**ORIGINAL SUBMISSION**

#563

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000563

December 12, 2014

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

Dear Sir or Madam,

We are hereby submitting one paper copy and one eCopy of a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' protease enzyme preparation produced by a genetically modified strain of *Fusarium venenatum*. The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. Novozymes has determined through scientific procedures that the protease is generally recognized as safe for use in the food industry as a processing aid for production of partly or extensively hydrolyzed proteins that in turn may be used for various applications as ingredients in a variety of food and beverage products. These hydrolyzed proteins can also be used in foods for special dietary use, medical foods, and infant formula.

Please contact me by direct telephone at 919 494-3151, direct fax at 919 494-3420 or email at lobg@novozymes.com if you have any questions or require additional information.

Sincerely,

(b) (6)

Lori Gregg
Manager, Regulatory Affairs

Enclosures

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OFFICE OF
FOOD ADDITIVE SAFETY

Novozymes North America, Inc.
Regulatory Affairs
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December 12, 2014

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R.§ 170.36 (c)(1) Novozymes North America Inc. hereby claims that protease preparations produced by submerged fermentation of a genetically modified *Fusarium venenatum* are Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

Novozymes North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

Protease preparation produced by a genetically modified *Fusarium venenatum* production strain

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use.*

The protease is used for partial or extensive hydrolysis of animal and vegetable proteins (such as casein, whey, gluten, and proteins from soy, corn, rice, peas, lentils, meat and fish) to be further used as ingredients in a variety of beverage and food products. These hydrolyzed proteins can also be used in foods for special dietary use, medical foods, and infant formula. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

Proposed §170.36 (c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times at Novozymes North America, Inc. or will be sent to FDA upon request.

(b) (6)

12-12-14

Lori Gregg
Manager, Regulatory Affairs

Date

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# A protease enzyme preparation produced by a genetically modified *Fusarium venenatum p*roduction strain

**Lori Gregg, Regulatory Affairs, Novozymes North America, Inc., USA**
December 2014

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## TABLE OF CONTENTS


| | | PAGE |
|---|---|---|
| **1.** | **GENERAL INTRODUCTION** | 3 |
| **2.** | **PRODUCTION MICROORGANISM** | 4 |
| 2.1 | Production Strain | 4 |
| 2.2 | Host Strain | 5 |
| 2.3 | Introduced DNA | 5 |
| 2.4 | Construction of the Recombinant Microorganism | 5 |
| 2.5 | Stability of the Introduced Genetic Sequences | 6 |
| 2.6 | Antibiotic Resistance Gene | 6 |
| 2.7 | Absence of the Production Organism in Product | 6 |
| 2.8 | Relevant Mycotoxins | 6 |
| **3.** | **MANUFACTURING PROCESS** | **7** |
| 3.1 | Raw Materials | 7 |
| 3.2 | Fermentation Process | 7 |
| 3.3 | Recovery/Purification Process | 8 |
| 3.4 | Quality Control of Finished Product | 8 |
| **4.** | **ENZYME IDENTITY** | **8** |
| **5.** | **COMPOSITION AND SPECIFICATIONS** | 9 |
| 5.1 | Quantitative Composition | 9 |
| 5.2 | Specifications | 9 |
| **6.** | **APPLICATION** | **10** |
| 6.1 | Mode of Action | 10 |
| 6.2 | Use Levels | 11 |
| 6.3 | Enzyme Residues in the Final Food | 12 |
| **7.** | **SAFETY EVALUATION** | 12 |
| 7.1 | Safety of the Production Strain | 12 |
| 7.2 | Safety of the Donor Organism | 14 |
| 7.3 | Safety of the Protease Enzyme | 14 |
| 7.4 | Safety of the Manufacturing Process | 15 |
| 7.5 | Safety Studies | 16 |
| 7.6 | Estimates of Human Consumption and Safety Margin | 17 |
| 7.7 | Results and Conclusion | 20 |
| **8.** | **LIST OF APPENDICES** | **21** |
| **9.** | **LIST OF REFERENCES** | **22** |

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## 1. GENERAL INTRODUCTION

The subject of this notification is a protease enzyme preparation produced by submerged fermentation of a *Fusarium venenatum* microorganism carrying the gene coding for serine protease from *Fusarium oxysporum.*

The enzyme is serine protease with trypsin specificity (EC 3.4.21.4, CAS 9002-07-7) which hydrolyzes peptide bonds in proteins with preferential cleavage at arginine and lysine resulting in smaller proteins and peptides of variable lengths. The serine protease is used for partial or extensive hydrolysis of animal and vegetable proteins (such as casein, whey, gluten, and proteins from soy, corn, rice, peas, lentils, meat and fish) to be further used as ingredients in a variety of beverage and food products. These hydrolyzed proteins can also be used in foods for special dietary use, medical foods, and infant formula.

The information provided in the following sections is the basis for our determination of general recognition of safety of this protease enzyme preparation. Our safety evaluation in Section 7 includes an evaluation of the production strain, the introduced DNA, the donor strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food [(1) (2)] (Appendix 1). The production strain is a genetically modified strain of *F. venenatum* expressing a serine protease from *F. oxysporum. Fusarium venenatum* is a relatively recently described species, established by Nirenberg [(3)] as a separate species within the F. sambucinum complex in the Discolor-section of the genus. It's known hosts include: *Zea mays, Humulus lupulus, Pinus radiata, Solanum tuberosum* and *Triticum aestivum*. In general these fungi are saprophytes found in soil and they are not considered as human pathogens [(4) (5).] No reports in the literature connecting any species within the Discolor-section with human or animal infections have been found. This *F. venenatum* expressing a heterologous xylanase has been accepted as GRAS in the US (FDA Agency Response Letter GRAS Notice No. GRN000054 and it received a positive evaluation at the JECFA (Joint FAO/WHO Expert Committee on Food Additives) meeting in June 2003 [(6)].

Furthermore, the safety of the serine protease preparation has been confirmed or is under consideration by external expert groups, as follows:

• Denmark: The enzyme preparation has been safety assessed according to the Guidelines for the evaluation of food enzymes (the Scientific Committee for Food, Commission of the European Communities, 1992). This resulted in the authorization of the enzyme product by the Danish authorities.

• France: The enzyme has been positively evaluated by the French Authorities and has been included in The French order of October 19, 2006 on use of processing aids in the manufacture of certain foodstuff, as amended.

• JECFA: The enzyme preparation has been positively evaluated in the 76th meeting of JECFA and has been allocated an Acceptable Daily Intake (ADI) “not specified”.

• Mexico: The enzyme has been positively evaluated by COFEPRIS, however the amendment to the positive list is awaiting the next official update.

• Brazil: The enzyme has been positively evaluated by ANVISA and is included in the latest update of the positive list.

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material [(7) (8) (9) (10) (11) (12)]. The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Section 2.

This notification includes information that addresses the safety of the enzyme source, the enzyme component, the manufacturing process and a consideration of dietary exposure which covers all the issues relevant to a safety evaluation of an enzyme preparation. Based on critical review and evaluation of its published and unpublished information, Novozymes concludes through scientific procedures that the subject of this notification, meeting appropriate food grade specifications and produced in accordance with current good manufacturing practices, is GRAS for the intended conditions of use.

Please note

## 2. PRODUCTION MICROORGANISM

### 2.1 Production Strain

The serine protease production strain designated WTY939-8-3, is a non-pathogenic, non-toxigenic genetically modified *Fusarium venenatum* strain. The production strain is marker-free, and it does not produce secondary metabolites of toxicological concern to humans as explained later in this section. It meets the criteria for a safe production microorganism as described by Pariza and Foster [(1)] and several expert groups [(7) (8) (9) (10) (11) (12)]. These criteria include the identification and characterization of the host strain, plasmid vectors, and inserted genetic sequences, all described below. This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms [(13)].

## 2.2 Host Strain

The host strain, designated *Fusarium venenatum* WTY842-1-11, was derived from the parental strain A3/5 a natural isolate. The classification is based on morphological, physiological, and molecular taxonomy characteristics. The taxonomic classification is as followed:

Name: *Fusarium venenatum*
Class: Sordariomycetes
Order: Hypocreales
Family: Nectriaceae
Genus: *Fusarium*
Species: *venenatum*

The identification of the parental strain A3/5 has been confirmed by the American Type Culture Collection.

<u>Genetic modifications</u>

The *F. venenatum* host strain WTY842-1-11 was constructed by transformation of A3/5 with a fragment containing a deleted version of the *F. venenatum tri5* gene, encoding trichodiene synthase, which catalyzes the first step in the trichodiene biosynthetic pathway. Therefore, the strain is incapable of producing secondary metabolites within the trichothecene biosynthetic pathway.

Southern blot analysis confirmed the disruption of the *tri5* gene and insertion of the *amdS* gene in strain WTY-842-1-11.

## 2.3 Introduced DNA

The DNA introduced in the final production strain originates from integration of a well characterized DNA fragment derived from plasmid pJRoy75, providing expression of the trypsin-like serine protease gene from *F. oxysporum* under control of the promoter of the *Fusarium venenatum* gene encoding glucoamylase and the terminator of the serine protease gene. The *bar* gene used as a selective marker in plasmid pJRoy75, encodes a phosphinothricin acetyltransferase and is derived from *Streptomyces hygroscopicus*.

