1998), the gene was isolated and multiplied by PCR, using the genomic DNA from the *Aspergillus niger* N400 strain (obtained from Wageningen University).

Carboxypeptidase expression plasmid

The carboxypeptidase 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* carboxypeptidase gene, and DNA from a well-characterized *Escherichia coli* vector pTZ18R.

The different elements of the plasmid are:

- The *glaA* promoter (P*glaA*) from the parental *Aspergillus niger* strain GAM-53.
- The entire genomic sequence encoding the carboxypeptidase protein of *Aspergillus niger* N400.
- A 3' flanking *glaA* terminator sequence from the parental *Aspergillus niger* strain GAM-53 for efficient termination of *pepG* gene transcription and targeting of the expression unit to the Δ *glaA* loci.
- DNA sequences from the *Escherichia coli* plasmid pTZ18R. These sequences are removed prior to transformation of the carboxypeptidase expression cassette into the host.

Selectable marker plasmid

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

The different elements of the plasmid are:

- The *glaA* promoter (P*glaA*) from the parental *Aspergillus niger* strain GAM-53.
- The *amdS* gene from *Aspergillus nidulans*.

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

Transformation and selection of the final production strain

The carboxypeptidase expression cassette and the selection marker cassette, both completely devoid of any *Escherichia 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 P*glaA* and 3'-*glaA* parts of the two expression units, they are preferentially targeted to one of the seven Δ *glaA* loci.

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

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

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

From the available recombinants a strain was chosen that contained sufficient gene copies to allow for commercial attractive expression levels of the carboxypeptidase enzyme: this strain was designated PEG-51A.

The expression unit is translated into a carboxypeptidase protein, which is glycosylated and secreted into the medium as the mature, active enzyme.

2.3 Stability of the Transformed Genetic Sequence

Strains belonging to the *Aspergillus niger* GAM-lineage - from which both the host ISO-528 and the recombinant carboxypeptidase production strain PEG-51A are derived - are genetically stable strains. The whole GAM-lineage has been 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 carboxypeptidase production strain does not differ from the parental GAM-strains or from production strains constructed by random integration.

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

2.4 Good Industrial Large Scale Practice (GILSP)

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

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

Therefore, consistent with the principles of Good Industrial Large Scale Practice (GILSP), as endorsed by the Organization of Economic Cooperation and Development (OECD), the carboxypeptidase production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. The production organism has been approved both by the Dutch 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, fermentations for the large-scale production of food and feed enzyme products are carried out below the GILSP level of physical containment.

2.5 Absence of transferable rDNA Sequences in the Enzyme Preparation

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

2.6 Absence of Production Organism in the Product

In accordance with the recommendations for safety evaluation by the International Food Biotechnology Committee (Coulston, 1990), all traces of the production organism are removed during the manufacturing process (see Section 4.4), ensuring that the enzyme preparations are free from the production organism *Aspergillus niger*.

2.7 Absence of Antibiotic Resistance Gene

As noted above, no antibiotic resistance markers or other heterologous markers are present in the strain. The enzyme preparations are tested to ensure the absence of antibiotic activity in accordance with the recommendation from the Joint Expert Committee of Food Additives of the FAO/WHO ("JECFA"). As is shown in Section 4.6 of this dossier, quality control testing of the finished carboxypeptidase 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 (6th edition) mentions the following: "*Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.*"

In the General Specifications for enzyme preparations laid down by JECFA in 2006, the following is said: "*Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.*"

Instead of analyzing the final enzyme preparations for mycotoxins, DSM therefore decided it more appropriate to test whether the carboxypeptidase-producing microorganism possesses the intrinsic capacity to produce mycotoxins. This was tested under fermentation conditions, as well as conditions that induce toxin production. The test showed that the production strain does not produce any known toxins under these conditions.

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5. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

- Systematic name : serine-type carboxypeptidase
- Enzyme Commission No. : 3.4.16.x

Carboxypeptidase belongs to the subclass of peptidases acting only at the near ends of polypeptide chains, also called exopeptidases (EC 3.4.11.x – 3.4.19.x).

3.2 Amino Acid Sequence

The carboxypeptidase described in this dossier is produced by *Aspergillus niger* as a glycoprotein with a primary sequence of 467 amino acids and a calculated molecular weight of 53 kDa. Based on SDS-PAGE, it can be concluded that the molecular weight of the enzyme is about 62 kDa. Annex 3.2.1 shows the amino acid sequence of the carboxypeptidase produced by *Aspergillus niger*.

3.3 Sequence Comparison to Other Carboxypeptidases

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

About 250 different sequences with significant ($E\text{-value} \leq 3 \cdot 10^{-23}$)² homology were found.

The most similar sequences are carboxypeptidases from related fungal species (ascomycetes) like *Aspergilli* (such as *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus phoenicis*) and *Penicillium marneffei* (each with an E-value of 0).

A second cluster of homologous fungal sequences is found around another peptidase in *Aspergillus niger* pepF (E-value of $1 \cdot 10^{-110}$), which has 41% identical amino acids to carboxypeptidase. This cluster contains the sequence of the maize plant *Zea mays*. Various other plant sequences (*Vitis vinifera* or winegrape, *Arabidopsis*, rice and *Sorghum*) are found in a cluster with more distant sequences, with BLASTp E-values around $1 \cdot 10^{-27}$. Another cluster consists of sequences from yeasts like (*Schizo-*) *Saccharomyces* ($3 \cdot 10^{-28}$).

The mammals possess distantly homologous sequences that cluster together, with an E-value around $1 \cdot 10^{-25}$ in the Blast comparison. The closest mammalian homolog of carboxypeptidase is a bovine cathepsin with an E-value of $1 \cdot 10^{-27}$. The human enzyme cathepsin A (which has been

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

positively identified as a serine carboxypeptidase by Matsuda and Misaka in 1975) has an E-value of 1×10^{-25} , and it shares 23% identity with carboxypeptidase.

3.4 Enzymatic Activity

Principal Enzyme Activity

The major enzymatic activity described in this dossier is a serine-type carboxypeptidase from *Aspergillus niger*, further called ‘carboxypeptidase’. The enzyme hydrolyzes C-terminal amino acids from proteins and peptides. The highest activity is found towards the amino acids phenylalanine, isoleucine, leucine, methionine and valine, however other amino acids can be hydrolyzed as well.

The activity of carboxypeptidase can be determined with the help of the substrate 3-(2-furyl)acryloyl (FA) linked to the di-peptide Phe-Ala. The enzymatic hydrolysis of the di-peptide from FA will result in a decrease of the optical density, which can be measured at a wavelength of 340 nm.

The activity of carboxypeptidase is expressed in so-called CPGU and is related to an officially assigned carboxypeptidase standard (Sibeijn et al., 2006). One CPGU is the amount of enzyme needed to decrease the optical density at 340 nm by one absorbency unit per minute under the conditions described for the carboxypeptidase assay.

The biochemical properties of carboxypeptidase from *Aspergillus niger* have been investigated extensively (see also Annex 3.4.1). The enzyme has shown to exhibit activity over a pH range between 2.5 and 6.5. The pH optimum is found around pH 4.0. The temperature optimum for carboxypeptidase activity is found at 40 - 45°C, while it is active in a range from 25 – 55°C. The enzyme is inactivated at temperatures above 60°C.

Subsidiary enzymatic activities

Enzyme preparations normally contain non-standardized amounts of other enzymes, which are also produced by the micro-organism. In the case of the carboxypeptidase preparation, some of these residual activities are unwanted for the applications. Therefore, the enzyme is purified using chromatography. The effectiveness of this step can be seen in Table 1.

Enzyme	Activity pre chromatography	Activity post chromatography
Fungal Amylase	39 FAU/g	0.018 FAU/g
Aspergillopepsin	289 APU/g	33 APU/g

Table 1: several subsidiary enzymatic activities pre and post chromatography

4. MANUFACTURING PROCESS

4.1 Overview

The carboxypeptidase described in this dossier is produced by a controlled submerged fermentation of a selected, pure culture of *Aspergillus niger* (see Section 2). The production process includes the fermentation process, recovery (downstream processing) and formulation of the product. An overview of the different steps involved is given in Annex 4.1.1.

4.2 Raw Materials

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

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

4.3 Fermentation Process

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

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

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

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

4.4 Recovery Process

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

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The cell material is separated from the enzyme 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). Finally, the product is further purified by chromatography.

4.5 Stabilization, Formulation and Standardization Process

After chromatography, the purified carboxypeptidase (eluate) is formulated to either a liquid or a dry preparation.

For the liquid formulation, the product is stabilized with glycerol to reach a carboxypeptidase activity of $900 \pm 10\%$ CPGU/ml, in accordance with the product specification. Finally the product is filter sterilized.

For the dry formulation, the product is spray-dried into a granulated form. After activity determination, the product is formulated with maltodextrin to reach a carboxypeptidase activity of $900 \pm 10\%$ CPGU/g, in accordance with the product specifications.

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 (6th edition), the final carboxypeptidase preparation from *Aspergillus niger* meets the following specifications:

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

The additional characteristics for the liquid formulation are:

<u>ITEM</u>	<u>NORM</u>
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Carboxypeptidase activity	900 ± 10% CPGU/g
Appearance	Yellow to brown, clear liquid
pH	4.5 ± 0.2

For the dry formulation they are:

<u>ITEM</u>	<u>NORM</u>
Carboxypeptidase activity	900 ± 10% CPGU/g
Appearance	Cream coloured granulate
Dry matter	≥ 90%

5. COMPOSITION AND SPECIFICATIONS

5.1 Formulation

The common starting material for all formulations is the eluate after chromatography. Typically, its composition falls within the following ranges:

<u>Item</u>	<u>Value</u>	<u>Unit</u>
Enzyme activity	1900 ± 200	CPGU/g
Water	95 - 98	%
Ash	0.1 – 0.3	%

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

The Total Organic Solids (“TOS”) of the carboxypeptidase preparations were calculated from 3 different batches of the eluate after chromatography:

Calculation of the TOS					
Batch number	Ash (%)	Water (%)	TOS (%)	Activity (CPGU/g)	CPGU/mg TOS
GRZ.0640	0.09	96.98	2.93	2080	71.0
GRZ.0712	0.14	96.74	3.12	2080	66.7
GRZ.0745	0.27	96.90	2.83	1739	61.4
Mean					66.4

Based on the above figures it can be calculated that the formulated commercial products with activities of 900 ±10% CPGU/g will have a TOS value of about 14 mg/g.

5.2 General Production Controls and Specifications (Good Manufacturing Practice)

Commercial demands require a strictly controlled fermentation process.

The enzyme fermentation factory at Seclin, France, which has fermentation experience since 1922, has acquired the ISO 9001-2000 certification.

Technical measures

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

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

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

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

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

Control measures

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

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

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

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

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

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

6. APPLICATION

6.1 Mode of Action

The technological function of carboxypeptidase is the release of C-terminal amino acids from proteins and peptides present in various foods.

6.2 Application

The carboxypeptidase in question is to be used in cheese, enzyme modified cheese or fermented meat.

Cheese

In cheese, the use of carboxypeptidase results in accelerated development of flavors as well as debittering during the ripening process.

During cheese production, lactic acid bacteria that are added to milk form lactic acid and flavor components. Formation of these flavor components is a slow process and takes several months. This period is referred to as “ripening of the cheese”. Cheese producers would like to shorten this ripening period.

When added to milk together with lactic acid bacteria, carboxypeptidase will be evenly distributed in the final cheese during cheese making. This enables the enzyme to act on the proteins present throughout the cheese and release the amino acids that act as precursors for the flavor components during the ripening period. In this way, the same types of flavor components are developed as during regular ripening, but the speed of the flavor development is accelerated resulting in a shorter ripening period of the cheese.

In Annex 6.2.1 the results of application trials are given. They confirm the benefits of using the carboxypeptidase in cheese ripening.

Enzyme modified cheese (EMC)

In EMC, carboxypeptidase is mainly used as debittering aid.

EMC is produced by hydrolyzing components (i.e., lipids and proteins) of young cheese (such as Cheddar) with a mixture of enzymes such as peptidases and lipases. During an incubation time of several hours at 30-40°C, the enzymes release several flavor components, giving the mixture the flavor of ripened cheese. After incubation, the mixture is pasteurized to inactivate the enzymes and often dried to an EMC powder.

The EMC-powder is used to create a cheesy-taste to several foods like soups and sauces. However, regular enzymes used in EMC production often also release bitter-tasting peptides, which are undesired in most applications. When carboxypeptidase is added to the regular enzyme mixture, the C-terminal amino-acids are split off from the bitter-tasting peptides, enhancing their taste.

Fermented meat

In fermented meat, carboxypeptidase can be used to speed up the development of flavor during ripening.

Fermented dry meat is produced by cutting and mixing pieces of meat and bacon together with lactic acid bacteria. The mixture is filled in foil or natural pig-intestine, and left to dry and ferment in a temperature- and moisture-controlled ripening cell. During the ripening period of three weeks, the moisture decreases by evaporation, the pH decreases because the lactic acid bacteria form lactic acid, and the taste develops because of flavor components formed by the lactic acid bacteria.

When added together with lactic acid bacteria during cutting and mixing of the meat and bacon, carboxypeptidase releases amino acids that stimulate lactic acids bacteria in their formation of flavor components. As a result, the meat reaches the desired taste 6 days earlier than standard (after 22 instead of 28 days, an improvement of 21%).

In Annex 6.2.2 the results of an application trial are given. They confirm the benefits of using the carboxypeptidase in fermented meat.

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. The levels of use expected to result in a beneficial effect are described below.

Cheese

The carboxypeptidase preparation will be used in an average dosage of 18-90 CPGU/liter milk. The maximal dosage will be 225 CPGU/liter milk. 1 liter milk will result in 100 g cheese and 900 ml whey.