## 2.4 Construction of the Recombinant Microorganism

The recombinant DNA molecule was introduced into the host *F. venenatum* strain WTY842-1-11, by incubating protoplasts with a digested and purified DNA fragment of plasmid pJRoy75 containing the expression cassettes for the protease gene and the selective marker. The introduced DNA fragment does not contain the Amp gene, encoding resistance to ampicillin or any part of the bacterial vector backbone. As WTY842-1-11 lacks the *bar* gene, it cannot grow in the presence of phosphinothricin.

Transformants are obtained upon the integration of multiple copies of the introduced DNA fragment into the chromosomal DNA. Selection of transformants was therefore achieved by growing on a medium with phosphinothricin and subsequent screening for expression of the protease. One transformant that showed high trypsin-like serine protease activity, strain WTY939-8-3, was selected as the final GM production strain.

## 2.5 Stability of the Introduced Genetic Sequences

The presence of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism. The transforming DNA is stably integrated into the *F. venenatum* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable.

## 2.6 Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified by Southern blot analysis using the relevant antibiotic resistance gene probes.

## 2.7 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC [(7)] is satisfactorily addressed.

## 2.8 Relevant mycotoxins

Strains of *Fusarium venenatum* are known to be potential producers of mycotoxins within the group of trichothecenes, like diacetoxyscirpenol [(14) (15)] (DAS). Other minor mycotoxins potentially produced by relevant members of the *Fusarium* genus include i) fusarin C and ii) “butenolide” (4-acetamido-4-hydroxy-2-butenoic acid $\gamma$-lactone), a metabolite that has been implicated in animal mycotoxicoses [(16)]. However, as noted above in section 2.2, the gene encoding trichodiene synthase (*tri5*), which catalyses the first step in the trichodiene biosynthetic pathway, was deleted in the host strain by means of site-directed gene disruption, thereby rendering it incapable of producing secondary metabolites within the trichothecene biosynthetic pathway.

This is further discussed below in section 7.1.1 and in the report in Appendix 2.

## 3. MANUFACTURING PROCESS

This section describes the manufacturing process for the protease preparation which follows standard industry practices [17] [18] [19]. The quality management system used in the manufacturing process for the protease preparation complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

### 3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the protease enzyme concentrate are standard ingredients used in the enzyme industry [17] [18] [19]. The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

The antifoams used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams in the product is less than 1%.

### 3.2 Fermentation Process

The protease preparation is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *Fusarium venenatum* 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.

#### 3.2.1 Production Organism

Each batch of the fermentation process is initiated with a stock culture of the production organism, *F. venenatum,* described in section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

#### 3.2.2 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermenter and the main fermenter before inoculation, at regular intervals during cultivation, and before

transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1. Infection is observed in 2 or more samples by microscopy

2. Infection is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

### 3.3 Recovery/Purification Process

The recovery process is a multi-step operation designed to separate the desired enzyme from the microbial biomass and consists of both the purification and the formulation processes.

#### 3.3.1 Purification process

The enzyme is recovered from the culture broth by a series of operations:

- Pretreatment
- Filtrations - vacuum drum filtration, polish filtrations and germ filtration
- Concentration - ultrafiltration and/or evaporation
- Preservation and Stabilization of the liquid enzyme concentrate.

#### 3.3.2 Formulation and standardization

After final concentration by evaporation and/or ultrafiltration, the concentrate is spray dried to result in a highly concentrated granulated product.

### 3.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in section 5.

## 4. ENZYME IDENTITY

Key enzyme and protein chemical characteristics of the protease are given below:

| | |
|---|---|
| Classification | Serine protease |
| IUBMB nomenclature: | Trypsin |
| EC No.: | 3.4.21.4 |
| CAS No.: | 9002-07-7 |
| Specificity: | This is an endoprotease with preferred cleavage sites at Arginine and Lysine residues in proteins. |
| Amino acid sequence: | the total nucleotide and amino acid sequences have been determined |

## 5. COMPOSITION AND SPECIFICATIONS

The protease enzyme preparation is presently available in a granulated form for use in food applications.

### 5.1 Quantitative Composition

The typical composition of the commercial protease enzyme preparation is as follows:

Enzyme solids (TOS) approx. 95 %
Water approx. 5 %
TOS = Total Organic Solids, defined as: 100% - water - ash - diluents

### 5.2 Specifications

The protease preparation complies with the purity criteria recommended for enzyme preparations as described in Food Chemicals Codex [(20).] In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications [(21).]

This is demonstrated by analytical test results of the protease enzyme preparation in table 1 below.

**Table 1**. Analytical data for one batch of protease enzyme preparation.

| Parameter | Specifications | Batch PFG10002 |
|---|---|---|
| Serine protease activity | Declared Activity Lower limit 1500 KMTU/g | 1660 KMTU/g |
| Total viable count | Upper limit 50,000 CFU/g | 500 CFU/g |
| Lead | Not more than 5 mg/kg | 0.50 mg/kg |

| Parameter | Specifications | Batch PFG10002 |
|---|---|---|
| Salmonella sp. | Absent in 25 g of sample | ND |
| Total coliforms | Not more than 30 per gram | <10 |
| Escherichia coli | Absent in 25 g of sample | ND |
| Antimicrobial activity | Not detected | ND |

ND: Not Detected

## 6. APPLICATION

### 6.1 Mode of Action

Since the 1970s proteases have been increasingly used in various industrial food applications for hydrolysis of proteins. Protein hydrolysates can also be produced by acid and alkaline hydrolysis as well as by heat treatment.

As compared with these alternatives, the benefits of the action of serine protease are:

- Higher yield of soluble proteins and peptides
- Mild process conditions
- Reduced amounts of salts compared to acid hydrolysed protein
- Protein hydrolysate with controlled peptide profile due to specificity of the enzyme
- Increased digestibility of proteins

The serine protease which is the subject of this dossier has preferential cleavage at arginine and lysine, thereby providing an alternative to using animal derived trypsin.

The enzyme can be used for partial or extensive hydrolysis of animal and vegetable proteins such as casein, whey, gluten, and proteins from soy, corn, rice, peas, lentils, meat and fish. The resulting peptides are used as ingredients in a variety of food products including foods for special dietary use, medical foods, and infant formula. The applicability of use as food ingredients are often determined by the functional properties of the processed proteins which to a large extent are governed by their molecular size and their distribution of hydrophobic amino acids. Enzymatic processing of proteins using selected proteases to hydrolyse specific peptide bonds is widely used to produce peptides with e.g. increased solubility, modified viscosity and altered foaming, gelling and emulsifying properties. Processing of proteins is also

used to improve the digestibility of proteins and thereby the nutritional value of the protein source.

This enzyme is useful when a moderate, controlled hydrolysis is desirable. Such moderate hydrolysis is well known to improve general functionality e.g. emulsifying capacity, solubility, foaming etc. compared to the native protein [22] [23].

Figure 1 shows the effect of the enzyme on milk protein (5% whey concentrate) when added in a dosage of 10800 KMTU/kg protein. The hydrolysis reaction was performed as a function of time (T=120 min), at 52 ºC and pH 7.5. The Degree of Hydrolysis (%DH) obtained was around 6.4. On average basis a %DH in the range of 6-10 is well known to result in the above mentioned functionalities.

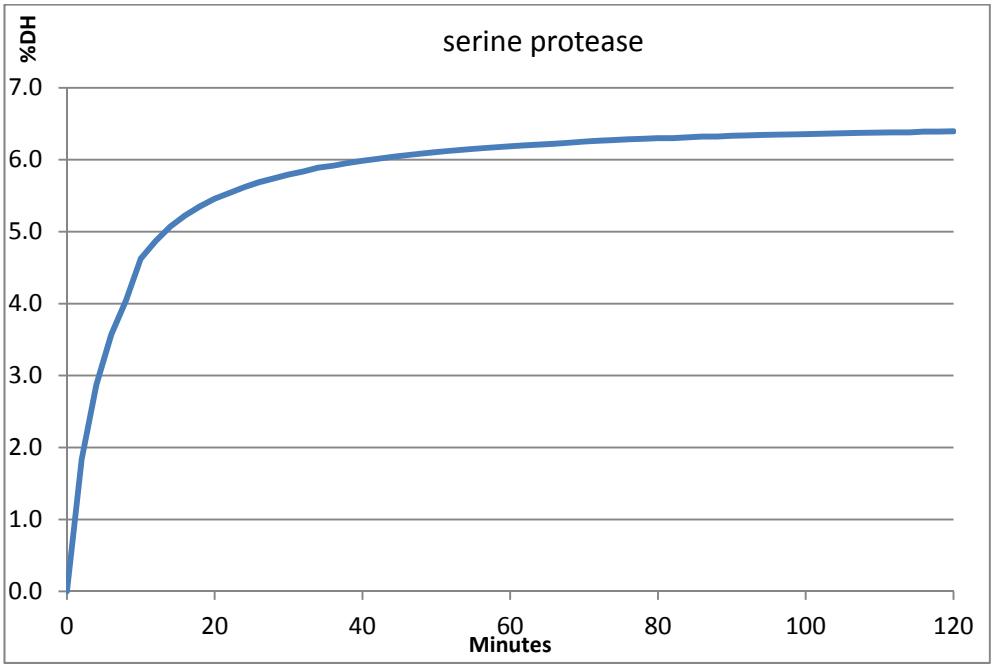


*Fig 1. Degree of hydrolysis (%DH) as a function of time (T= 0-120 min).*
*Enzyme dosage: 10800 KMTU/kg protein; substrate: 5 % whey concentrate; pH 7.5; temp. 52°C.*

## 6.2 Use Levels

The protease enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP.