EMC

The carboxypeptidase preparation will be used in an average dosage of 400-1600 CPGU/kg EMC. The maximal dosage will be 4000 CPGU/kg EMC.

Fermented meat

The carboxypeptidase preparation will be used in an average dose of 80-180 CPGU/kg meat. The maximal dosage will be 400 CPGU/kg meat.

6.4 Enzyme Residues in the Final Food

6.4.1 Residues of inactive enzyme

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

Final food	Enzyme use level in food ingredient	Residual amount of enzyme in final food	Amount of TOS in final food
Cheese	18-225 CPGU/ L milk	180-2250 CPGU/kg	2.7-33.9 mg/kg
EMC	400-4000 CPGU/kg	400-4000 CPGU/kg	6.0-60.2 mg/kg
Meat	80-400 CPGU/kg	80-400 CPGU/kg	1.2-6.0 mg/kg
Total			9.9 – 100.1 mg/kg

Fate of Carboxypeptidase in cheese and meat products

The ripening or maturation of cheese is a complex process that involves several reactions with milk components like glycolysis, proteolysis and lipolysis. These chemical reactions are catalyzed partly by enzymes added to milk (e.g., rennet and chymosin). The enzymes produced by the different microbial cultures added or naturally present in milk also play a vital role in the ripening process.

The type of enzymes involved in ripening depends very much on the type of cultures used. Fungal cultures are known for the production of carboxypeptidases.

The ripening time differs considerable between cheeses and may be as long as two years, e.g., for Parmesan. All enzymatic activities evolve during ripening. Variation in enzymatic activity is illustrated by both Gobbetti (1997) and Spettoli (1985), who describe the increase of carboxypeptidase activity during maturation in Gorgonzola, while in Provolone the activity decreases and in Montasio it vanishes during ripening. The functionality of the enzymes is determined by the pH, availability of substrate, proteolytic enzymes originating from lactic acid bacteria and pasteurization. At the end of the ripening process, the enzymes are no longer functional.

The principles of the ripening of cheese and meat are the same. Therefore, it can be concluded that enzymes will no longer be functional at the end of the ripening process in meat. In EMC carboxypeptidase is inactivated by means of pasteurization.

6.4.2 Possible Effects on Nutrients

Many microorganisms used in food fermentation produce serine-type carboxypeptidases. Examples are *Aspergillus oryzae* (Kobayashi et al., 2007), a fungus with a long history in food in Japan, and various *Lactobacilli* (Kleerebezem et al., 2003; Bolotin et al., 2001) which are used as starter cultures for the production of cheese and fermented meat.

It can therefore be concluded that the enzyme, as well as its reaction products, are normal constituents of food and that the addition of extra carboxypeptidase will not lead to new or unintended reaction products in cheese, meat or EMC.

Based on this information, there are no reasons to expect any possible effects on the nutrients other than those already known.

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7. SAFETY EVALUATION

7.1 Safety of the Donor and Production Strain

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the IFBC, food or food ingredients are safe to consume if they have been produced, according to current Good Manufacturing Practices, from a nontoxicogenic and nonpathogenic organism (Coulston, 1990). A nontoxicogenic 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 is not toxicogenic. It is known to naturally occur in foods. The fungus is commonly present in products like rice, seeds, nuts, olives and dried fruits.

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

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

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

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

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

decision in 1990 and change the ADI for enzyme preparations derived from *Aspergillus niger* into “not specified”. See Annex 7.1.4 to this notification for JECFA’s 1990 review.

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

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

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

The recombinant carboxypeptidase strain PEG-51A (DS 55577) has been classified by both the Dutch and French competent authorities as a Group I safe micro-organism. In addition, the strain was approved by the same authorities as a self-cloned GMO. Consequently, the strain was approved for large scale production of carboxypeptidase in the DSM factory in Seclin, France.

Based on the genetic modification performed (see Section 2.2), there are no reasons to assume that the recombinant production strain should be less safe than the original GAM-53 (DS 3045) strain. In fact, it has been shown that the DSM GAM/ISO lineage of *A. niger* strains are safe hosts for the over-expression of enzymes to the extent that for new enzymes the safety is already covered by the safety studies performed on other enzyme production strains derived from this lineage and consequently new safety studies are superfluous (van Dijck et al., 2003, see Annex 2.1.1).

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

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

7.2 Safety of the Carboxypeptidase Enzyme

As noted above, enzymes produced by *Aspergillus niger* have already been used for food production for several decades. In the USA, FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *Aspergillus niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132), and lipase (GRN 000111 and GRN 000158). The FDA recently summarized the safety of microorganisms, including *Aspergillus niger*, used

as a host for enzyme-encoding genes (Olempska-Beer et al., 2006). In addition, a phospholipase A₂ (GRN 000183), an asparaginase (GRN 000214) and a lipase (GRN 000296) preparation from genetically modified *Aspergillus niger* strains, which were derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier, have been notified as GRAS. FDA had no objections to each of these notifications.

The Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO has evaluated several enzymes from *Aspergillus niger* for their safety (WHO Food Additives Series 6 (1975) and 22 (1988)). All these enzymes have received an ADI “not specified” by JECFA (see also Section 7.1).

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

The enzyme preparation carboxypeptidase produced by *Aspergillus niger* strain PEG-1A, over-expressing the carboxypeptidase gene from *Aspergillus niger*, was evaluated according to the Pariza & Johnson Decision Tree. The decision tree is based on the safety evaluation methodology published by Pariza and Foster in a 1983 article, which was extended by the IFBC into the decision tree format and published in 1991. In 2001, Pariza and Johnson published an update. DSM's decision tree analysis, based on the most recent update of the decision tree, is described in Annex 7.2. Based on the Pariza and Johnson decision tree analysis, DSM concludes that the carboxypeptidase preparation is safe.

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

- Dose-range finding (14-day) oral toxicity study
- Sub-chronic (90 day) oral toxicity study
- Ames test
- Chromosomal aberration test, *in vitro*
- Micronucleus assay, *in vivo*

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

7.2.1 Allergenicity

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

Enzymes are proteins with highly specialized catalytic functions. They are produced by all living organisms and are responsible for many essential biochemical reactions in micro-

organisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are natural protein molecules that act as very efficient catalysts of biochemical reactions.

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

Because exposure to enzymes used as processing aids in food is very low, even if they were potentially allergenic by the oral route, the likelihood of allergic sensitization of consumers to these proteins is virtually 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 found of people that have been sensitized or that reacted allergic by ingestion of food prepared with various enzymes (see Annex 7.2.1). Even people who ingest high daily doses of enzymes as digestive aids are not reported to have gastrointestinal allergy to enzymes even after many years of daily intake.

The amino acid sequence of carboxypeptidase has been compared with sequences of known food allergens. Using the SwissProt database (last updated December 4, 2007) and the WHO-IUIS database (last updated December 21, 2007) within the Allermatch database³, and using a FASTA algorithm, it was established that the amino acid sequence for the DSM carboxypeptidase preparation (Accelerzyme® CPG) does not have 35% or more overlap with known food allergens using a window of 80 amino acids.⁴ Accordingly, DSM concludes that the carboxypeptidase preparation is not a potential allergen and no further allergenicity studies are necessary.

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

The safety of the production organism is the point of focus as to the safety of the enzyme which will be used in food processing. In this case, the production organism *Aspergillus niger*, has

³ See Fiers MWEJ, Kleter GA, Nijland H, Peijnenburg AACM, Nap J-P, Van Ham RCHJ (2004) Allermatch™, a webtool for the prediction of potential allergenicity according to current FAO/WHO Codex alimentarius guidelines. BMC Bioinformatics 5:133 (16 Sep 2004). <http://www.biomedcentral.com/1471-2105/5/133>. See also www.allermatch.org™.

⁴ According to the FAO/WHO method, a protein is a potential allergen when there is more than 35% homology between the amino acid sequence of the protein and a known allergen using a window of 80 amino acids and a suitable gap penalty. See Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 22-25 (January 2001).

been demonstrated to be nontoxicogenic and nonpathogenic and any food ingredient (enzyme) from that organism will exhibit the same safety properties if manufactured under current Good Manufacturing Practices (“cGMPs”). Pariza and Foster (1983) noted that a nonpathogenic organism was very unlikely to produce a disease under ordinary circumstances. In their publication, *Aspergillus niger* is included in the authors’ listing of the organisms being used in the industry.

The FDA has also accepted the Enzyme Technical Association’s GRAS Notifications stating that carbohydrase, pectinase, protease, glucose oxidase and catalase (GRN 000089), lipase (GRN 000111, GRN 000158 and GRN 000296), lactase (GRN 000132), phospholipase A2 (GRN 000183) and asparaginase (GRN 000214) enzyme preparations from *Aspergillus niger* are generally recognized as safe. *Aspergillus niger* is listed as a production organism for enzymes (Pariza and Johnson, 2001) and has a long history of safe use (see Annex 7.1.1).

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

The evaluation of the safety of the genetic modification should be examined based on the concepts outlined in the Pariza and Foster (1983) paper. Their basic concepts were further developed by the IFBC in 1990, the EU Scientific Committee for Food in 1991, the OECD in 1991, ILSI Europe Novel Food Task Force in 1996 and FAO/WHO in 1996. Basically, the components of these evaluations start with an identified host strain, descriptions of the plasmid used and the source and fraction of the material introduced, and an outline of the genetic construction of the production strain. This information is found in Section 2.

Carboxypeptidases are ubiquitous in nature and can be found in fungi, plants and animals (including humans) (Breddam, 1986). They have been recognized as an individual category by the International Union of Biochemistry and Molecular Biology (IUBMB) since 1972.

Carboxypeptidases are proteolytic enzymes (“peptidases”, also known as “proteases”) that only cleave the C-terminal peptide bond in polypeptides. Proteases have been affirmed as GRAS by FDA in 2002 (GRN 000089 and GRN000090). Proteases have been included in the safety evaluation by Pariza and Johnson (2001). Besides, specifically serine carboxypeptidase has been found in various fruits and seeds (Breddam, 1986) and mammals (rat (Matsuda & Misaka, 1975), pig (Kawamura et al., 1977) and humans (Ody and Erdös, 1981)).

As Enari described in 1980, the carboxypeptidases from germinated barley are important in the production of beer. Also, watermelon contains carboxypeptidase (Matoba and Doi, 1975) and as such, naturally-occurring carboxypeptidases are consumed as they will be in the applications described here.

7.2.3 Substantial Equivalence

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

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

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

Close similarities exist between the carboxypeptidase that is the subject of the present GRAS notification and other peptidases that have been safely marketed for years, including those that are the subject of GRAS notifications.

Carboxypeptidases (IUB 3.4.16) are part of the peptidases (IUB 3.4). Several other peptidases have been notified as GRAS by the FDA: Aspartic proteinase (GRN 000034: IUB 3.4.23.x) derived from *Aspergillus oryzae*, carrying a gene encoding aspartic proteinase from *Rhizomucor miehei*; protease enzyme preparations from *Aspergillus oryzae* (GRN 000089: IUB 3.4.23.6); and protease from *Aspergillus niger* (GRN000090: IUB 3.4.21.14, 3.4.23.6 and 3.4.24.4). To each of those notifications, FDA responded with a letter stating they had no questions.

In addition to the safety of the carboxypeptidase enzyme itself, the current production strain is derived from a safe strain lineage of *Aspergillus niger*. *Aspergillus niger* is a common food constituent of products like rice, seeds, nuts, olives and dried fruits. In addition, *Aspergillus niger* has been used for several decades for the production of organic acids and enzymes to be used in the food industry. The FDA recently summarized the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Bier et al., 2006).

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

The Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO has evaluated several enzymes from *Aspergillus niger* for their safety (WHO Food Additives Series 6 (1975)

and 22 (1988)). All these enzymes have received an ADI “not specified” by JECFA (see also Section 7.1).

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

Moreover, a phospholipase A₂ (GRN 000183), an asparaginase (GRN 000214), and a lipase (GRN 000296) preparation from genetically modified *Aspergillus niger* strains, derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier and processed according to the mentioned concept, were the subject of GRAS notifications to which FDA has no questions.

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

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

7.3 Safety of the Manufacturing Process

Carboxypeptidase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 5.2, the carboxypeptidase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

7.4 Safety Studies

This section describes the studies performed to evaluate the safety of using DSM’s carboxypeptidase preparation.

7.4.1. Safety Studies in Summary

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

The safety studies were performed with the non-purified and non-formulated UF concentrate of batch number CPG.GRZ.0704, referred to as 'tox-batch'. The tox-batch was produced according to the procedure used for commercial production and contained an activity of 4441 CPGU/g.

Levels used in the 90-day oral gavage studies were based on the results of the 14-day dose range-finding study to provide a sufficient margin of safety towards expected exposure (see Section 7.5).

1 14-day dose range-finding

The range-finding study was performed at Advinus, India with four groups of 6 male and 6 female Wistar rats. The rats received daily the tox-batch by gavage at 1000, 3000 and 10,000 mg/kg bw/day at a volume of 10 ml/kg bw/day. The control group received double distilled water. Clinical signs, body weight, food consumption, hematology, clinical chemistry, organ weights of principal organs (kidney, liver, spleen, heart, brain, thymus, adrenals, epididymis and ovaries), macroscopy at necropsy and microscopy of the stomach were studied.

Results

The administration of the tox-batch at levels up to 10,000 mg/kg bw/day did not result in treatment-related effects in the rats.

2 90-day oral toxicity

The sub-chronic oral toxicity of the tox-batch was examined in a 90-day toxicity study at Advinus, India, in accordance with the following guidelines:

- OECD Guideline for the Testing of Chemicals 408. Repeated Dose 90-day Oral Toxicity Study in Rodents, adopted 21st September 1998.
- B.26. Sub chronic oral toxicity test. Repeated dose 90-day oral toxicity study in rodents. Annex 5D to Commission Directive 2001/59/EC, Official Journal of the European Communities L225, 21.8.2001.

The study consisted of four groups of 10 male and 10 female Wistar rats. The rats received daily the tox-batch by oral gavage at a level of 1000, 3000 and 11,000 mg/kg bw/day, corresponding with 96, 288 and 1056 mg TOS/kg bw/day or 4441, 13332 and 48851 CPGU/kg bw/day, respectively, at a volume of 10 ml/kg bw/day. The control group received double distilled water. General clinical observations, neurobehavioral testing, ophthalmoscopic examination, body weight, food consumption, hematology, clinical chemistry, organ weights of principal organs, macroscopic examination, histopathology of organs (control and high-dose group only) and microscopy of all lesions were studied.

Results

At the highest dose tested (11,000 mg/kg bw/day which is equivalent to 1056 mg TOS/kg bw/day or 48851 CPGU/kg bw/day), no treatment-related effects were observed; therefore this dose is considered the NOAEL.

3 Mutagenicity tests

AMES test

A bacterial reverse mutation test (Ames) was conducted with the tox-batch at NOTOX, The Netherlands, in accordance with the following guidelines:

- OECD guideline no. 471, Genetic toxicology: Bacterial Reverse Mutation Test, adopted 21 July 1997.
- B.13/14. Mutagenicity: Reverse Mutation Test using bacteria. Directive 2000/32/EC published June 8, 2000.

The tox-batch was assessed for mutagenic activity in four selected strains of *Salmonella typhimurium*, TA 1535, TA 1537, TA 98 and TA 100, as well as in the *Escherichia coli* mutant WP2 uvrA. All were tested in both the absence and presence of a metabolic activation system (S9-mix).

Two bacterial reverse mutation tests were performed. In the first assay the tox-batch was tested at a concentration range of 100 to 5000 µg TOS/plate in the absence and presence of 5% (v/v) S9-mix. In the second assay the test substance was tested at the same concentration range as in the first assay in the absence and presence of 10% (v/v) S9-mix in all tester strains. Positive and negative controls were run accordingly.

The bacterial background lawn was not reduced at any of the concentrations tested and no biologically relevant decrease in the number of revertants was observed.

The negative and strain-specific positive control values were within the laboratory historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

The tox-batch did not induce a significant dose-related increase in the number of revertant colonies in each of the tester strains both in the absence and presence of S9-metabolic activation.

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

Chromosomal aberration test

A chromosomal aberration *in vitro* test was performed with the tox-batch in human lymphocytes at NOTOX, The Netherlands in accordance with the following guidelines:

- OECD guideline 473, Genetic toxicology: In vitro Mammalian Chromosome Aberration Test, adopted 21 July 1997.
- B.10. Mutagenicity: *In vitro* Mammalian Chromosome Aberration Test. Directive 2000/32/EC published June 8, 2000.

In the first chromosomal aberration test cells were treated with the tox-batch for 3 hours (pulse treatment) in the presence and the absence of S9-mix. The harvesting time of the cells was 24 hours after treatment. Concentrations analyzed were 1000, 3330 and 5000 µg TOS/ml in the presence of S9-mix and 1000, 4330 and 5000 µg TOS/ml in the absence of S9-mix.

In the second chromosomal aberration test, the treatment and harvest times were 3/48 hours (pulse treatment) in the presence of S9-mix. In the absence of S9-mix the treatment/harvest times were 24/24 hours and 48/48 hours (continuous treatment). Analyzed concentrations were 1000, 3330 and 5000 µg TOS/ml for pulse treatment. In the continuous treatment group concentrations analyzed were 300, 600 and 1000 µg TOS/ml for 24/24 hours and 300, 1000 and 2000 µg TOS/ml for 48/48 hours. Due to toxicity, higher dose levels could not be analyzed.

Negative controls were run accordingly and showed the number of chromosome aberrations to be within the laboratory historical control data range. Positive control chemicals, mitomycin C and cyclophosphamide, both produced a statistically significant increase in the incidence of cells with chromosome aberrations, indicating that the test conditions were adequate and that the metabolic system (S9-mix) functioned properly.

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

In the first cytogenic assay it was noted that the tox-batch increased the number of polyploid cells both in the absence and presence of S9-mix at the two highest doses. This may indicate that the tox-batch has the potential to disturb mitotic processes and cell cycle progression.

The data obtained in the two chromosomal aberration tests support the conclusion that, under the conditions used in these tests, the tox-batch is not clastogenic to cultured human lymphocytes.

Micronucleus test *in vivo*

In order to elucidate the findings in the chromosomal aberration assay regarding the possible potency to disturb mitotic processes and cell cycle progression, the tox-batch was subjected to a micronucleus test *in vivo*. This test is more appropriate to detect damage induced to the mitotic apparatus than a chromosomal aberration assay.

The micronucleus test was performed with the tox-batch in mice at NOTOX, The Netherlands in accordance with the following guidelines:

- OECD guideline 474, Genetic toxicology: Mammalian Erythrocyte Micronucleus Test, adopted 21 July 1997.
- B.12. Mutagenicity: *In vivo* Mammalian Erythrocyte Micronucleus Test. Directive 2000/32/EC published June 8, 2000.

In the dose range finding study three male and three female mice (NMRI BR mice (SPF)) were dosed at 2000 µg TOS/kg bw once by oral intubation. None of the animals showed any abnormality during the three days of the study.

000037

Since no sex difference was observed in the dose-range finding study, five male animals were used in each of the six treatment groups. The tox-batch was dosed at 500, 1000 and 2000 mg TOS/kg bw in 5.2, 10.4 and 20.8 mg/kg bw, respectively. Animals of the low- and mid-dose groups were sampled at 24 hours, and the high-dose groups were sampled at 24 and 48 hours. Negative control animals (physiological saline) received 20.8 mg/kg bw and were sampled at 24 hours. Positive control animals were dosed 50 mg/kg bw cyclophosphamide in 10 ml/kg bw and were sampled at 48 hours.

The incidence of micronucleated polychromatic erythrocytes in the bone marrow of all negative control animals was within the historical solvent control data range. The positive control substance induced a statistically significant increase in the number of micronucleated polychromatic erythrocytes. Hence, both criteria for an acceptable assay were met.

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

Two animals of the mid-dose group showed a high incidence of 7 micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes. Since the high incidence was only observed in two out of five animals, which was at the intermediate dose, and the mean number of micronucleated polychromatic erythrocytes of this group was within the laboratory historical control data range, the finding was considered not biologically relevant. Moreover, the quality control of these slides scored by a different scorer revealed no increase in the number of micronucleated polychromatic erythrocytes above the historical data range (0-6).

Therefore, it is concluded that the tox-batch is not clastogenic or aneugenic in the micronucleus test under the experimental conditions described.

7.5 Estimates of Human Consumption and Safety Margin

The EDI was calculated based on the maximal dose levels and consumption data in the United States of America (Wilson et al., 1997). Based on the data given in Section 6.1 and a body weight for a person of 60 kg, the following calculation can be made:

Food category	Residual amount of enzyme in food (CPGU/kg)	Mean consumption of food (g/person/day)	90th percentile intake level (g food/person/day)⁵	Estimated daily intake of enzyme (CPGU/kg bw)
Cheese/EMC ⁶	2250-4000	16	32	2.1
Meat	400	21	42	0.3
Total				2.4

⁵ 90th percentile is 2 times the intake level (CFSAN, 2006).

⁶ It is assumed that all of the enzyme preparation ends up in the cheese and not in the whey (see Section 6.1). This is a 'worst case' situation, since the consumption of cheese is generally higher than that of whey.

The Margin of Safety (MoS) can be calculated by dividing the NOAEL by the EDI. With an overall NOAEL of 48851 CPGU/kg body weight/day the MoS will be $48851/2.4 = 20355$.

Regarding the height of the MoS, it was concluded that further testing of the safety of the product is not meaningful.

7.6 Results and Conclusion

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

8. LIST OF ANNEXES

- 2.1.1 Dijck, P.W.M. van, Selten, G.C.M., Hempenius, R.A., On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains, Regulat. Toxicol. Pharmacol. 38:27-35 (2003)
- 2.4.1 Surviving studies of *Aspergillus niger* strains in soil, surface water, and waste water
- 3.2.1 Amino acid sequence of carboxypeptidase from *Aspergillus niger*
- 3.4.1 Biochemical characterization of carboxypeptidase from *Aspergillus niger*
- 4.1.1 Flow diagram of manufacturing process
- 6.2.1 Application trials of carboxypeptidase in cheese production
- 6.2.2 Application trial of carboxypeptidase in fermented meat production
- 7.1.1 Schuster, E., Dunn-Coleman, N., Frisvad, J.C., Dijck, P.W.M van, *On the Safety of Aspergillus niger – a Review*, Appl. Microbiol. Biotechnol. 59:426-435 (2002)
- 7.1.2 JECFA safety evaluation of *Aspergillus niger* as a source of enzymes to be used in food, 1987
- 7.1.3 Expert reports of Prof. J.W. Bennett and Dr. M.O. Moss on the probability of mycotoxins being present in industrial enzyme preparations obtained from fungi (September 1988)
- 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 carboxypeptidase from *Aspergillus niger* PEG-1A
- 7.2.1 Literature search on allergenicity by ingestion of food prepared with enzymes

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Pages 000045-000053 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

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

Aspergillus niger was inoculated in three different environments, namely: soil, surface water and sewage (waste water of Gist-brocades production plant in Delft (The Netherlands)).

As positive control, that is to say to judge whether the environment contained sufficient substrates for the organisms to survive in the absence of competitive (micro)organisms, and that there were no toxic components present, additional inoculations were performed in sterile environments. Sampling was performed during 6 months.

Experimental results are shown in this annex (see figures 1,2 and 3, in which the data are presented for each environment at 8°C as well as 25°C.

In independent incubations the wild-type *Aspergillus niger* (NRRL 3122) and an industrial strain (GAM-53) were compared in sterile and non-sterile environments.

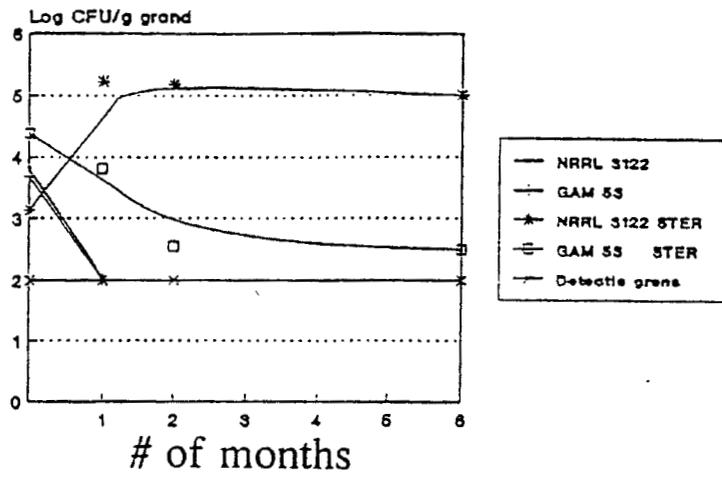
In all sterile environments at 25°C inoculated with *A. niger* spores, having a titre between 10^4 - 10^5 , colony forming units/gram sample were found. At 8°C the wild-type strain was surviving well in soil as well as in surface water, whereas in waste water it was decreased beneath the detection level.

In the surface water environment the industrial strain decreased rapidly (within 2 months) below the detection level, whereas in soil instabilisation occurred when inoculated with a low (10^2 /g soil) titre.

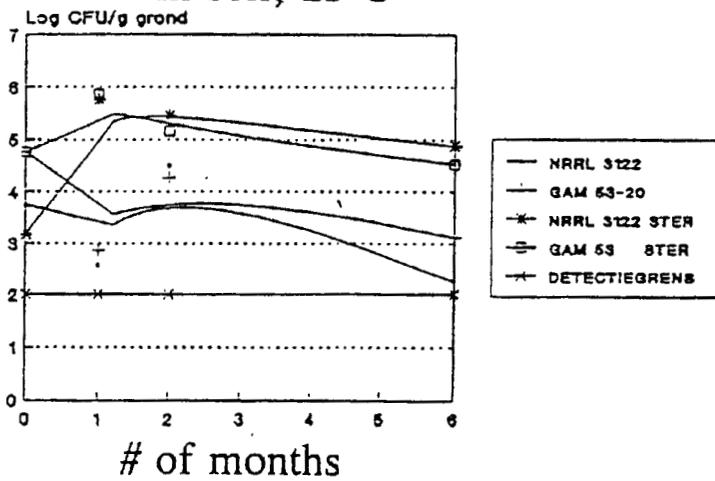
In natural environments and in the presence of competitive micro-organisms surviving *Aspergillus niger* strains were found after 6 month only in soil samples at 25°C, inoculated with at least 10^3 spores/g. In both aqueous environments at 8°C and 25°C and in soil of 8°C a fast decline of surviving strains was observed: within three months and often even faster titres decreased below the detection level.

During optimal conditions (when there is no competition) *Aspergillus niger* survives well in different environments, and the industrial strain usually less compared to the wild-type. Under natural condition *Aspergillus niger* survives only in soil at 25°C (after 6 month titre is decreased only 50 times). In the other environments under different conditions both tested *Aspergillus* strains were not detectable any more within 1 to 3 months, showing that the industrial strain *A. niger* GAM-53 is biological restricted with respect to growth and environmental surviving.