The maximum recommended dosage for production of solid and liquid food is 10,800 KMTU/kg protein (dry matter) raw materials.

The dosage applied in practice by a food manufacturer depends on the particular process. It is based on an initial recommendation by the enzyme manufacturer (cf. below) and optimised to fit the process conditions.

### 6.3 Enzyme Residues in the Final Food

The enzyme is active during the processing of the protein. When the desired degree of hydrolysis has been achieved, the enzyme reaction is terminated by high temperature treatment, causing an inactivation of the enzyme activity.

## 7. SAFETY EVALUATION

### 7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food [(1) (2) (24)] . If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume [(7)]. Pariza and Foster [(1)] define a non-toxigenic organism 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”.

*Fusarium venenatum* is a relatively newly described species, established by Nirenberg [(3)] as a separate species within the F. sambucinum complex in the Discolor-section of the genus. It’s known hosts include *Zea mays, Humulus lupulus, Pinus radiata, Solanum tuberosum* and *Triticum aestivum*. In general these fungi are saprophytes found in soil and they are not considered as human pathogens [(4) (5).] No reports in the literature connecting any species within the Discolor-section with human or animal infections have been found.

This *F. venenatum* expressing a heterologous xylanase has been accepted as GRAS in the US (FDA Agency Response Letter GRAS Notice No. GRN000054 and it received a positive evaluation at the JECFA (Joint FAO/WHO Expert Committee on Food Additives) meeting in June 2003 [(6)]

An evaluation of the genetically modified *F. venenatum* production microorganism according to the concepts initially outlined by Pariza and Foster [(1)] and further developed by IFBC in 1990 [(7)] the EU SCF in 1991 [(8)] the OECD in 1993 [(10)] FAO/WHO in 1996 [(12),] ILSI Europe Novel Food Task Force in 1996 [(9),] and updated by Pariza and Johnson in 2001 [(2)] demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the introduced DNA sequences, the sources and functions of the introduced genetic material, an outline of the genetic

construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Sections 2 and 4.

7.1.1 Potential for Production of Secondary Metabolites

Strains of *Fusarium venenatum* are known to be potential producers of mycotoxins within the group of trichothecenes, like diacetoxyscirpenol (DAS) [(14)] [(15)] Therefore, the gene encoding trichodiene synthase (tri5), which catalyses the first step in the trichodiene biosynthetic pathway, was deleted in the host strain by means of site-directed gene disruption, thereby rendering it incapable of producing secondary metabolites within the trichothecene biosynthetic pathway. Other minor mycotoxins potentially produced by relevant members of the *Fusarium* genus include i) fusarin C and ii) "butenolide" (4-acetamido-4-hydroxy-2-butenoic acid $\gamma$ -lactone), a metabolite that has been implicated in animal mycotoxicoses [(16).] The report "Analysis of selected *F. venenatum* strains for production of mycotoxins" was used in order to demonstrate that we have successfully removed the ability to produce trichothecenes and that *F. venenatum* does not produce other relevant mycotoxins under different inducing conditions. (Appendix 2).

Absence of production of the secondary metabolites under enzyme production conditions was confirmed by analytical tests of four batches of serine protease. The results are shown in Table 2 below:

**Table 2.** Analytical data for four serine protease batches, including a tox test batch.

| **Parameter** | **Unit** | **PFFR 118** | **PFFR 119** | **PFFR 121** | **Tox test batch PPF 26813** |
|---|---|---|---|---|---|
| DAS (Diacetoxyscirpenol) | ppm | ND (DL <0.020) | ND (DL <0.020) | ND (DL <0.020) | ND (DL <0.020) |
| Fusarin C | ppm | ND (DL <0.020) | ND (DL <0.020) | ND (DL <0.020) | ND (DL <0.020) |
| Butenolide | ppm | ND (DL <0.5) | ND (DL <0.5) | ND (DL <0.5) | ND (DL <0.5) |
| Antimicrobial activity | - | ND | ND | ND | ND |

## 7.2 Safety of the Donor Organism

As noted above, it is the safety of the production strain that should be the primary concern when assessing the safety of an enzyme used for food. The donor strain for the protease gene is *Fusarium oxysporum*.

*Fusarium oxysporum* belongs to the section Elegans of the genus *Fusarium* within the class of imperfect fungi Hyphomycetes. In general these fungi are not regarded as primary human pathogens. *F. oxysporum* is a common plant-pathogen and has been connected with opportunistic mycosis in humans [(3) (4) (5)] *F. oxysporum* has mainly been associated with eye infections in humans and specific strains of this species have been reported to produce various secondary metabolites, including fusaric acid, monoliformine and zearalenone.

The pathogenic traits or the metabolic potential for the particular strain used as donor for the protease gene has not been investigated. This is not considered to be of particular importance to the safety of the protease enzyme preparation because only well characterized DNA fragments, limited solely to the protease coding sequence from the donor strain, are used in the construction of the genetically modified strain. The introduced DNA does not code for any known harmful or toxic substances.

## 7.3 Safety of the Protease Enzyme

A wide variety of enzymes are used in food processing [(1) (2)]. Enzyme proteins do not generally raise safety concerns [(1) (2) (25) (26).] Exceptions could include enzymes that produce substances that are not ordinarily digested and metabolized or that produce toxic substances [(27)] Pariza and Foster [(1)] note that very few toxic agents have enzymatic properties. The safety of the protease was assessed using the Pariza and Johnson (2001) decision tree (Appendix 3).

As indicated in section 4, the subject of this GRAS notification is a serine protease EC 3.4.21.4. Enzymes including proteases have a long history of use in food [(1) (2).]

In 2010, Nielsen reported that enzymatic processing of proteins using selected proteases to hydrolyse specific peptide bonds have been used for many years to produce peptides with improved functional properties [(28)]. Microbial protease enzyme preparations are the subject of GRN No. 10, 34, 89, 90, 333 and 345.

### 7.3.1 Consideration of the Allergenic Potential of the Protease Enzyme

The ingestion of a food enzyme protein is not considered a concern for food allergy. This is based on the following considerations:

- Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions.

- The majority of proteins are not food allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal based foods, and based on previous experience, food enzymes are not homologues to known allergens, which make it very unlikely that a new enzyme would be a food allergen.

- Exposure to enzymes via food is always very low, and food allergens are generally recognized to be abundant in their allergenic food source.
    - First, enzymes in foods are always added in concentrations in the low range of part per millions.
    - Second, the enzyme is typically removed or denatured during food processing, and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system.
    - Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in native unprocessed form.

The above statements are further supported by Bindslev-Jensen *et al*, 2006 [(29).] The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bees or wasps. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

In order to further evaluate the possibility that the protease will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known food allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 [(30)] and modified by Codex Alimentarius Commission, 2009 [(31)] the protease was compared to allergens from the FARRP allergen protein database (http://allergenonline.org) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (http://www.allergen.org). A search for more than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a suitable gap penalty identified 13 mite related allergens, 3 insect venom related allergens and 1 canine allergen.

The report, in Appendix 4, addresses these findings and concludes that an oral intake of the protease, which is the subject of the notification, is not anticipated to pose any food allergenic concern.

## 7.4 Safety of the Manufacturing Process

The protease meets the purity specifications for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 3, the protease preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for

general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes [17] [18] [19].

## 7.5 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the enzyme preparation which is the subject of this notice.

### 7.5.1 Description of Test Material

The toxicological testing of the protease was conducted on the serine protease test batches PPF 26813 and PPF 32126, both produced according to the description given in Section 3. The test batch was a protease enzyme concentrate without addition of additives or other standardization or stabilization ingredients.

PPF 32126 is identical to PPF 26813 apart from that the water content has been partly removed by evaporation. The resulting toxicology test batch PPF 32126 is 3 times more concentrated compared to the original batch PPF 26813 (which allows more of the test substance to be fed to the rats).

### 7.5.2 Studies

The following studies were performed:

<u>Tox test batch PPF 26813:</u>

- Ames Test. Test for mutagenic activity
- In vitro Chromosome aberration test
- 90-days oral (gavage) toxicity study in rats

<u>Tox test batch PPF 32126:</u>

- 25-days oral (gavage) toxicity study in rats

This 25-day study was performed as a bridging study in order to establish a higher NOAEL for serine protease than what could be obtained in the 13 weeks toxicity study due to the inability to feed the rats any more of the test substance.