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

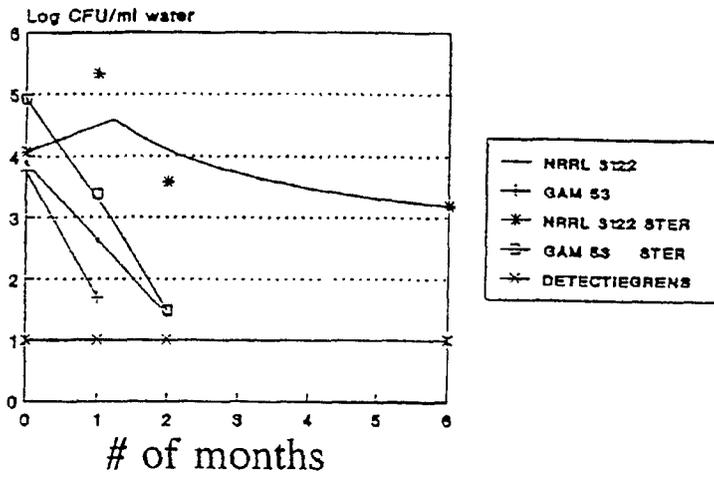


Incubation of *A. niger* mycelium
in soil, 25°C

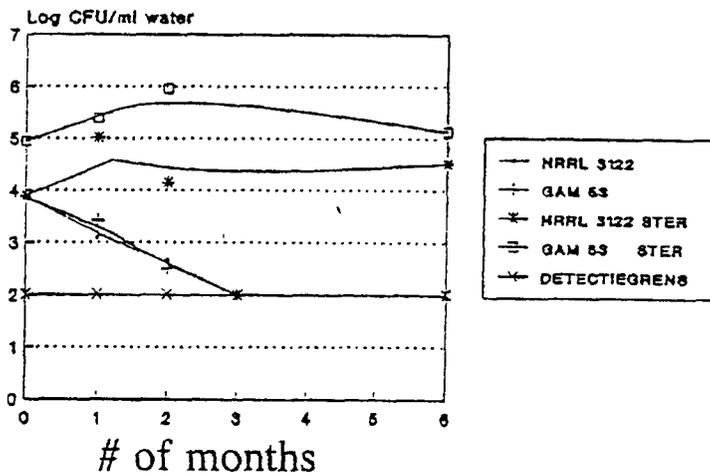


Surviving of *Aspergillus niger* I

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



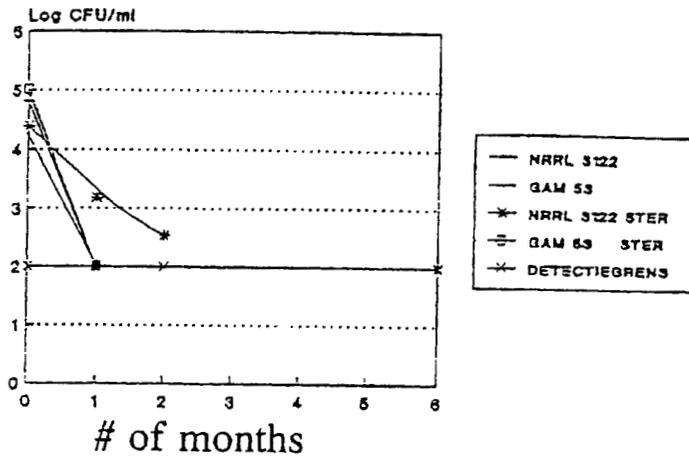
Incubation of *A. niger* mycelium
in surface water, 25°C



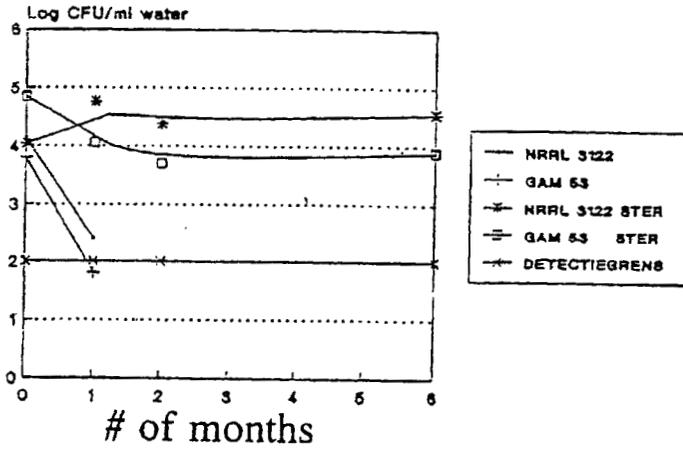
Surviving of *Aspergillus niger* II

000057

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



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



Surviving of *Aspergillus niger* III

Amino acid sequence of the carboxypeptidase protein of *Aspergillus niger*.

MRVTT AIASL LLVGS ATSLQ NPHRR AVPPP LSHRS VASRS VPVER RTTDF EYLTN **KTARF**
LVNGT SIPEV DFDVG ESYAG LLPNT PTGNS SLFFW FFPSQ NPEAS DEITI WLNGG PGCSS
LDGLL QENGP FLWQP GTYKP VPNPY SWTNL TNVVY IDQPA GTGFS PGPST VNNEE DVAAQ
FNSWF KHFVD TFDLH GRKVY ITGES YAGMY VPYIA DAMLN EEDTT YFNLK GIQIN DPSIN
SDSVM MYSPA VRHLN HYNNI FQLNS TFLSY INAKA DKCGY NAFLD KAITY PPPSP FPTAP
EITED CQVWD EVVMA AYDIN PCFNY YHLID FCPYL WDVLG FPSLA SGPNN YFNRS DVQKI
LHVPP TDYSV CSETV IFANG DGSDP SSWG P LPSVI ERTNN TIIGH GWLDY LLFLN GSLAT
IQNMT WNGKQ GFQRP PVEPL FVPYH YGLAE LYWGD EPDPY NLDAG AGYLG TAHT E RGLTF
SSVYL SGHEI PQYVP GAAYR QLEFL LGRIS SLSAK GNYTS

Amino acid sequence of the PepG precursor protein. The mature PepG protein runs from position 53 until position 520 (indicated in blue)

000060

Biochemical characterization of carboxypeptidase from *Aspergillus niger*

1. Summary

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

- pH-curve
- temperature-curve
- Molecular weight and purity of the UF concentrate, based on SDS-PAGE

The results are given below.

2. Methods

pH and temperature profiles were made using an adaptation of the standard protocol for measuring carboxypeptidase activity. In order to cover a pH range from 2 to 6, the standard buffer was replaced by a range of pH buffers containing sodium phosphate, citric acid, acetic acid and Tris. Contradictory to the standard assay, 8 mmol/l FA-Phe-Ala was added as substrate. For the pH range the activities were measured at 25°C at pH 2, 3, 4, 5 and 6. For the temperature profile the activities were measured at pH 4.5 at temperatures of every multiple of 5°C from a minimum of 5 to a maximum temperature of 65°C. Incubation time was 10 minutes. Activities were measured by measuring the absorption at 377 nm.

In order to determine the molecular weight of carboxypeptidase, an SDS-PAGE was performed with enzyme containing UF concentrate before and after chromatography. The SDS-PAGE therefore also shows the purity of the product.

3. Results and Conclusions

3.1 pH profile

As can be seen in Figure 1, the optimum carboxypeptidase activity is at pH 4.0.

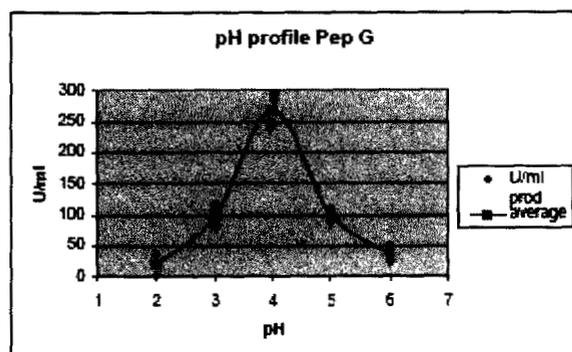


Figure 1. Relative activity of carboxypeptidase between pH 2 - 6, at 25°C

3.2 Temperature profile

As is shown in Figure 2, carboxypeptidase is active between 25 and 60°C and has a temperature optimum between 40 and 45°C.

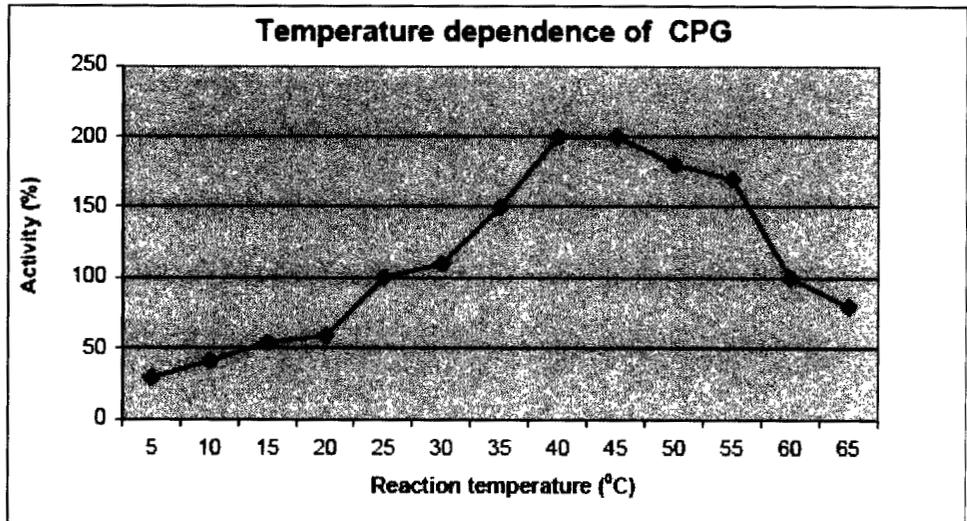


Figure 2. Relative activity of carboxypeptidase at temperatures between 20-65 °C at pH 4.5.

As can be seen in Figure 3, carboxypeptidase is rapidly inactivated at temperatures above 60°C.

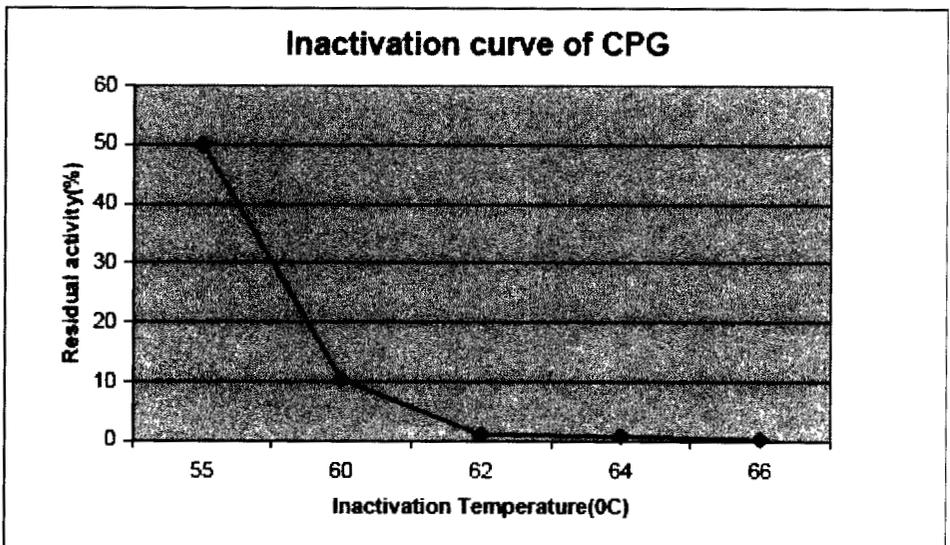


Figure 3. Inactivation of carboxypeptidase at temperatures between 55 and 66°C after 20 seconds at pH 4.5.

3.3 Molecular weight of the carboxypeptidase, and purity of the UF concentrate

From the amino acid sequence of the carboxypeptidase, a theoretical molecular mass of the protein part of the enzyme of 53 kD, can be deduced.

When analysed by SDS PAGE, the purified carboxypeptidase eluate shows a band of 62 kDa (Fig. 4). The difference can be explained by the fact that the enzyme is glycosylated.

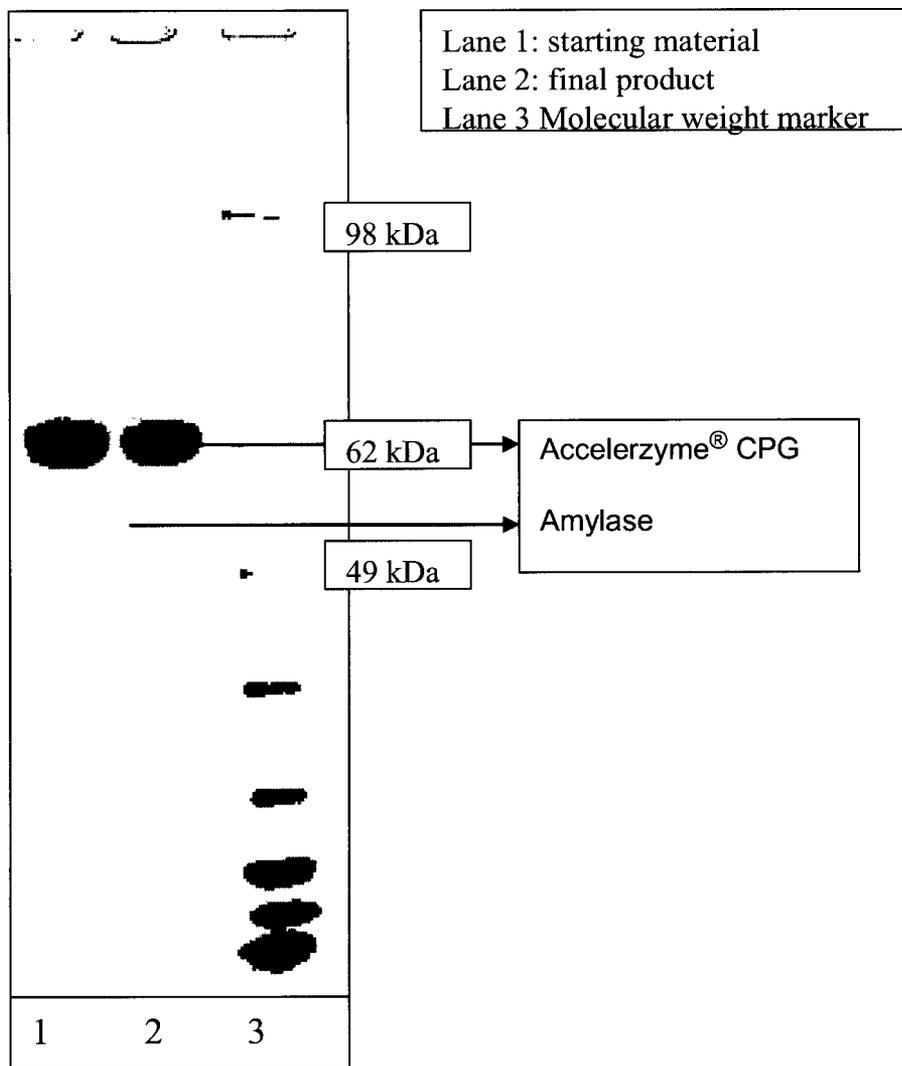
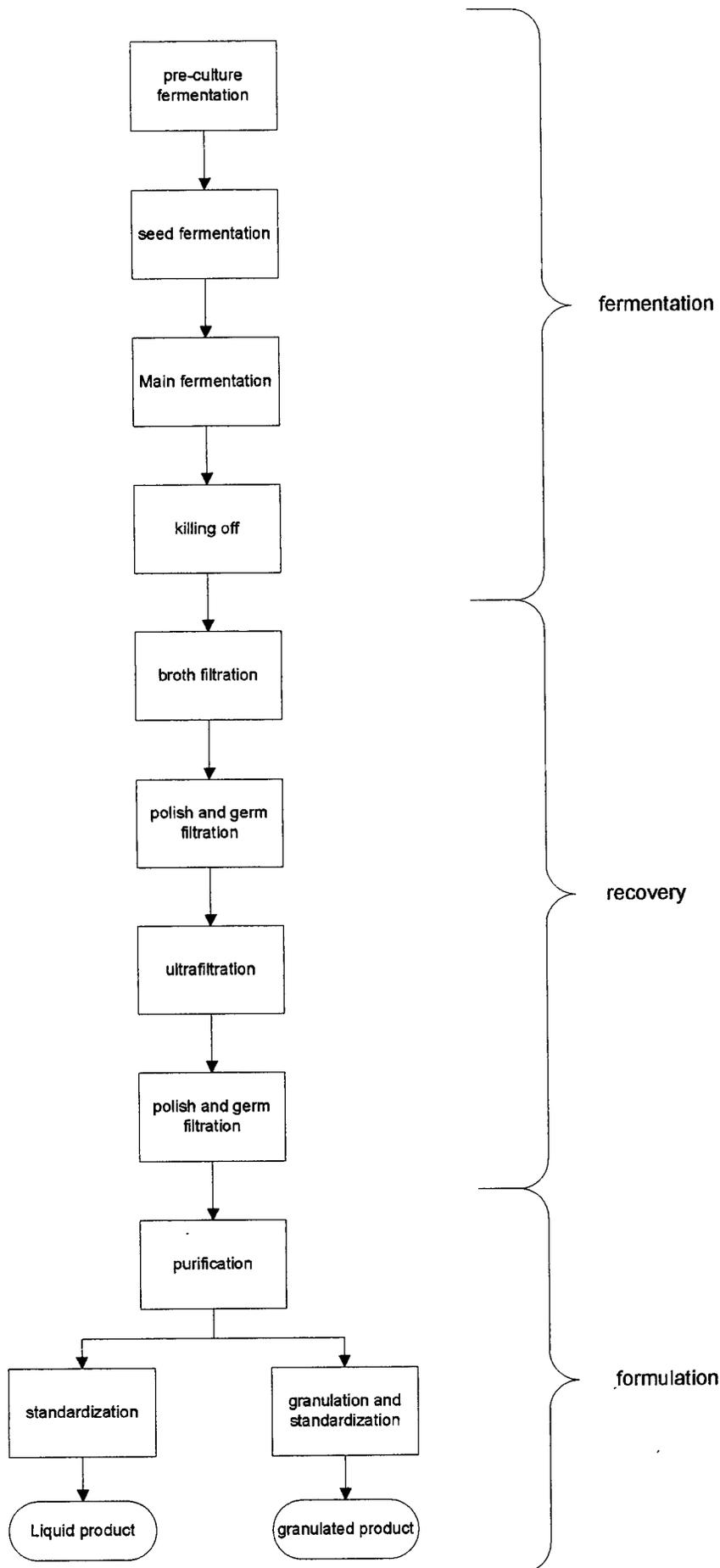


Figure 4. SDS-PAGE analysis of the carboxypeptidase before (lane 1) and after (lane 2) chromatography on 4-12% Bis-Tris Gel with runningbuffer MOPS during 50 minutes at 200V, stained with Simply Blue Safe Stain (Coomassie G250)



000066

Application trials of carboxypeptidase from *Aspergillus niger* in cheese production

Summary

Cheese trials were performed using carboxypeptidase in the production of Cheddar cheese. When compared to standard Cheddar cheese, cheese prepared with carboxypeptidase contained increased levels of free amino acids and key flavor compounds after a ripening period of 4 and 16 weeks. Moreover, after a ripening period of 6 weeks, the cheese prepared with carboxypeptidase was found to be less bitter and sour, and had an increased 'bouillon' taste and increased 'ripeness'.

It is concluded that Cheddar cheese prepared with carboxypeptidase develops flavor and ripens faster when compared to standard Cheddar cheese.

Materials and Methods

Two Cheddar cheeses of approximately 10 kg were produced with and without the addition of carboxypeptidase (39.6 CPGU/l milk).

After a ripening period of 4 and 6 weeks, free amino acids in the cheese were measured by HPLC.

In addition, several key flavor compounds in the cheeses were measured by head space GC-MS.

After a ripening period of 6 weeks, the cheeses were tasted by an external descriptive panel. Samples were offered randomly, and results were analyzed using FIZZ sensory analysis software from Biosystems.

Results

After a ripening period of 4 and 16 weeks, cheddar cheese prepared with carboxypeptidase contained higher levels of free amino acids than standard cheese, as is shown in Figure 1.

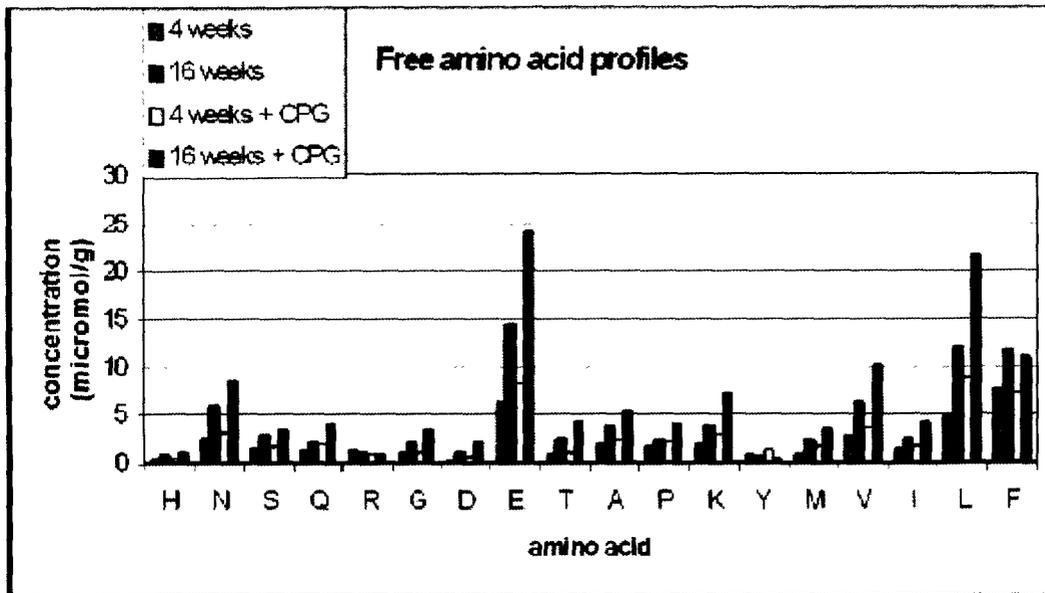


Figure 1. Free amino acid profiles in cheddar cheese prepared with and without carboxypeptidase after a ripening period of 4 and 16 weeks

Moreover, several key flavor compounds were analyzed in Cheddar cheese (Table 1) and found to be in greater quantities in cheese prepared with carboxypeptidase, as is shown in Figure 2.

Compound	Odor	Origin
Diacetyl	Butter-like	Lactose
2-Butanone (methylethylketone)	Butterscotch	Fatty acids
Ethylhexanoate	Fruity, sweet	Free fatty acids + alcohols
Ethylbutyrate	Fruity, bubble gum	Lactose + amino acids
3-Hydroxy-2-butanone (AMC, acetoin)	Buttery, sour	Lactose
1-Octene-3-one	Mushroom	Fatty acids
3-Methylbutanal	Malt, dark chocolate	Leucine

Table 1. Key volatile flavor compounds in Cheddar cheese that were analyzed

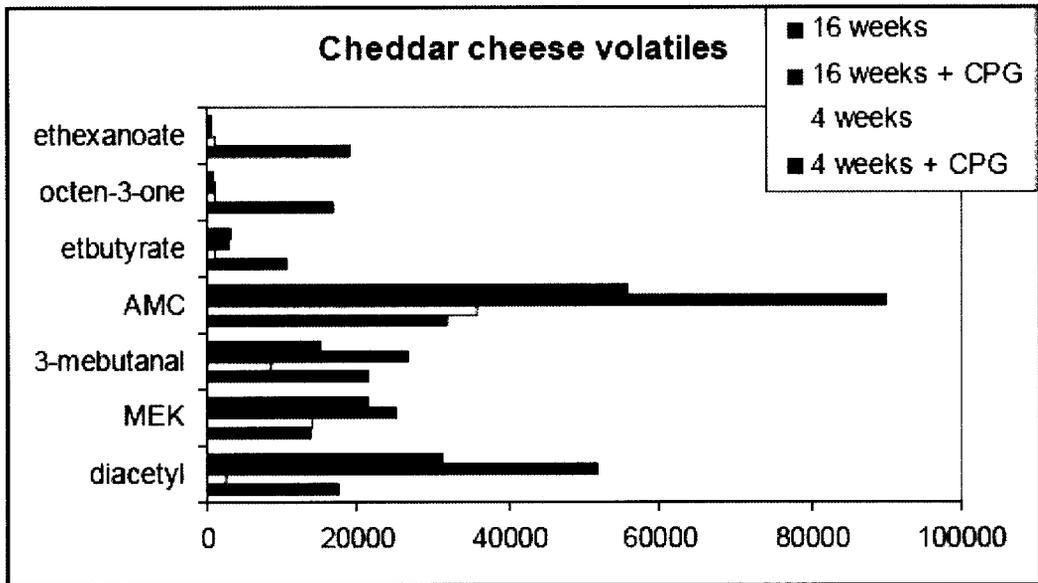


Figure 2. Key flavor compounds in Cheddar cheese prepared with and without carboxypeptidase after a ripening period of 4 and 16 weeks

After a ripening period of 6 weeks, the taste panel judged the cheese prepared with the carboxypeptidase to be less bitter, less sour, and more 'ripe' (i.e., the overall odor and flavor intensity was found to be higher than that of the cheese without the addition of carboxypeptidase) and to have a more 'bouillon' (i.e., savory) taste, as is shown in Figure 3.

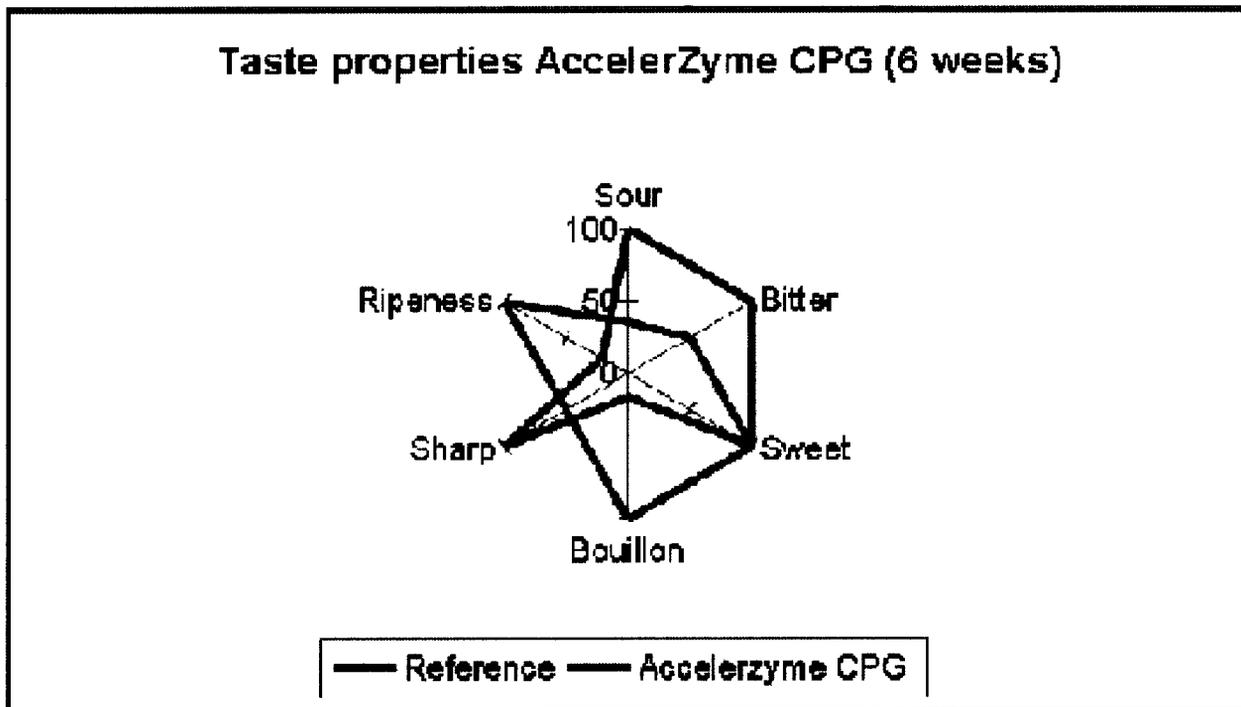


Figure 3. Sensory evaluation of Cheddar cheese prepared with and without carboxypeptidase after a ripening period of 6 weeks

Application trial of carboxypeptidase from *Aspergillus niger* in fermented meat production

Summary

The effect of carboxypeptidase was tested in the preparation of fermented meat sausages. After a ripening period of 22 days, sausages that were prepared with carboxypeptidase already had developed the texture, appearance and taste comparable to a regular dry sausage that had ripened in 28 days.