A summary of the safety studies is enclosed in Appendix 5. (Note: the internal naming convention SP387/TL1 used in this report represents batches PPF 26813 and PPF 32126.)

The main conclusions of the safety studies can be summarized as follows:

- Serine protease, PPF 26813 did not induce gene mutations in the Ames test, neither in the presence or absence of S-9 mix.

- Serine protease toxicology test batch PPF 26813 under the conditions of the test, did not induce chromosome aberrations in cultured human blood lymphocytes.
- 90 days of oral (gavage) treatment of rats with serine protease toxicology test batch PPF 26813 at dose levels up to 581 mg TOS/kg bw/day caused no dose-related findings. The NOAEL of this study is the highest administered dose level, i.e. 581 mg TOS/kg bw/day, equivalent to 618.3 KMTU/kg bw/day
- Daily administration of up to 3605 mg TOS/kg bw/day of serine protease toxicology batch PPF 32126 by oral gavage to Sprague Dawley rats for 25 days did not cause any treatment related toxicological effects. Consequently, 3605 mg TOS/kg bw/day (corresponding to an enzyme activity of 4005 KMTU/kg bw/day) was considered the NOAEL of this study.

Based on the present toxicity data it can be concluded that the serine protease enzyme preparation, represented by batch PPF 26813 and PPF 32126, exhibits no toxicological effects under the experimental conditions described.

7.6 Estimates of Human Consumption and Safety Margin

As the processed proteins are used as ingredients in a variety of beverage and food products, the exposure assessment for adults was performed according to the Budget Method [32] [33] [34]. The exposure assessment for infants 0-12 months was performed using data provided by the CDC (Centers for Disease Control), WHO (World Health Organization) and the FDA (Food and Drug Administration).

Budget Method and Infant Formula Calculations

The Budget Method and Infant Formula assumptions represent a "maximum worst case" situation of human consumption, in which the food enzyme, the subject of this notice, would be used at its maximum recommended dosages in all processed food and all processed beverages including infant formula.

The Budget Method and Infant Formula calculations also assume that all of the food enzyme will end up in the final food. This assumption is exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted. Therefore, the safety margin calculation derived from this method is highly conservative.

**Assumptions in the Budget Method**

**Solid Food:** The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight (b/w) /day. Fifty kcal corresponds to 25 g food. Therefore, adults ingest 25 g food per kg body weight per day.

Assuming that 50% of the food is processed food, the daily consumption of processed food will be 12.5 g processed foods per kg body weight.

It is further assumed that, on average, all processed food contains 10% protein hydrolysate dry matter = 1.25 g protein hydrolysate dry matter per kg bw per day.

**Liquids:** The maximum intake of liquids (other than milk) is 100 ml/kg body weight day. Assuming that 25% of the non-milk beverages are processed, the daily consumption will be 25 ml processed beverages per kg body weight.

It is further assumed that all processed beverages contain 3.5% protein hydrolysate = 0.875 g protein hydrolysate dry matter per kg bw per day.

It is assumed that the densities of the beverages are ~ 1.

**Assumptions in the Infant Formula**

The EER (Estimated Energy Requirements) were calculated based on the United States Dietary Reference Intakes [(35)]. The average weight of infants at 0 (zero) and 12 months for both girls and boys was taken from WHO growth charts and the CDC [(36) (37).] The range for protein content in infant formula is 1.8 – 4.5 g protein/100 kcal [(38).] The maximum protein content, or 0.045g protein/kcal, was used for the TMDI calculations.

7.6.1 Estimate of Human Consumption

<u>TMDI (Theroretical Maximum Daily Intake)</u>
The maximum recommended dosage of the enzyme is 10800 KMTU per kg protein (dry matter) raw material.

The activity/TOS ratio based on typical composition data provided in Section 5 is:

| | |
|---|---|
| Declared activity: | 1500 KMTU/g enzyme product |
| TOS content: | 95 g TOS/100g enzyme product |

10,800 KMTU/kg protein raw materials x 1g enzyme product/1500 KMTU x 95gTOS/100g enzyme product = 6.84 g TOS/kg protein raw material.

Therefore a dosage of 10800 KMTU per kg protein raw material corresponds to 6840 mg enzyme TOS (total organic solids) per one kg protein raw material.

TMDI solid food
Based on the maximum recommended dosage, 1.25 g protein hydrolysate dry matter per kg bw per day in solid food will contain a maximum: 6840 mg TOS per kg / 1000 g per kg * 1.25 g = 8.55 mg TOS/kg bw/day.

TMDI liquids
Based on the maximum recommended dosage, 0.875 g protein hydrolysate dry matter per kg bw per day in liquids will contain a maximum: 6840 mg TOS per kg / 1000 g per kg * 0.875 g = 5.99 mg TOS/kg bw/day

TMDI solid food + liquids
The theoretical maximum daily intake (TMDI) of consumers of the food enzyme via various beverage and food products is: 8.55 + 5.99 = 14.5 mg TOS/kg bw/day.

TMDI Infant Formula - Zero Months
The theoretical maximum daily intake (TMDI) of infants at 0 months of the food enzyme via infant formula is: 0.045 g protein/ kcal * 112 kcal/kg bw/day * 6840 mg TOS per kg / 1000 g per kg = 34.5 mg TOS/kg bw/day.

TMDI Infant Formula - 12 Months

The theoretical maximum daily intake (TMDI) of infants at 12 months of the food enzyme via infant formula is: 0.045 g protein/ kcal * 80 kcal/kg bw/day * 6840 mg TOS per kg / 1000 g per kg = 24.6 mg TOS/kg bw/day.

7.6.2 Safety Margin

The NOAEL in the 25 day oral toxicity study in rats was concluded to be 3605 mg TOS/kg bw/day (taken from the dossier). The safety margin can be calculated as the NOAEL divided by the TMDI, as seen below.

| | |
|---|---|
| NOAEL (mg TOS/kg bw/day) | 3605 |
| TMDI (solid food+ liquids)<br>(mg TOS/kg bw/day) | 14.5 |
| TMDI for infant formula (mg TOS/kg bw/day) | 34.5 (0 months)<br>24.6 (12 months) |
| **Safety margin (Budget Method)** | **249** |
| **Safety margin (Infant Formula)** | **104** (0 months)<br>**146** (12 months) |

Based on the calculations above, it can be concluded that the serine protease is safe for its intended use. This includes cases where the produced protein hydrolysates are being used in products specifically designed for infants 0-12 months or young children.

## 7.7 Results and Conclusion

On the basis of the evaluation contained in Section 7, a review of the published literature, the history of safe use of *F. venenatum* and the limited and well defined nature of the genetic modifications used in the construction of the production organism, it is concluded that this protease can be manufactured and used safely in the food industry for production of partly or extensively hydrolyzed proteins that in turn may be used for various applications as ingredients in food and beverages including foods for special dietary use, medical foods, and infant formula.

## 8. LIST OF APPENDICES

1. Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186, 2001.

2. Analysis of selected *F. venenatum* strains for production of mycotoxins

3. Pariza and Johnson Decision Tree analysis

4. Toxicity and allergen risk assessment report. Novozymes Report No. 2013-15937.

5. SP387/TL1: Summary of toxicity data Novozymes Report No.: 2013-15851.

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Pages 000030-000043 have been removed in accordance with copyright laws. The removed reference is:

Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186, 2001.

# Appendix 2

## Analysis of selected *F. venenatum* strains for production of mycotoxins

## INTRODUCTION

*Fusarium venenatum* belongs to a taxonomic group of fungi with the potential to produce several toxic secondary metabolites (Thrane and Hansen 1995) such as type A trichothecenes (diacetoxyscirpenol, DAS), and other minor mycotoxins (fusarin C, FUC) and metabolites that have been implicated in animal mycotoxicoses like "butenolide" (4-acetamido-4-hydroxy-2-butenoic acid $\gamma$-lactone, Fig. 1; Desjardins and Proctor 2007, BUT).

The present investigation was undertaken to determine the metabolic potential of *F. venenatum* with particular attention to DAS, FUC and BUT. The analysis of secondary metabolites was performed for the *F. venenatum* parental strain A3/5 and the host strain WTY842-1-11 cultured under strong inducing conditions for the production of secondary metabolites.

## TAXONOMY AND THE SECONDARY METABOLITE POTENTIAL OF *F. venenatum*

*Fusarium* species are common saprophytes on plant debris and in soil. Many species are important plant pathogens. The taxonomy of the genus *Fusarium* has been the subject of controversy for many years. Correct identification of strains based on morphological traits has been cumbersome and it is essential when evaluating the potential production of secondary metabolites. In fact, the *F. venenatum* type strain (ATCC 20334) was originally classified as a *F. graminearum* strain, a grass pathogen but both toxin and molecular data confirmed the wrong taxonomic classification (O'Donnell et al., 1998). In a specific European research project, the type species of *F. sambucinum* was studied in detail (Nirenberg 1995). As a result of these investigations, that included a combination of morphological, molecular and metabolic characteristics, the different strains of this species were re-classified in three different new species: *F. sambucinum*, *F. torulosum* and *F. venenatum* (Nirenberg 1995). This classification has been confirmed using DNA fingerprinting methods and has shown that the Novozymes parental strain A3/5 (ATCC 20334) is indeed a *F. venenatum* (O'Donnell et al., 1998; Yoder and Christianson 1998).