It can therefore be concluded that the ripening period can be shortened by at least 6 days if sausages are prepared with carboxypeptidase.

Materials and methods

Two batches of 200 kg dry sausage were prepared by cutting and mixing meat, bacon, spices and lactic acid bacteria. One batch, the reference, was prepared according to the standard recipe. To the second batch, carboxypeptidase (144 CPGU/kg) was added at the start of cutting and mixing of the ingredients.

As soon as the mixtures reached a desirable particles size, the meat-doughs were transferred to the filling machine, where the mixtures were transferred into foil. Subsequently, the sausages were placed on racks where air could freely flow around each sausage. The racks were placed in a ripening chamber in which the temperature was kept at 17°C for 22 days.

Subsequently, the ripening of the sausages was evaluated by professional sausage graders of a large Dutch dry sausage producer.

Results

As can be seen in Table 1, after 22 days, ripened sausages prepared with carboxypeptidase had developed the texture, appearance and taste which were comparable to a regular dry sausage that had ripened 28 days.

	Without CPG	With CPG
Texture	Soft	Firm
Colour	Light red	Deep red
Taste	Like it should be after 22 days ripening without CPG	Comparable to 28 days ripening without CPG

Table 1. Evaluation of sausages prepared with and without carboxypeptidase after a ripening period of 22 days.

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Pages 000086-000095 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

DATE: September 14, 1988

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

multistep pathways with many genes and many enzymes involved.

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

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

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

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

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

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

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

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

Specifically, there is no reason to test Aspergillus niger, Penicillium funiculosum, Trichoderma harzianum or T. reesei for aflatoxin B₁, sterigmatocystin, ochratoxin, T-2 toxin or zearalenone. Since some strains of Aspergillus alliaceus are known ochratoxin producers, enzyme preparations from this species might be tested for this one toxin. It would not be necessary to test A. alliaceus preparations for aflatoxin B₁, sterigmatocystin, T-2 toxin, or zearalenone.

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Sep. 1988

THE OCCURRENCE AND SIGNIFICANCE
OF MYCOTOXINS

000105

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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 B₁, ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone). The major justification for looking for these mycotoxins in products from species not associated with their production must presumably be concern for carry over from contaminated raw materials, or a failure to maintain a pure culture during the manufacturing process.

5. Effect of mutations on mycotoxin production

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

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

The growth and morphological and biochemical differentiation of filamentous fungi involve the sequential induction, formation and repression of many hundreds of enzymes, some of which are involved in the biosynthesis of mycotoxins.

However, the relatively small number of enzymes of industrial interest are usually associated with the earlier stages of vigorous growth and their production is directly growth related. This is in contrast to the production of mycotoxins most of which occurs during the later stages of development and their optimum production is often associated with some form of stress on growth processes.

In a limited study of strains of *Aspergillus flavus* and closely related species at the University of Surrey, it was found that an inverse correlation occurs between the ability of strains to produce aflatoxin and the ability to produce and secrete high levels of growth related catabolic enzymes such as amylases. Such observations are entirely compatible with the suggestion that *Aspergillus oryzae* and *A. sojae* are "domesticated" forms of *A. flavus* and *A. parasiticus* respectively (Wicklow, 1984).

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

The enzymes of particular interest in the food industry are globular proteins which are high molecular weight water soluble compounds in contrast to the low molecular weight secondary metabolites many of which are more soluble in organic solvents than in water.

If secondary metabolites, including mycotoxins, were present in the production liquors from which enzyme are obtained, it is highly probable that some stages in down stream processing, such as ultra filtration, will effect a partial removal.

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

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

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

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

* recognised as mycotoxins

APPENDIX 1

Search Strategy Used

Set	Items	Description
S1	5328	MYCOTOXIN
S2	16258	AFLATOXIN
S3	195	DIHYDROXYFLAV?
S4	736	DIACETOXYSCIRPENOL
S5	2352	OCHRATOXIN
S6	238	LUTEOSKYRIN
S7	0	EPOXY(W)TRICOTHECENE
S8	1226	STERIGMATOCYSTIN
S9	172721	TOXIN? ?
S10	2352	T(2W)2(2W)TOXIN
S11	2094	ZEARALENONE
S12	36	TRICOTHECENE
S13	394	RUBRATOXIN
S14	1506	PATULIN
S15	22846	S1 OR S2 OR S3 OR S4 OR S5
S16	173438	S6 OR S7 OR S8 OR S9 OR S10
S17	3802	S11 OR S12 OR S13 OR S14
S18	183474	S15 OR S16 OR S17
S19	1274181	ENZYME? ?
S20	261508	MANUFACTUR?
S21	2117	S19 AND S20
S22	80623	DEEP
S23	885707	CULTURE? ?
S24	2056	S22 AND S23
S25	4156	S21 OR S24
S26	77	S18 AND S25

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

APPENDIX 2 - FILE SEARCHED

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

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SAFETY EVALUATION using the PARIZA & JOHNSON DECISION TREE of Carboxypepidase from a genetically modified strain of *Aspergillus niger* (PEG-1A)

Introduction

The "Decision Tree for evaluation of the relative safety of food and food ingredients derived from genetically modified organisms" from the International Food Biotechnology Council (IFBC) was published in 1990¹. This publication was an extension based on an earlier publication by Pariza and Foster in 1983². Recently, an update of the 1991 IFBC Decision Tree was prepared by Pariza and Johnson and is published in the April issue of the Regulatory Toxicology and Pharmacology of 2001³.

The enzyme preparation of carboxypepidase from a genetically modified strain of *Aspergillus niger* PEG-1A was evaluated according the Pariza and Johnson Decision Tree. The result is described below.

Decision Tree

1. Is the production strain genetically modified?

YES

The strain *Aspergillus niger* PEG-1A is derived from host ISO-502, which is a genetically modified strain (GMO self-clone) derived from the DSM GAM lineage of *A. niger* strains.

If yes, go to 2. ~~If no, go to 6.~~

2. Is the production strain modified using rDNA techniques?

YES

If yes, go to 3. ~~If no, go to 5.~~

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

- 3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?

NO

~~If yes, go to 3c.~~ If no, go to 3b

While peptidases/proteases have a long history of safe use, the caboxypeptidase from GMO *A. niger* does not have a published history supporting safe use. There are three peptidase enzyme preparations that are the subject of GRAS notifications to which FDA had no objections (GRN 34, 89, and 90).

- 3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?

YES

If yes, go to 3c. ~~If no, go to 12.~~

This test article is made by a new generation of DSM *A. niger* production strains, for which the safety has been covered by safety studies on previous test articles from the

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

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

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

same safe strain lineage⁴; a 90-day study on the current carboxypeptidase ccUF did not show any effects resulting in a sufficient high NOAEL⁵ in relation to the proposed use.

- 3c. Is the test article free of transferable antibiotic resistance gene DNA?

YES

If yes, go to 3e. ~~If no, go to 3d.~~

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

NA

~~If yes, go to 12. If no, go to 3e.~~

- 3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?

YES

If yes, go to 4. ~~If no, go to 12.~~

The final production strain is complete marker gene free and devoid of any uncharacterized heterologous DNA.

4. Is the introduced DNA randomly integrated into the chromosome?

NO

~~If yes, go to 5. If no, go to 6.~~

The introduced DNA is targeted when integrated.

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

NA

Nevertheless, the strain has been analyzed with respect to its potential to produce secondary metabolites, including mycotoxins. The strain PEG-1A showed no potential to produce secondary metabolites or mycotoxins of importance in food. This was supported by analyses on broth and concentrated UF samples (REG#51656).

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.

⁴ P.W.M. van Dijck et al. Reg. Toxicol. Pharmacol. Vol. **38** (2003) 27-35.

⁵ P.M. Sathish. Enzyme preparation of *Aspergillus niger* containing carboxypeptidase G activity (PEG-1A): 90-day oral toxicity study by gavage in Wistar rats – study no. G4903

WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

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Copenhagen, August 1998

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Summary

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

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

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

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

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

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

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

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

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1.0. Introduction

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

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

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

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

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

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

2.0 Background

2.1 General

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

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

2.2 Occupational respiratory allergy

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

Fungal enzymes, bacterial enzymes and extracted plant and animal enzymes are equally capable of inducing respiratory allergy - Papain and Bromelain^{2,4}, Trypsin⁵, protease's from the skin yeast *Candida albicans*⁶, from bacteria/ subtilisins^{7,8}, fungal amylases^{9,10}, bacterial amylases¹¹, fungal hemicellulases¹², lipases¹³, xylanases and cellulases^{14,15} are all examples of industrial enzymes known to induce allergic sensitisation and respiratory occupational allergy. This is a feature characterised by highly purified enzyme protein products rather than the origin or the methods of production.

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

The classical food allergens are also capable of inducing respiratory allergy when they are brought into a dust- or aerosol form and inhaled. Soya¹⁶, eggs^{17,18}, milk¹⁹ and fish²⁰ are just examples. Soya may be one of the best described examples of epidemic inhalation allergy to an allergen also well recognised as a food allergen²¹.

3.0. Food allergy

3.1. Allergy caused by ingestion of proteins in foods

Eight percent of children under 3 years of age are allergic to food²². In, and in this age group, milk, egg, fish and soya are examples of common allergens. Many of these allergies disappear with age, but food allergy is seen also in older children and in adults. The overall frequency of verified food allergy is 1 - 2% of the population²²⁻²⁵.

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

Symptoms

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

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

Types of food allergens

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

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

Properties of food allergens

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

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

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

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

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

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

Doses at which food allergy occurs

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

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

Steinman²⁹ wrote a leading article in the August 1996 issue of *J. Allergy Clin. Immunol.* regarding hidden allergens in food. It is representative of the concern in the medical profession and in the public. He suggested a number of preventive measures including labelling in clear language. His article does not mention enzymes.

Food produced by GMO's

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

Scientists in the fields of gene technology³⁰⁻³³ and allergy seem to agree that gene technology and the results thereof expressed in foods should not cause concern with regard to allergy risk. However, gene technology does bring about new proteins, and it is important to be aware that some of these new proteins may be allergenic.

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

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

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

3.2. Epidemiology of Food Allergy

In a survey of 5000 households in the USA carried out in 1989, 1992 and again in 1993²⁵ it was found that 13.9 - 16.2% of the households reported at least one member to be allergic to foods.

A study of food allergy in a random sample of 1483 adults in Holland²³ showed that 12.4% reported allergy to foods, but by controlled tests only 2.4% could be confirmed by Double Blind Placebo Controlled Food Challenge (DBPCFC).

In Spain, 3034 patients from the outpatient allergy clinics at two hospitals were tested for food allergy²⁴. The patients were tested by skin prick, RAST and open food challenge. They found 0.98% positive to one or more foods.

When looking at food additives, the same pattern emerges. In a survey of a population sample in the UK, 7% claimed to have reactions to food additives. Double blind challenge tests could verify only 0.01 - 0.23% to be true reactions to food additives³⁴.

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

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

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

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

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

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

3.3. Enzymes in food

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

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

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

In 1983 Mansfield and co-workers³⁷ published a case story of a person who had allergic symptoms after ingestion of papain used as a meat tenderiser. - Later, in 1985 they reported a study of 475 patients³⁸ with allergy of which 5 had a positive skin prick test to Papain.

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

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

In one other case story by Binkley³⁹, described below in the section 3.6.2, it can't be totally excluded that sensitisation took place by ingestion of a food product containing relatively high amounts of industrial produced enzymes.

A recent review by Wüthrich⁴⁰ of enzymes in food concluded that orally ingested enzymes are not potent allergens and that sensitisation to ingested enzymes is rare as is also the case of reactions to bread in bakers with occupational allergy to enzymes.

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

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

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

3.4. The Theory of cross reactions

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

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The theory that people with allergy to common moulds which are related to those used for the fermentation of enzymes might react to enzyme residues in food was one of Schata's¹ claims and was given relatively high coverage in the media.

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

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

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

A study by Cullinan⁴¹ was conducted with the objective of testing if patients with a well-documented allergy to the widely distributed common mould *Aspergillus fumigatus* reacted upon the ingestion of bread prepared with enzymes of *Aspergillus* origin. The study was a double blind placebo controlled food challenge study on 17 *Aspergillus* allergic people.