Early studies on the potential for secondary metabolite production by *F. sambucinum* and its interpretation in light of the new taxonomical structure of this species concluded that members of the new *F. venenatum* species may include producers of DAS and other DAS-derived trichothecenes, FUC and BUT (Thrane and Hansen 1995).

As an example, *F. venenatum* produces a DAS-derivative compound not identified in *F. sambucinum* while the latter species produces two unique metabolites only identified by HPLC analysis that are not produced by any of the *F. venenatum* strains studied (Thrane and Hansen 1995). The structure of relevant secondary metabolites potentially produced by *F. venenatum* is shown below (Fig. 1).

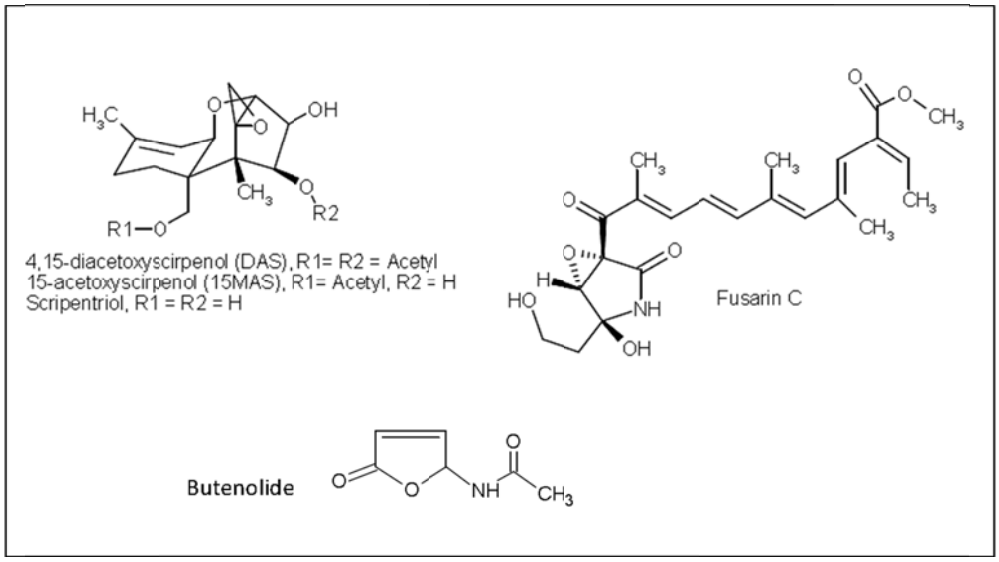


**Fig.1.** Relevant secondary metabolites potentially produced by *F. venenatum*

## MATERIALS AND METHODS

### Strains and culturing conditions for the measurement of secondary metabolites

The strains tested in this study are shown in Table 1.

| Strain # | Strain Name | Description |
|---|---|---|
| 1 | A3/5 | Parental |
| 2 | WTY842-1-11 | Host |

**Table 1.** *F. venenatum* strains tested in this study.

*F. venenatum* strain A3/5 (parental) and WTY842-1-11 (host) were revived from storage in 10% glycerol at -140°C by plating onto PDA plates. The two strains were subsequently inoculated onto the following media:

- Raulin-Thom (RT) agar, 5 ml in a 50 ml blue-cap bottle; RT liquid (still), 5 ml in a 50 ml blue-cap bottle; and RT liquid (shake), 5 ml in a 25 ml blue-cap bottle.
- MYRO (Miller et al. 1991) liquid (still) 5 ml in a 50 ml blue-cap bottle; and MYRO liquid (shake), 5 ml in a 25 ml blue-cap bottle
- YES agar, 5 ml in a 50 ml blue-cap bottle; YES liquid (still), 5 ml in a 50 ml blue-cap bottle; and YES liquid (shake), 5 ml in a 25 ml blue-cap bottle (Frisvad 1981)
- PDA agar in standard Petri dish (Samson et al., 2000).

The plates and cultures were incubated at 25°C for 8 days.

Whole cultures (PDA only 10 $cm^2$) and media-blanks were extracted with 2 x 14 ml ethyl acetate (after 30 min. shaking). The combined ethyl acetate phases were evaporated to dryness in a rotational vacuum concentrator (RVC) from Christ (Germany), redissolved in 500 µl MeOH and filtered through a 0.45 µm syringe filter, ready for LC-MS analysis. This was performed by our standard C18 LC-DAD-ESI+-MS method (Nielsen and Smedegaard 2003), except that the gradient was started at 10% $CH_3CN$, and that two scan functions were used, one with 29 V and one with 9 V in between the skimmers (for detection of the labile trichothecenes).

### Analysis of LC-DAD-MS data

The C18 LC-DAD-ESI+-MS data files were inspected for:

1. MS Scan function
2. Diacetoxyscirpenol (DAS), [M+NH4]+ at m/z 384.15-384.25, RT 11.9 min (see Fig. 2); mono-acetoxyscirpenol, [M+NH4]+, at m/z 342.16-342.23, RT 7.1 min; and triacetoxyscirpenol, [M+NH4]+ at m/z 426.15-426.25, RT 15.8 min. 2.
3. From the UV trace 350-370 nm, fusarin C (poor sensitivity in MS) and analogues, as well as MS scan function 2 for m/z 432 (fusarin C and D) and 416 (fusarin A).
4. All major peaks in the UV and MS traces were matched against the internal metabolite database (~ 600 metabolites).

Detection limits (LOD) were roughly estimated by:
**LOD=(IC x Q)/(s/n x noise x recovery)**, where **IC** is the ion count of the peak apex of the detection ion, **Q** is the quantity of the injected reference standard, **s/n** is the signal-to-noise ratio used to accept a detection (in this case, 10), **noise** is the base line level of the detection ion and **recovery** is the % of the metabolite extracted from sample, set at 50%. In case of SPE this was multiplied by the recovery of SPE column.

## RESULTS

### Trichothecenes

Strain A3/5 produced DAS on all media and culturing conditions investigated, whereas the host strain WTY842-1-11 showed no indication of production of DAS or other trichothecenes on any of the media and conditions studied. This is in accordance with the previous work published on *F. venenatum* (O'Donnell et al., 1998). From the data in Table 1, it is clear that incubation with shaking stimulated

DAS production. Also, the data indicate that MYRO was the best induction medium for DAS production and for production of trichothecenes derived from DAS in *F. venenatum* (Table 1). These derivatives were only detected under conditions that resulted in high levels of DAS production for parental strain A3/5 (Table 1).

For the host strain WTY842-1-11, no DAS or other trichothecene derivatives were detected even in cultures grown in MYRO medium with shaking (Table 1), demonstrating the lack of potential for the production of DAS in a *F. venenatum* strain with a deletion of the *tri5* gene involved in the biosynthesis of trichothecenes like the host strain (see Annex B2).

The ion chromatograms for cultures grown in MYRO medium with shaking for strain A3/5, WTY842-1-11, and the medium alone as a control are shown below (Fig. 2). A major peak corresponding to DAS is only observed for strain A3/5 (Fig. 2A). The chromatograms of strain WTY482-1-11 and of the medium do not show any trace of DAS, only background (noise) signals (Fig. 2B and C, respectively).

| **Trichothecene profiling of *F. venenatum* strains grown on different media and conditions** | | | | |
|---|---|---|---|---|
| Strain | Medium | Peak area (ion counts)[1] | | |
| | | 15-MAS | DAS | TAS |
| A3/5 | RT liquid | ND | 681 | ND |
| | RT shake | ND | 737 | ND |
| | RT agar | ND | 534 | ND |
| | YES liquid | ND | 3017 | ND |
| | YES shake | 82 | 5513 | ND |
| | YES agar | ND | 3119 | ND |
| | MYRO liquid | ND | 2650 | ND |
| | MYRO shake | 457 | 13104 | 1732 |
| | PDA agar | 70 | 4581 | ND |
| WTY842-1-11 | RT liquid | ND | ND | ND |
| | RT shake | ND | ND | ND |
| | RT agar | ND | ND | ND |
| | YES liquid | ND | ND | ND |
| | YES shake | ND | ND | ND |
| | YES agar | ND | ND | ND |
| | MYRO liquid | ND | ND | ND |
| | MYRO shake | ND | ND | ND |
| | PDA agar | ND | ND | ND |

**Table 1.** Analysis of trichothecene production in *F. venenatum* parental strain A3/5 and host strain WTY842-1-11 grown in different induction media (RT, YES, MYRO and PDA) and conditions. 15-MAS = 15-mono-acetoxyscirpenol; DAS = diacetoxyscirpenol; TAS = triacetoxyscirpenol; ND: not detected. [1]Detection limit for DAS, TAS and 15-MAS (LOD) was 5-20 ppb (parts per billion).

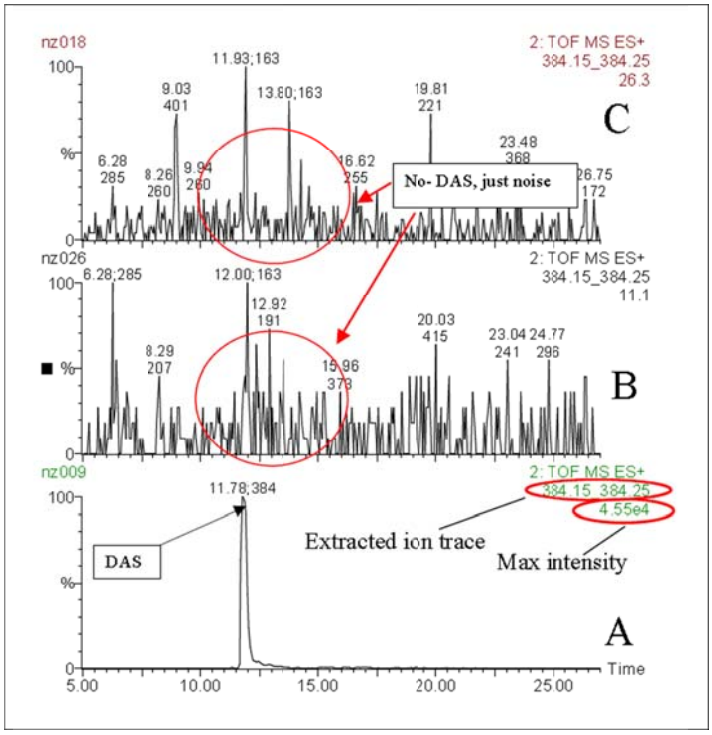


**Fig. 2.** Extracted ion chromatograms of m/z 384.15-384, [M+NH4]+, comparison of MYRO shake cultures, showing a large (overloaded) DAS peak in strain A3/5 (A), and no detection of DAS in WTY842-1-11 (B) or in the medium (C). The multitude of peaks in B and C do not represent individual metabolites but just noise.

**Fusarin C and Butenolide**

Fusarin C, as well as fusarin A (tentatively identified) were detected in low quantities in strain A3/5 grown on PDA agar but were not detected in WTY842-1-11 grown on PDA (Table 2). Cultures grown in the other media did not contain detectable quantities of these metabolites for either strain A3/5 or WTY842-1-11 (Table 2). Thus, the host strain does not produce detectable amounts of FUC under strong inducing growth conditions.

Similarly, no butenolide was detected for any of the strains investigated in any of the media/conditions investigated (Table 2). This is consistent with published data showing the lack of production of this secondary metabolite in *F. venenatum* (Thrane and Hansen 1995).

| **Minor secondary metabolite profiling of *F. venenatum* strains** | | | |
|---|---|---|---|
| Strain | Medium | FUC | BUT |
| A3/5 | RT liquid | ND[1] | ND[2] |
| | RT shake | ND | ND |
| | RT agar | ND | ND |
| | YES liquid | ND | ND |
| | YES shake | ND | ND |
| | YES agar | ND | ND |
| | MYRO liquid | ND | ND |
| | MYRO shake | ND | ND |
| | PDA agar | (+) | ND |

| **Minor secondary metabolite profiling of *F. venenatum* strains** | | | |
|---|---|---|---|
| Strain | Medium | FUC | BUT |
| WTY842-1-11 | RT liquid | ND | ND |
| | RT shake | ND | ND |
| | RT agar | ND | ND |
| | YES liquid | ND | ND |
| | YES shake | ND | ND |
| | YES agar | ND | ND |
| | MYRO liquid | ND | ND |
| | MYRO shake | ND | ND |
| | PDA agar | ND | ND |

**Table 2.** Analysis of Fusarin C (FUC) and Butenolide (BUT) production in *F. venenatum* parental strain A3/5 and host strain WTY842-1-11 ("WTY") grown in different induction media (RT, YES, MYRO and PDA) and conditions; FUC: Fusarin C; BUT: Butenolide; (+) low level; ND: not detected. [1]Detection limit for FUC (LOD) was 20 ppb (parts per billion); [2]LOD for BUT was 0.5 ppm (parts per million).

## CONCLUSION

An investigation of production of relevant secondary metabolites potentially produced by *F. venenatum* was performed. The data generated demonstrate that:

- The *F. venenatum* host strain WTY842-1-11 does not produce DAS and related trichothecenes. The lack of DAS production is the expected result from the inactivation of the biosynthetic pathway that follows the gene disruption of the *tri5* gene performed during strain construction to obtain the host strain WTY842-1-11. The data generated conform to the expected result and demonstrate the lack of potential for production of these compounds in the Novozymes *F. venenatum* strain lineage.
- The *F. venenatum* host strain WTY842-1-11 does not produce any other known mycotoxin or secondary metabolite of concern (FUC, BUT) under the different growth conditions tested
- The parental strain A3/5 produces DAS under all conditions
- The parental strain produced low amounts of FUC under a single growth condition
- The parental strain does not produce BUT

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**Appendix 3-** Pariza & Johnson Decision Tree analysis of a protease produced by a genetically modified strain of *Fusarium venenatum*.

This protease produced by a genetically modified strain of *Fusarium venenatum* was evaluated according to the decision tree published in Pariza and Johnson, 2001. The result of the evaluation is presented below.

Decision Tree

1. Is the production strain genetically modified?
   **YES**
   *If yes, go to 2.*

   The production strain Fusarium venenatum designated WTY939-8-3 was derived from the parental strain ATTC 20334 (A3/5) a natural isolate.

2. Is the production strain modified using rDNA techniques?
   **YES**
   *If yes, go to 3.*

3. Issues relating to the introduced DNA are addressed in 3a-3e.
   a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
      **YES**

   According to Pariza and Foster 1983, enzymes including proteases have a long history of use in food. Animal derived trypsin (protease) has been used for processing milk proteins since before 1958 (60FR32906). In 2010, Nielsen reported that enzymatic processing of proteins using selected proteases to hydrolyse specific peptide bonds have been used for many years to produce peptides with improved functional properties. Microbial protease enzyme preparations are the subject of GRN No. 10, 34, 89, 90, 333 and 345.

      *If yes go to 3c.*

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

      **YES**

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified.
*If yes go to 3e.*

e. 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 products?
**YES**

The genetic modifications are well characterized and specific and the incorporated DNA does not encode and express any known harmful or toxic substances.

*If yes, go to 4.*

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

*If yes go to 5.*

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?
If yes go to 6. If no go to 7.
**YES**

The strain has been characterized to verify the introduction of DNA in the two GM-steps from parental to production strain. The modifications resulted in the expected changes in the genome. The genetic changes have been shown to be stably maintained in the strain for a number of generations that exceed the production process. Together with the toxicological investigations performed, this indicates that the modifications performed to construct the production strain do not result in unintended pleiotropic effects.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?

**NO**

**However,** the production strain is a *F. venenatum* derived via the parental strain ATTC 20334 (A3/5). A *Fusarium venenatum* strain derived from the same

parental strain was used as the production organism for a xylanase enzyme preparation which is the subject of GRAS Notice No. 54. A 13-week oral toxicity in rats, an Ames test, and a human lymphocyte cytogenetic assay were performed on that xylanase enzyme preparation, demonstrating the safety of the enzyme and the production strain.

Furthermore, strain ATCC 20334 is the main ingredient in Quorn™, a commercial non-animal protein product that has been in the market for more than forty years (Trinci 1992). Here, the product is actually the fungal biomass. Selection of the original *F. venenatum* production strain was performed after extensive toxicological trials in animals and humans. The *F. venenatum* strain proved non-toxic and of excellent nutritional value (Anderson and Solomons 1984).

*If no go to 7.*

7. Is the organism nonpathogenic?

**YES**

None of the species described in opportunistic mycosis or systemic infections of humans is a member of the *Fusarium*-section within the genus *Fusarium*, the section where *F. venenatum* is taxonomically classified. There are no reports connecting *F. venenatum* with pathogenesis in animals or humans.

*If yes go to 8.*

8. Is the test article free of antibiotics?

**YES**

*If yes go to 9*

9. Is the test article free of oral toxins known to be produced by other members of the same species?

**YES**

The potential for production of secondary metabolites is addressed in section 7.1.1 in the notice. The gene encoding trichodiene synthase (*tri5*), which catalyses the first step in the trichodiene biosynthetic pathway, was deleted in the host strain by means of site-directed gene disruption, thereby rendering it incapable of producing secondary metabolites within the trichothecene biosynthetic pathway. The

recipient (host) strain was shown unable to produce diacetoxyscirpenol (DAS) and related compounds under different inducing conditions and media and is described in the report in Appendix 2.