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

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

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

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

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

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

3.5. Food related reactions in occupationally sensitised people

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

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

Kanny & Moneret-Vautrin,⁴² and Baur & Czuppon⁴³ each describes one patient who since late childhood, has had asthma and occupational asthma with allergy to flour and enzymes for several years. Both patients were tested for elicitation of symptoms by ingestion of bread baked with and without enzymes. Kanny & Moneret-Vautrin's patient was tested in a blinded design, Baur's patient in an open, non-controlled programme. In both cases the result was elicitation of respiratory symptoms after challenge with bread baked with enzymes. Baur's patient also had a slight reaction to bread without enzymes, however not as pronounced as the reaction after the enzyme containing bread.

Losada et al⁴⁴ investigated occupational allergy to α -amylase in a pharmaceutical plant and found a number of employees sensitised to α -amylase. None reported reactions related to ingestion of bread. Five patients, all positive to α -amylase were given oral doses of native α -amylase in doses up to 10 mg.

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

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

Tarlo and co-workers⁴⁶ reported results of testing for papain allergy in 330 allergy patients. - Seven had positive RAST and Skin prick test but none of them had any gastrointestinal or other allergic symptoms to papain.

The elicitation of gastrointestinal symptoms upon respiratory sensitisation is also reported for flours. One example is reported by Vidal et al⁴⁷ and describes a man with occupational asthma after exposure to flours and other grain dusts. He was sensitised to barley, and experienced gastrointestinal reaction upon ingestion of foods and beverages made from barley.

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

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

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

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

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

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

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

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

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

3.6.1. Medical uses:

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

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

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

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

We have not been able to find any evidence of sensitisation or allergy symptoms caused by the ingestion of enzymes from these enzyme preparations. One example is the use of enzymes given as tablets for the treatment of non-articular rheumatism. Uffelmann⁵¹ describes a double blind study of 424 patients, of which 211 received enzyme treatment. The daily doses of the mixed enzyme preparations was 240 mg Lipase, 240 mg Amylase, 1,44 g Papain, 1,08 g Bromelain and 2.4 g Pancreatin. This dosage was given for 8 weeks and no serious adverse effects and no allergy reactions were reported.

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

and hospital staff who have become sensitised by inhalation of dust from these enzyme preparations⁵²⁻⁵⁴. This of course might also happen to the Cystic fibrosis patients when they handle the enzyme preparations themselves. However no cases of enzyme allergy in Cystic Fibrosis patients have been described, but there are reports of allergy to common food allergens⁵⁵.

An informal telephone survey on unpublished cases of enzyme allergy to European Cystic fibrosis Centres, resulted in only one possible case. The patient was a boy who reacted with vomiting after administration of the enzyme preparation containing amylase, protease and lipase. - The enzyme treatment had been stopped because of suspected allergy to the enzymes. However, testing for specific allergy antibodies by Maxisorp RAST⁵⁶ did not confirm sensitisation to any of the enzymes. Challenge tests have not been performed⁵⁷

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

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

Binkley³⁹, described a case of allergic reaction to ingested lactase. This patient had a respiratory allergy with positive skin prick test reaction to *Aspergillus sp.*

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

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

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

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

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

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

4.0. Conclusion

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

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

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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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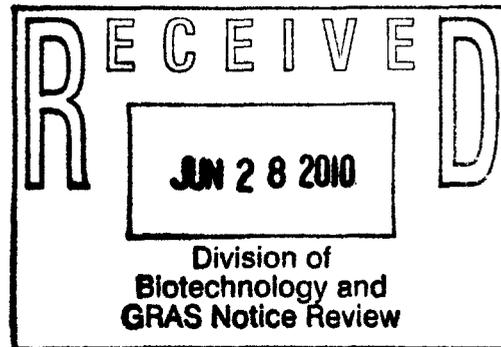
June 25, 2010

Gary L. Yingling

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Via Federal Express

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



Re: Additional copy for FSIS review of GRAS Notification for carboxypeptidase from genetically modified *Aspergillus niger*

Dear Dr. Robert Martin:

We are submitting an addition copy of the GRAS notification that was originally submitted on June 15, 2010 by K&L Gates LLP on behalf of DSM Food Specialties (DSM). DSM notified FDA of its determination through scientific procedures that a carboxypeptidase preparation from genetically modified *Aspergillus niger* is generally recognized as safe for use in cheese, enzyme modified cheese, and fermented meat as a processing aid to accelerate the development of flavors as well as debittering during the ripening process.

The enclosed document responds to FDA's request for a copy to forward onto FSIS. Please let us know if you have additional questions.

Sincerely,

(b) (6)

Gary L. Yingling

Enclosure

cc/without attachment: Marina Ramos
DSM Food Specialties

SUBMISSION END

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Reference List for Industry Submission, GRN 000345

<i>Pages</i>	<i>Author</i>	<i>Title</i>	<i>Publish Date</i>	<i>Source</i>	<i>BIB_Info</i>
000045- 000053	van Dijck, Piet W. M.; Selten, Gerard C.M.; Hempenius, Rixta A.	On the safety of a new generation of DSM Aspergillus niger enzyme production strains	2003	Regulatory Toxicology and Pharmacology	Volume 38, pgs 27-35
000075- 000084	Schuster, E.; Dunn- Coleman, N.; Frisvad, J.C.; van Dijck P.W.M.	On the safety of Aspergillus niger - a review	2002	Appl Microbiol Biotechnol	Volume 59, pgs 426 - 435
000086- 000095	JECFA and Joint FAO/WHO Expert Committee on Food Additives	Enzymes derived from aspergillus niger. Cambridge Univ Pr. Report nr WHO food	1988		Series 22
000115- 000120	Joint FAO WHO Expert Committee on Food Additives	Toxicological Evaluation of Certain Additives, WHO Food Additives	1988	Joint FAO WHO Expert Committee on Food Additives	Series 22

NA- Not applicable

From: Yingling, Gary L.
To: Srinivasan, Jannavi;
cc: Mariella Kuilman (mariella.kuilman@dsm.com); Zega, Robert; "hans.vloet@dsm.com";
Subject: RE: GRN 345
Date: Wednesday, September 22, 2010 10:39:57 AM

Dear Jannavi: With this email, I am copying the three people who will be joining from DSM. Ms Kuilman and Mr. Vloet will be calling from Delft, Netherlands and Mr. Zega from the US. If it is easier for FDA to arrange the call in number that is fine. I was not sure about being an international call. We will look forward to receiving the call in number or numbers. While we would like to have had some understanding as to the questions, we can proceed without that. It is not a problem. gary

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From: Srinivasan, Jannavi [mailto:Jannavi.Srinivasan@fda.hhs.gov]
Sent: Wednesday, September 22, 2010 10:14 AM
To: Yingling, Gary L.
Subject: RE: GRN 345

Gary

I will send a meeting request out for 9:00 AM on Sep 27th. Please let me know email information of the

people attending from the clients' side. I am planning to set up a call-in number so that folks from USDA can dial-in as well. Would that work for you?
Since the purpose of the call is to communicate the review points, and it will be mainly from the USDA side, I am not in a position to give that to you ahead of time. I hope you understand.
Thank you.
Jannavi

From: Yingling, Gary L. [mailto:Gary.Yingling@klgates.com]
Sent: Wednesday, September 22, 2010 8:39 AM
To: Srinivasan, Jannavi
Cc: Mariella Kuilman (mariella.kuilman@dsm.com)
Subject: RE: GRN 345

Dear Jannavi:

I have heard from my client and they would prefer a call on Monday, September 27th. I would suggest 9 am EDT for the call but if another time is better, please let me know. I will send you a call in number later this morning. My client will be calling from Delft in the Netherlands. Could you give me an idea to the questions that that FDA and USDA may have or the page numbers of the Notification where there is a question so we can be prepared to respond. Look forward to speaking with you on Monday. gary

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From: Srinivasan, Jannavi [mailto:Jannavi.Srinivasan@fda.hhs.gov]
Sent: Tuesday, September 21, 2010 1:42 PM
To: Yingling, Gary L.
Subject: RE: GRN 345

Thank you, Gary.

FYI, I just heard back from our USDA counterparts and it seems like they (and us too) will be available Thu this week and Mon next (both in the mornings, 8-noon) for now. Just thought I should pass this info on to you.

Jannavi

From: Yingling, Gary L. [mailto:Gary.Yingling@klgates.com]
Sent: Tuesday, September 21, 2010 1:38 PM
To: Srinivasan, Jannavi
Subject: RE: GRN 345

Dear Doctor Srinivasan:

I want to thank you for the email. I am in the process of contacting my client and I will get back to you as soon as I can with possible times and dates for the remainder to this week and next. gary

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From: Srinivasan, Jannavi [mailto:Jannavi.Srinivasan@fda.hhs.gov]
Sent: Tuesday, September 21, 2010 11:43 AM
To: Yingling, Gary L.
Subject: GRN 345

Dear Mr. Yingling,

I hope all is well with you. I am writing to you with regards to GRN 345, carboxypeptidase enzyme preparation from modified *Aspergillus niger* for use as an enzyme in processing of cheese and fermentation of meat. FSIS/USDA and FDA has completed the initial review of the notice and would like to discuss it with you. Would you be available for a phone conference with USDA and FDA this week or the next?

Please get back to me about your availability at the earliest so that I can set up a teleconference.

Thank you and have a nice day!

Jannavi

*Jannavi R. Srinivasan Ph. D
Chemistry Reviewer
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October 8, 2010

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

Dear Sir/Madam:

In a phone conference with USDA/FDA on September 27, 2010, USDA/FDA requested additional information and clarification in connection with DSM's submission of GRAS Notification GRN No. 345 ("GRN 345"). By this letter, DSM responds to USDA/FDA's request.

To further substantiate the application of carboxypeptidase (CPG) in meat, new data with CPG in meat (salami) are being submitted. In short, an Italian-type of dry salami was prepared using two different recipes, with and without CPG. During ripening and in the final sausage, several parameters were analyzed. The final sausages underwent sensory and ripening evaluation. It was concluded that the sausages prepared with CPG resulted in a more rounded and balanced flavor, which developed at a faster rate. While CPG increases the rate of flavor development, DSM acknowledges that fermented meats must still meet the ripening times specified in any applicable Standard of Identity. The experiment shows that CPG improves the flavor of the fermented meat product, and the effect of CPG is immediate and terminal (flavor development occurs only in the fermentation phase). Therefore, CPG is a processing aid that both helps to develop flavors and increases the rate of the flavor development.

USDA

1. **Amount of CPG:** USDA noted that from GRN 345, page 23, it appears that the level of CPG when it is added to the meat product is equal to the level in the final food. The following provides information as to the level added during manufacture vs. the level in the final commercial food product.

As discussed in Sections 1.3 and 6.2, CPG is proposed to be used in the manufacture of fermented meat products. For example, for fermented sausages, the product is manufactured by mixing meat, dry ingredients, flavorings, starter cultures, salts and enzyme. The mixture is ground and stuffed into fibrous casings. Some types of products

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are placed in a smokehouse until they are ready to be sold and consumed. (Note—the salami that was the subject of the subsequent trial is not smoked or processed in a smokehouse.) None of the ingredients are removed after being added to the batter. Therefore, the amount of enzyme added to the batter as a catalyst is the same amount as that present in the final food product. This would be true for all fermented meat products in which CPG is used.

As a processing aid, the CPG enzyme is added in a small amount and its catalytic activity occurs during the fermentation phase of the meat. The enzyme is still present in the final product in a small amount. However, it has no observable impact on any further flavor development in the final product. Given that the enzyme does not contribute to any meaningful flavor development in the finished product, it has no functional or technical effect in the finished food.

2. CPG as a processing aid: USDA noted that the enzyme level is the same at the use level and in the final food. DSM provides the basis for considering CPG a processing aid.

CPG is added during processing to increase the rate of and to facilitate the development of flavor characteristics. As discussed in Section 1.3.2 and 6.3 of GRN 345, CPG is added at very low levels and would be present in the finished food at insignificant levels. Also, as discussed in GRN 345, the impact on flavor occurs only during fermentation. In fact, the salami experiment indicates that the effect is immediate and occurs directly after blending of the enzyme and the meat. No further organoleptic changes were observed after the fermentation period, thus the enzyme no longer influences development of flavors. Since CPG does not contribute to flavor development in the finished product, the enzyme does not have any technical or functional effect in the finished food.

Therefore, CPG fulfills the requirements of the definition of processing aid under 21 C.F.R. § 101.100(a)(3)(ii)(c): Substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food.

3. Pasteurization process: DSM provides clarification on the reference to pasteurization mentioned in GRN 345.

Functionality of an enzyme is determined by, among others factors, pasteurization of the final food. GRN 345 mentions pasteurization in the context of enzyme-modified cheese in Sections 1.3, 6.2, and 6.4.1. Pasteurization is mentioned generally on page 23, as one of many means to influence enzyme activity without specifically referring to a product group. We clarify that the enzyme-modified cheese will be pasteurized, but the other products referenced in GRN 345 will not be.

4. Ripening of cheese and meat: USDA noted that GRN 345 makes a comparison between the ripening of meat and the ripening of cheese. DSM provides an explanation on how these two items correlate with each other.

The principle of ripening cheese and meat are the same in the sense that lactic acid bacterial cultures are used to form lactic acid, which lowers the pH, and the cultures' proteolytic activities are responsible for development of flavor components. When added together with lactic acid bacteria, CPG releases amino acids that stimulate lactic acid bacteria in their formation of flavor components. Carboxypeptidase is a carefully selected exoprotease, which has a broad spectrum of activity but specifically releases only C-terminal located amino acids from proteins and peptides. The enzymatic mechanism (release of C-terminal amino acids) of this carboxypeptidase is expected to be independent of the application. Amino acids are essential precursors for flavor development in both cheese and meat. In cheese, the liberated amino acids will directly contribute to flavor development and during early ripening, the cheese cultures will benefit from these released amino acids allowing an increased and improved flavor development. In meat applications, the same model of flavor development applies. Starter cultures gain a easier source of amino acids instead of having to break down proteins themselves and are therefore capable of faster growth and faster acid production. This results in a faster decrease in pH and an improved flavor development. In both cheese and meat applications, release of amino acids by carboxypeptidase activity accelerates the development of flavors in the end-product.