*If yes go to 11.*

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

**YES**

A 90 day oral (gavage) treatment of rats with serine protease tox test batch PPF 26813 at dose levels up to 581 mg TOS/kg bw/day caused no dose-related findings. The NOAEL of this study was the highest administered dose level, i.e. 581 mg TOS/kg bw/day. A 25-day study was performed with serine protease toxbatch PPF 32126 (a more concentrated batch) in order to establish a higher NOAEL for serine protease than what could be obtained in the 13 weeks toxicity study due to the inability to feed the rats any more of the test substance. Daily administration of up to 3605 mg TOS/kg bw/day of serine protease toxbatch PPF 32126 by oral gavage to Sprague Dawley rats for 25 days did not cause any treatment related toxicological effects. Consequently, 3605 mg TOS/kg bw/day was considered the NOAEL of this study. The NOAEL is sufficiently high to ensure safety as indicated in section 7.6.2.

*If YES the test article is **ACCEPTED.***

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# Toxicity and Allergen Risk Assessment report

By ELRo, EPF
31 October 2013

# Protease produced by NZYM-FG

**Assessment of sequence homology to known toxins**

The homology between protease from NZYM-FG and known toxins was assessed on the basis of the information present in the UNIPROT database (24-Jan-2013). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries did not exceed 30% indicating that the homology to any toxin sequence in this database is random.

**Sequence homology assessment:**

Analysis of protease from NZYM-FG according to the EFSA scientific opinion recommended allergen analysis using allergen on-line and allergen.org databases, identified 13 mite (Dermatophagoides, Blomia, Tyrophagus and Euroglyphus spp.) related allergens, 3 insect (Polistes (wasp) and Apis (bee) spp.) venum related allergens and 1 canin allergen having an identity with protease produced by strain NZYM-FG above the treshold of 35%.

The mite related allergens (e.g. Blo t 3, Der f 3, Der p 3, Der f 6, Tyr p 3) were revealed to have up to - 60.2 % (unscaled) and 57.5% (scaled) identity with protease from NZYM-FG over 80 amino acids windows using the allergen on-line and allergen.org databases. The venum allergens (upto 44.8% (43.8% (scaled)) and canin allergen (39.0% (37.5% (scaled)) revealed less identity.

Similarities with other allergens were below 35%. Since it is generally accepted that proteins with such a low identity rarely share epitopes they were not considered in this report (Pearson, 2000; Aalberse et al., 2001; Hileman et al., 2002; Ladics et al., 2007).

**Allergen risk assessment:**

Mite allergens well recognized and a variety of these allergens has been characterized in great detail. They are well-known to trigger respiratory sensitization and asthma. Prevalences of 25% to 30%

were reported (Bessot and Pauli, 2011; http://www.aaaai.org/ask-the-expert/house-dust-mite-allergy-in-united-states.aspx; Sönmez and Caliskan, 2009).

*Respiratory allergy:*

The analysis suggests similarities between protease from NZYM-FG and known respiratory allergens. The risk for respiratory sensitization and for acquiring asthma associated with the handling of enzymes has been recognized for many years, and has resulted in clearly described measures for assuring occupational safety.

*Food allergy:*

There is compelling evidence that vast majority of adults affected by food-induced occupational asthma can ingest the allergen without symptoms, suggesting that inhallation is not likely to result in food allergy (Brisman, 2002; Armentia et al., 2009).

This is backed up by a study using the generally recognized guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC). This study included 400 patients with diagnosed allergy to one or more of inhalation allergens, food allergens, bee or wasp allergens. The study concluded that no cases of IgE-mediated food allergy to commercial enzymes (incl. proteases) could be found. There were further no indications of cross-reactivity between the tested enzymes used in food and the main known allergens represented by the patients included in the study (Bindslev-Jensen et al., 2006).

**Conclusion:**

On the basis of the available evidence it can be concluded that oral intake of protease from NZYM-FG is not anticipated to pose any food allergenic concern, and that the current measures for assuring occupational safety are sufficient.

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**Toxicology & Product Safety**

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# SUMMARY OF TOXICITY DATA

# SP387/TL1

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## CONTENTS

PAGE

1. Abstract ..... 3

2. Test Substance ..... 3

   2.1 General information on the test material ..... 3
   2.2 Characterization ..... 3

3. Toxicity Data ..... 4

   3.1 General toxicity ..... 4

      3.1.1. 13-week Oral (gavage) Toxicity Study in Rats ..... 4
      3.1.2 25-Day Oral (Gavage) Toxicity Study in Rats ..... 5
      3.1.3 Overall conclusion of the General Toxicity Studies ..... 6

   3.2 Mutagenicity ..... 6

      3.2.1. Bacterial Reverse Mutation Assay (Ames Test) ..... 6
      3.2.2. Chromosome Aberration Assay – cultured human lymphocytes ..... 7

4. References ..... 9

   4.1 Studies ..... 9
   4.2 Literature and guidelines ..... 9

Last page ..... 9

# 1. ABSTRACT

SP387/TL1 is a liquid microbial enzyme concentrate. The present summary covers the toxicological studies relevant for the evaluation of SP387/TL1 when used as a processing aid in the production of food products.

All studies were carried out in accordance with current EU and OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were carried out at Novozymes in Denmark, Covance in UK and LAB Scantox in Denmark during the period April 2007 to August 2011.

The main conclusions of the safety studies can be summarized as below:

- 13 weeks of oral (gavage) treatment of rats with SP387/TL1 caused no dose related findings. Therefore the highest dose level 581 mg TOS/kg bw/day was considered the NOAEL of the study.

- In order to establish a higher NOAEL than obtained in the previous 13 week toxicity study, a 25 day oral gavage study in rats was performed as a *bridging study* on a more concentrated batch of SP387/TL1 and with a higher dose volume (10 mL/kg bw instead of 5 mL/kg bw) than in the previous 13 week toxicity study. No treatment related toxicological effects were seen at any dose level. Therefore, the highest dose level, 3605 mg TOS/kg bw/day, was considered the NOAEL of the study. This NOAEL is considered the overall NOAEL for the two general toxicity studies.

- SP387/TL1 gave no indication of mutagenic activity when tested in the bacterial reverse mutation assay in the presence or absence of S9 mix.

- SP387/TL1 did not induce chromosome aberrations in cultured human blood lymphocytes when assessed in the chromosome aberration assay.

# 2. TEST SUBSTANCE

## 2.1 General information on the test material

SP387/TL1 belongs to the peptidase family S1 and is capable of hydrolysing proteins at arginine and lysine residues.

SP387/TL1, batch PPF 26813 and batch PPF 32126, are the liquid enzyme concentrates used as the test substances in the present toxicological program. Batch PPF 26813 is a mixture of three identically produced fermentation sub-batches, recovered by purification/concentration of the fermented culture broth according to the same procedures as are used for the production of commercial SP387/TL1 preparations, except that formulation/standardization is omitted. Batch PPF 32126 was prepared by up-concentration by evaporation of batch PPF 26813.

The test substances do not contain the production strain and its absence is part of the complete specification of the product.

## 2.2 Characterization

The two batches, PPF 26813 and PPF 32126, used for the toxicological studies are characterized as described in Table 1.

**Table 1. Characterization of batch PPF 26813 and batch PPF 32126**

| | Batch PPF 26813 | Batch PPF 32126 |
|---|---|---|
| Activity KMTU/g | 117 | 340 |
| Water (KF) % w/w | 86.7 | 62.5 |
| Dry matter % w/w | 13.3 | 37.5 |
| Ash (600°C) % w/w | 2.3 | 6.9 |
| Total Organic Solids (TOS[1]) % w/w | 11.0 | 30.6 |
| Specific gravity (g/ml) | 1.057 | 1.178 |

[1] %TOS = 100% ÷ % water ÷ % ash.

# 3. TOXICITY DATA

## 3.1 General toxicity

### 3.1.1. 13-week Oral (gavage) Toxicity Study in Rats

The study was carried out in accordance with the OECD guideline 408 (adopted on September 1998). It was conducted in accordance with Good Laboratory Practice.

Forty male and forty female SPF Sprague Dawley rats of the Ntac:SD strain from Taconic Europe A/S were used in this study. The animals were randomly allocated to 4 groups (10 males and 10 females each). Group 1 was treated with tap water. Groups 2, 3 and 4 were treated with 58, 192 and 581 mg TOS per kg body weight (corresponding to 62 KMTU, 204 KMTU and 618 KMTU per kg body weight), respectively. The animals were treated orally (gavage) for 13 weeks. The dose volume was 5 ml/kg bw.

Clinical signs were recorded daily and detailed clinical observations were performed once weekly. Body weights and food consumption were recorded once weekly and water consumption twice weekly. All animals were examined with respect to reactivity to different types of stimuli, grip strength and motor activity. Ophthalmoscopy was performed before start of treatment and before termination of treatment. Before termination of treatment, blood samples were taken for hematology and clinical chemistry. The animals were killed and subjected to a macroscopic necropsy. Specified organs and tissues were weighed, fixed and prepared for histopatho-logical examination.

Analysis of achieved concentration was performed on samples taken once during weeks 1, 6 and 13. Achieved concentration was evaluated by measurement of enzyme activity, KMTU/g.

The oral administration of SP387/TL1, PPF 26813, for 13 weeks was well- tolerated. One male and one female in the intermediate group died (days 35 and 69, respectively). No *ante mortem* signs were observed and the cause of death was not identified at necropsy and both deaths were considered incidental.

No treatment related findings were recorded at the clinical and behavioural examinations, on food and water consumption, body weights or at the ophthalmoscopic examination.

No treatment related findings were observed on the parameters for serum biochemistry, haematology and on organ weights.

Necropsy and the following microscopic examination revealed no treatment related effects.

The results from the content check analysis showed that in the samples from week 1, 6 and 13, there were no significant differences between the intended activity and the results.

In conclusion, thirteen weeks of oral (gavage) treatment of rats with SP 387/TL1, batch PPF 26813 at dose levels up to 581 mg TOS/kg bw/day caused no dose-related findings. The NOAEL of the study is therefore 581 mg TOS/kg bw (equivalent to 618.3 KMTU/kg bw/day).

### 3.1.2 25-Day Oral (Gavage) Toxicity Study in Rats

The study was performed in compliance with Good Laboratory Practice. The study was carried out in accordance with the OECD guideline 407 (adopted on October 2008) with the one exception that the rats were dosed for 25 day instead of 28 days due to shortage of test substance.

This study was performed as a *bridging study* in order to establish a higher NOAEL for SP 387/TL1 than obtained in the previous 13 weeks toxicity study (study no. 65860, summarized in section 3.1.1) in which no toxicity occurred even at the highest dose level. The dosing volume in this study was increased from 5 to 10 mL/kg/day and the enzyme activity of the batch tested in this study was increased by a factor 3 compared to the previous 13-weeks study.

The study was conducted in 20 male and 20 female SPF Sprague Dawley rats of the Ntac:SD strain, in a parallel group design with 4 groups of 10 rats. The rats received daily oral treatment with SP387/TL1, batch PPF 32126 for 25 days at dose levels of 0 (vehicle control = tap water), 360, 1190 and 3605 mg TOS/kg bw/day for Groups 1, 2, 3 and 4, respectively (corresponding to 401, 1322 and 4005 KMTU/kg bw/day).

Clinical signs of ill health and behavioural changes were recorded daily including a more detailed observation of physical appearance once weekly. A functional observation battery consisting of an open field test and stimuli-induced tests was performed at termination.

Body weight, food consumption and water consumption were recorded on a regular basis. Ophthalmoscopy was performed before start and before termination of treatment. Blood and urine samples were collected from all animals before termination of treatment in Week 13 for evaluation of clinical chemistry (blood and urine) and haematology (blood) parameters. A macroscopic evaluation was performed on all animals. Selected tissues and organs were fixed, trimmed and subjected to microscopic examination.

Dose formulation analysis results showed that no activity above the detection limit was found for the control group, whereas the results of the enzyme activity for Groups 2-4 showed that the dosing formulations did not differ significantly from the expected values for all groups.

No treatment related clinical signs were recorded. One control animal (No 7, female) was killed on Day 23 due to adverse clinical signs and weight loss, caused by a mis-dosing with the gavage tube.

A slight tendency towards a reduction in food intake and body weight gain and an increased water intake in the high dose group was observed. These findings

indicate that the highest dose (3605 mg TOS/kg bw) of enzyme SP387/TL1 might not be palatable for the rat.

No treatment related findings were recorded at the ophthalmoscopic examination or in the functional observation battery and stimuli induced tests.

An increased serum urea level was observed as well as a lowering of pH in urine in the high dose females, which could be an effect of the increased protein intake from the test article and thereby increased protein catabolism. This was considered not to be of toxicological importance.

The test item did not have any treatment related effects on the haematology, coagulation parameters or on the urine microscopy.

At necropsy, at the organ weight analysis and at the histopathological examination, no treatment related findings were observed.

In conclusion, daily administration of up to 3605 mg TOS/kg bw/day of SP387/TL1 by oral gavage to Sprague Dawley rats for 25 days did not cause any treatment related toxicological effects. Consequently, 3605 mg TOS/kg bw/day (corresponding to an enzyme activity of 4005 KMTU/kg bw/day) was considered the NOAEL of this study.

### 3.1.3 Overall conclusion of the General Toxicity Studies

No treatment related changes of toxicological significance were seen when SP387/TL1 was tested at dose levels up to 581 mg TOS/kg bw (equivalent to 618.3 KMTU/kg bw/day) and 3605 mg TOS/kg bw/day (corresponding to an enzyme activity of 4005 KMTU/kg bw/day) in a 13 week oral (gavage) toxicity study in rats and a 25 day oral gavage study, respectively.

3605 mg TOS/kg bw/day (corresponding to an enzyme activity of 4005 KMTU/kg bw/day) is therefore considered the overall NOAEL of these two studies.

## 3.2 Mutagenicity

### 3.2.1. Bacterial Reverse Mutation Assay (Ames Test)

The procedures used in this test complied in principle with OECD Guideline for Testing of Chemicals No. 471 (1997).

The test material SP387/TL1, batch PPF 26813 was examined for mutagenic activity in the bacterial reverse mutation assay using *Salmonella typhimurium* strain TA1535, TA100, TA1537, TA98 and *Escherichia coli* WP2uvrA.
Crude enzyme preparations, like the present batch of SP387/TL1, contain the free amino acid L-histidine, most often in an amount, which exceeds the critical concentration for incorporation in the direct standard assay.

To overcome this problem, all *Salmonella* strains were exposed to the test substance in liquid culture ("treat and plate assay"). Bacteria were exposed to 6 doses of the test substance in a phosphate buffered nutrient broth for 3 hours with 5 mg (dry matter) per ml as highest concentration. After incubation the test substance was removed by centrifugation prior to plating.

Usually the content of tryptophan in enzyme preparations is low and insignificant. Therefore the part of the study comprising *Escherichia coli* was conducted with the strain WP2uvrA using the direct plate incorporation assay. 6 doses of the test substance

were applied with 5 mg (dry matter) per plate as the highest dose level followed by successive bi-sections between doses. The study was conducted with and without the metabolic activation system S9 - a liver preparation from male rats, pre-treated with Aroclor 1254, and the co-factors required for mixed function oxidase activity (S9 mix).

Two identical and independent experiments were conducted.

We consider a test substance as positive when it has induced at least a doubling in the mean number of revertants per plate compared to the appropriate solvent control in one or more of the strains, in the presence or absence of S9, if this response is dose related and reproducible. If a dose related numerical increase below a doubling but at least 50% higher than the solvent control is observed then the result is considered as equivocal and need further clarification.

No treatments of any of the *Salmonella* and *E .coli* strains with the test substance, either in the presence or absence of S-9 mix, resulted in any increases in revertant numbers that meet these criteria for a positive response.

It was concluded, that the results of the experiments, described in this report, give no indication of mutagenic activity of SP387/TL1, batch PPF 26813 in the presence or absence of metabolic activation, when tested under the conditions employed in this study.

### 3.2.2. Chromosome Aberration Assay–cultured human lymphocytes

The effects on chromosomal structure of exposure to SP387/TL1, batch PPF 26813 were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (1995).

Heparinised whole blood cultures from three female donors were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.

Two independent experiments were performed both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from animals induced with Aroclor 1254.

Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls (–S-9: 4-Nitroquinoline 1-oxide, +S-9: Cyclophosphamide). Treatments with the test substance covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5000 µg/mL.

The lymphocyte cultures were exposed to the test substance for three hours and cells were harvested 17 hours later. The second experiment included a continuous exposure for 20 hours in the absence of S-9. The test substance dose levels for chromosome analysis were selected by evaluating the effect of the test substance on mitotic index.

Chromosome aberrations were analyzed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from test substance treatments and negative controls. Slides were scored blind and aberrations classified according to the scheme described by ISCN (ISCN 1985).

The proportion of cells with structural aberrations in all cultures of the solvent controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with structural aberrations, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.

In the first experiment, the highest concentration of the test substance chosen for analysis, 5000 µg/mL, produced no cytotoxicity (reduction in mitotic index) in the absence and presence of S-9 respectively.

In the second experiment, the treatment in the absence of S-9 was continuous for 20 hours. The highest concentration chosen for analysis, 5000 µg/mL, approximately 33% and 0% mitotic inhibition in the absence and presence of S-9 respectively.

Cells treated with the test substance either in the absence or presence of S-9, had similar numbers of aberrations to those observed in concurrent solvent controls. There were no increases in aberration frequency that were significantly higher than those observed in the negative controls.

Normal frequencies of cells with numerical aberrations were seen under all treatment conditions.

It was concluded that SP387/TL1, batch PPF 26813 under the conditions of the test, did not induce chromosome aberrations in cultured human blood lymphocytes.

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## 4.1 Studies

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SP387/TL1: A 25-Day Oral (Gavage) Toxicity Study in Rats. NZ Reference No.: 20116015. LAB Research (Scantox) Study No.: 73488. LAB Research (Scantox), August 2011. File No. 2011-23302.

SP 387/TL1: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocytes. NZ Reference No.: 20076031. Covance Study No. 1974/63. Covance, Sept 2007. File No.: 2007-38804-01.

SP 387/TL1, PPF 26813: Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli.* NZ Study No.: 20078062. Novozymes, November 2007. File No.: 2007-46872-01.

## 4.2 Literature and Guidelines

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SUBMISSION END