5. Sensory/Organoleptic properties: DSM submits additional data on the sensory/organoleptic effect of CPG in meat.

Both the supplemental data and revised GRN 345, Annex 6.2.2 describe the sensory/organoleptic effect of CPG in meat. See Attachments 1 and 2.

The trial described in revised Annex 6.2.2 was performed by DSM in collaboration with meat companies. Organoleptic evaluation was conducted by application specialists of DSM as well as meat specialists (butchers) at different meat companies.

The supplemental trial was performed at the facilities of and by personnel of Technical Ingredient Solutions, LLC, an independent research and development company managed by Jim Bacus, Ph.D.

The results from the both the Annex 6.2.2 trial and the supplemental trial indicate that the effect of the enzyme is very quick and terminal. Specifically, in the Annex 6.2.2 trial, after adding the enzyme and allowing fermentation, the formulation was tasted, and it was deemed to have the characteristic full meat flavor typically observed after fermentation for a longer period of time. This characteristic flavor did not change in evaluations conducted after an additional 22 days after the fermentation period ended. In the supplemental trial, the samples with CPG were described as "rounder" with "more balanced savory" flavors as compared to

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the controls, and this flavor profile did not change between the initial taste test immediately after the preparation of the sausages and at either evaluation at the end of the fermentation (after 20 days from preparation) and that which was performed 7 days thereafter.

In both trials, sausages containing the enzyme resulted in a rounder, more balanced savory taste, which was preferred by the tasters.

DSM believes that the trial data for fermented meat sausages would be applicable to other types of fermented meat products as the mechanism of action of CPG would be the same in all types of fermented meat products.

FDA

1. **Allergenicity: FDA stated that it appears that DSM searched one database only and that database was last updated in January 2006. DSM submits the results of an additional search in a more recently updated, peer-reviewed allergenicity database.**

A sequence comparison between the amino acid sequence of CPG and known food allergens was performed using SDAP database (last updated May 28, 2010) in September 2010. See Attachment 3.

For the comparison, a FASTA algorithm was used. It was established that the amino acid sequence for CPG does not have 35% or more overlap with known food allergens using a window of 80 amino acids. Accordingly, DSM concludes that the carboxypeptidase preparation is not a potential food allergen, and no further allergenicity studies are necessary.

Sincerely,

(b) (6)

Gary L. Yirgling

cc: Jannavi Srinivasan, FDA

Attachments

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ATTACHMENT 1

Application trial of carboxypeptidase from *Aspergillus niger* in fermented meat production II

Most dried sausages and meats are fermented by microbial starter cultures. These starter cultures have various functions including reducing product pH, enhancing cure color development, improving product safety and stability, facilitating the drying process to produce higher quality sausages, and contributing to the characteristic flavors associated with the various products. The unique flavor of dried meats results from a combination of the acids produced during fermentation, spicing, enzymatic hydrolysis of the meat, and the proteolytic and lipolytic characteristics of the starter cultures. Generally, the meat products have to be "aged" under specific environmental conditions for long periods of time to achieve the optimum combination of the above reactions.

Many of the flavor compounds developed during the aging of dried meats and sausages have been identified, but the use of starter cultures with specific formulation and processing conditions remains the primary method to achieve the desired characteristics. These traditional methods require long periods of time, which requires high capital investment in meat product inventories.

This experiment shows that DSM's carboxypeptidase can achieve accelerated flavor development in dried sausages.

Italian Dry Salami was chosen as the test system since microbial flavor development is more unique and requires longer processing periods than both semi-dried products and dried US Pepperoni.

The experiment was performed at the facilities of and by personnel of Technical Ingredient Solutions, LLC, an independent research and development company managed by Jim Bacus, Ph.D. Dr. Bacus led the experiments and evaluations. He has spent 40 years working in the area of processed meats and meat microbiology, and he is an Adjunct Professor in the Meat Science Department at the University of Florida and a member of the Institute of Food Technologists, American Meat Science Association and the International Association of Food Protection.

Materials & Methodology:

Four samples were evaluated.

- 1) containing starter cultures but no carboxypeptidase (control)
- 2) containing both starter cultures and carboxypeptidase
- 3) containing chemical acidulants instead of starter cultures and no carboxypeptidase (control)
- 4) containing chemical acidulants and carboxypeptidase, but no carboxypeptidase

All samples were from the same meat block with the ingredients as outlined in following formulation:

Dry Salami Manufacture:

1. Grind all pork through ½ -inch plate. Mix meat, dry ingredients, flavorings, starter culture, cure salt and salt in ribbon mixer.
2. Re-grind meat batter through 3/16-inch plate.
3. Stuff meat batter into approximately 2.5-inch diameter fibrous casings.
4. Place product on rack to ripen under monitored conditions.

The salami was evaluated three times: directly after preparation of the sausages, after 20 days of fermentation, and 7 days thereafter.

Table 1. Formulation per 100 lbs meat

	Sample 1	Sample 2	Sample 3	Sample 4
Starter Cultures	yes	yes	-	-
CPG 32 ml/200 kg	-	7.26 g	-	7.26 g
Chemical Acidulant (encapsulated GDL)	-	-	yes	yes

Results and Conclusion

	Sample 1	Sample 2	Sample 3	Sample 4
Initial pH	6.51	6.44	6.07	5.96
Final pH	4.72	4.78	4.39	4.49
Final Average Dry Yield %	71.90	72.14	72.69	72.14
Water Activity (aw)	0.918	0.917	0.912	0.908
Protein	23.37	22.99	21.60	22.18
Moisture	44.04	43.84	44.22	42.15
Fat	27.34	27.75	27.55	28.49
Moisture/Protein Ratio	1.88:1	1.90:1	2.04:1	1.90:1

Sample 1 (control) (containing starter cultures but no carboxypeptidase) exhibited flavor notes of porky-meaty, savory, salty, and slight acidic sour notes typical of North American produced salami. Final evaluation of salami was identical to flavor observation noted immediately post fermentation. Based on Brookfield texture analysis, product texture may have been slightly harder/firmer than the other three samples, but no instrumental texture difference was noted in sensory comparisons.

Sample 2 (containing both starter cultures and carboxypeptidase) exhibited typical flavor notes similar to sample 1, but it appeared less salty with more rounded/balanced savory flavor (less "acid"). Evaluation of the salami post preparation showed a "rounder" with "more balanced savory" flavor as compared to the control. The flavor profile post preparation did not change between the preparation evaluation and the two other evaluations.

Sample 3 (control) (containing chemical acidulants instead of starter cultures and no carboxypeptidase) exhibited typical European flavor notes of sweet-fruity and acidic fermented type flavors, unlike the sour acidic flavors noticed in samples 1 and 2. Sample 3 also appeared less porky-meaty, making it more similar to Northern European produced salami. Final evaluation was identical to flavor observation noted immediately post fermentation.

Sample 4 (containing chemical acidulants and carboxypeptidase, but no starter cultures) exhibited typical European flavor notes similar to those observed in test 3, but less sweet-fruity and acidic fermented with more rounded savory type flavor notes. Evaluation of the salami post preparation showed a "rounder" with "more balanced savory" flavor as compared to the control. The flavor profile post preparation did not change between the preparation evaluation and the two other evaluations. This

combination of ingredients did yield a more fermented flavor typical to Northern American salamis and similar to flavor notes in samples 1 and 2.

In conclusion, the addition of CPG improved the flavor development of the samples as compared to the controls, and the flavor profile of each sample was unique. The addition of the carboxypeptidase resulted in an improved, less acidic, savory flavor note, which appeared more rounded and better balanced as compared to the controls. For both the samples using starter cultures and the samples using chemical acids, the enzyme enhanced the "fermented flavor" notes observed with the traditional use of starter culture, even when those were not part of the ingredients. These "fermented flavor notes" are not typically seen when using chemical acids alone. Using carboxypeptidase at this usage level, the resulting flavor development was improved and appeared very controllable in practical application.

Also, because of the moisture/protein ratio requirement established by USDA food standards, which is independent of flavor and/or texture development, carboxypeptidase did not appear to shorten the maturing and drying time. However, flavor development was finished immediately after preparation. This could mean a shorter flavor development time or alternate tools for flavor development are feasible. For products where a moisture/protein ratio requirement does not exist, carboxypeptidase could facilitate shorter ripening time. In addition, for each sample with CPG, as there was no difference between the taste profile immediately post fermentation and at final evaluation (and in fact there was no difference between the taste profile between the three points of evaluation), it was concluded that the enzyme no longer effected development of flavors after preparation of the sausage.

ATTACHMENT 2

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Application trial of carboxypeptidase from *Aspergillus niger* in fermented meat production

Summary

The effect of carboxypeptidase was tested in the preparation of fermented meat sausages. After a ripening period of 22 days, sausages that were prepared with carboxypeptidase already had developed the texture, appearance and taste comparable to a regular dry sausage that had ripened in 28 days.

It can therefore be concluded that the development of flavors can be shortened by at least 6 days if sausages are prepared with carboxypeptidase.

Materials and methods

Two batches of 200 kg dry sausage were prepared by cutting and mixing meat, bacon, spices and lactic acid bacteria. The first batch, the control, was prepared according to the standard recipe. To the second batch, carboxypeptidase (144 CPGU/kg) was added at the start of cutting and mixing of the ingredients.

As soon as the mixtures reached a desirable particles size, the formula was transferred to the filling machine, where the mixtures were transferred into foil. Subsequently, the sausages were placed on racks where air could freely flow around each sausage. The racks were placed in a ripening chamber in which the temperature was kept at 17°C for 22 days.

The sausages were evaluated on the 22nd day by professional sausage graders of a large Dutch dry sausage producer. After another three week period (during which the sausages were stored at 17°C), the sausages were evaluated again.

The sausages were evaluated on different aspects of quality. A visual inspection of the sausages was conducted to evaluate the drying process as dried sausages are higher quality. Specifically, smooth and uniform sausage cases indicate that product did not dry enough, and pearl-like structures or firmness indicate that the product successfully dried. A red color on the inside is a sign of fast souring (fast decrease in pH) during ripening. This would reflect increased

Annex 6.2.2 (revised)

microbiological stability and fast removal of internal liquids. And finally, the taste is tested.

Results

As can be seen in Table 1, after 22 days, ripened sausages prepared with carboxypeptidase had developed the texture, appearance and taste comparable to a regular dry sausage that had ripened 28 days.

Table 2 shows that after an additional 21 days (total 22 days of ripening and 21 days of storage), the sausages with carboxypeptidase tasted better than the sausages without carboxypeptidase.

Physical parameters (pH and water activity (A_w)) are similar and acceptable in all samples. Since flavour is a limiting component for shelf life, and sausages treated with carboxypeptidase enjoy enhanced flavors, the shelf life of carboxypeptidase-treated sausages can potentially increase.

	Without CPG	With CPG
Outside	Smooth	Pearled
Texture	Soft	Firm
Colour	Light red	Deep red
Taste	Like it should be after 22 days ripening without CPG	Comparable to 28 days ripening without CPG

Table 1. Evaluation of sausages prepared with and without carboxypeptidase after a ripening period of 22 days.

	Without CPG	With CPG
Texture	Firm	Firm
Colour	Good	Good
Taste	Acceptable	Full meat flavour

Table 2. Evaluation of sausages prepared with and without carboxypeptidase after a ripening period of 22 days and a subsequent 21 day shelf life period.

ATTACHMENT 3

Memo

Date:
October 1st 2010

Subject: Potential allergenicity CPG update

Introduction

A comparison of the amino acid sequence of the enzyme protein of Carboxypeptidase (CPG) with sequences of known food allergens was performed in 2008 using www.Allermatch.com. Per FDA's request, a new comparison was made using the SDAP™ database.

Amino acid sequence comparison with known allergens

For the comparison, use was made of the database SDAP™ (Structural Database of Allergenic Proteins, last updated the 28th of May 2010, ref. 1). SDAP™ allows the search in SwissProt, PIR, GenBank, PDB and the SHO-IUIS databases using a FASTA algorithm. The WHO-IUIS list is set up by the I.U.I.S. Allergen Nomenclature Sub-committee operating under the auspices of the International Union of Immunological Societies and the World Health Organization. The objectives of the I.U.I.S. Allergen Nomenclature Sub-committee are to maintain a unique and unambiguous nomenclature for allergen molecules and maintain the 'official list of allergens'.

The search was performed following the guidelines from the FAO/WHO (ref. 2). According to the guideline, a protein is a potential allergen when there is more than 35% homology between the amino acid sequence of the protein and a known allergen using a window of 80 amino acids and a suitable gap penalty.

The results of the SDAP™ database analysis indicate that when using a window of 80 amino acids, CPG protein sequence shows more than 35% homology with only one known allergen: Api m 9.0101 (a serine carboxypeptidase of *Apis mellifera* or honey bee).

Discussion

By using a window of 80 amino acids, CPG protein sequence shows more than 35% homology (specifically, 43.75% identity) with a known allergen: Api m 9.0101. This protein is a serine carboxipeptidase of *Apis mellifera* or honey bee. Although this allergen is included in the database of recognized allergenic proteins from the WHO-IUIS, it is not a food allergen. This match is therefore not regarded relevant in the present assessment.

It is therefore concluded that DSM Carboxypeptidase amino acid sequence is not likely to produce any allergenic or sensitization reactions by oral consumption.

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DSM Food Specialties
Regulatory Affairs

Reference

1. <http://fermi.utmb.edu/SDAP/index.html>
2. Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 22-25 January 2001, Rome Italy

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