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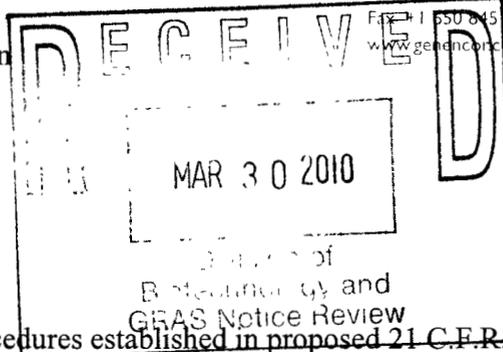
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March 29, 2010

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Deputy Division Director
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
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Dear Dr. Martin:

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor, a Danisco Division, ("Danisco") has determined that its Acid Fungal Protease enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding Acid Fungal Protease from *T. reesei* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. We are hereby submitting, in triplicate, a Generally Recognized As Safe ("GRAS") Notification, in accordance with proposed 21 C.F.R. § 170.36, informing FDA of the view of Danisco that the Acid Fungal Protease enzyme preparation is GRAS, through scientific procedures, for its uses in grain processing (corn steeping), alcoholic beverages manufacture, noncitrus juice manufacture (i.e., apple juice), and degumming of membranes during orange juice manufacture. In these applications the enzyme is considered a processing aid.

If you have questions or require additional information, please contact me at 650-846-7557 or fax at 650-845-6505.

Sincerely,

(b) (6)

Alice J. Cadow
Vice President of Regulatory
& Sustainability

Enclosures (3 binders)

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**Acid Fungal Protease Enzyme
Preparation Derived from
*Trichoderma reesei***

Expressing the Acid Fungal Protease Gene

from

***T. reesei* Is Generally Recognized As Safe**

For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

March 26, 2010

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1. GENERAL INTRODUCTION

The Acid Fungal Protease (AFP) enzyme preparation under consideration is produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding the native Acid Fungal Protease enzyme, also called Aspergillopepsin I from *T. reesei*.

The enzyme product is intended for use in grain processing (corn steeping), alcoholic beverages manufacture, noncitrus juice manufacture (i.e., apple juice), and degumming of membranes during orange juice manufacture. In all of these applications, AFP will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food. It is assumed that the enzyme used for degumming of membranes will be washed away after the degumming and will not be present in the orange juice.

Other proteases currently in use are derived from other microorganisms, most notably *Aspergillus oryzae*, *A. niger* and *Bacillus subtilis*. The protease from *T. reesei* has been in commerce as a minor component of other *T. reesei* derived enzymes, but was not produced at commercially viable levels until the development of this production organism and process. It replaces these other proteases currently marketed for the intended uses as well as some of the SO₂ currently used in corn steeping.

The accepted name of the principle enzyme activity is aspergillopepsin I. Other names used are *Aspergillus acid protease*; *Aspergillus acid proteinase*; *Aspergillus aspartic proteinase*; *Aspergillus awamori acid proteinase*; *Aspergillus carboxyl proteinase*; (see also Comments); carboxyl proteinase; *Aspergillus kawachii aspartic proteinase*; *Aspergillus saitoi acid proteinase*; pepsin-type aspartic proteinase; *Aspergillus niger acid proteinase*; sumizyme AP; proctase P; denapsin; denapsin XP 271; proctase; and Acid Fungal Protease.

The enzyme catalyses hydrolysis of proteins with broad specificity. It generally favors hydrophobic residues in P1 and P1', but also accepts Lys in P1, which leads to activation of trypsinogen. Aspergillopepsin I does not clot milk.

The EC number of the enzyme is 3.4.23.18, and the CAS number is 9025-49-4.

The information provided in the following sections is the basis of our determination of GRAS status of this AFP enzyme preparation

Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as a determination of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for food use.^{1,2} The safety of the production organism for this AFP, *T. reesei*, is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material.³⁻⁸ The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no

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evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor, a Danisco Division, (“Danisco”) has determined that its Acid Fungal Protease enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding Acid Fungal Protease from *T. reesei* is a Generally Recognized as Safe (“GRAS”) substance for the intended food application and is, therefore, exempt from the requirement for premarket approval.

1.2 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.3 Common or Usual Name of Substance

Acid Fungal Protease enzyme preparation from *Trichoderma reesei* expressing the gene encoding the Acid Fungal Protease, Aspergillopepsin I, enzyme from *T. reesei*.

1.4 Applicable Conditions of Use

Acid Fungal Protease is used as a processing aid in the for grain processing (corn steeping), alcoholic beverages manufacture, noncitrus juice manufacture (i.e., apple juice), and degumming of membranes during orange juice manufacture.

1.5 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.6 Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

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2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism is a strain of *Trichoderma reesei*, which has been genetically modified by deletion of several cellulase genes and by overexpression of a gene for the production and secretion of AFP isolated from *T. reesei* (see Section 2.2). *T. reesei* is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U. S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees, and is also considered as Good Industrial Large Scale Practice (GILSP) worldwide. It also meets the criteria for a safe production microorganism as described by Pariza and Johnson¹. It contains the *T. reesei* gene encoding AFP under the regulation of a native *T. reesei cbhI* (cellobiohydrolase 1) gene. The *A. nidulans amdS* gene was used as a selectable marker. The inserted DNA was integrated into the recipient chromosome.

2.2 Host Microorganism

The host organism *T. reesei* strain RL-P37 was obtained from Dr. Montenecourt. The derivation and characterization of strain RL-P37 has been published by Sheir-Neiss and Montenecourt⁹. Strain RL-P37 is a cellulase over-producing strain that was obtained through several classical mutagenesis steps from the wild-type *Trichoderma reesei* strain (QM6a). Strain QM6a is present in several public culture collections, e.g. in the American Type Culture Collection as ATCC 13631. *T. reesei* has more recently been identified as a clonal derivative or anamorph of *Hypocrea jecorina*.^{10,11}

2.3 Acid Fungal Protease Expression Vector

For construction of the vector, the DNA encoding the *T. reesei* mature secreted AFP protein was fused to the DNA encoding the *T. reesei* CBHI signal peptide. This open reading frame is flanked by the promoter and terminator sequences of the *T. reesei cbhI* gene. The vector also contains the *Aspergillus nidulans amdS* gene, encoding acetamidase, as a selectable marker for transformation of *T. reesei*.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *T. reesei* strain had been made.

2.4 Stability of the Introduced Genetic Sequences

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by AFP production.

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2.5 Antibiotic Resistance Gene

No antibiotic resistance genes were used in the construction of the production microorganism.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of 1CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by the International Food Biotechnology Council (IFBC)³ is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

| | |
|------------------------------------|---|
| IUBMB Nomenclature ¹² : | Aspergillopepsin I |
| IUBMB Number: | 3.4.23.18 |
| CAS Number: | 9025-49-4 |
| Reaction catalyzed: | Hydrolysis of proteins with broad specificity. Generally favours hydrophobic residues in P1 and P1', but also accepts Lys in P1, which leads to activation of trypsinogen. Does not clot milk. |
| Other Names: | <i>Aspergillus</i> acid protease; <i>Aspergillus</i> acid proteinase; <i>Aspergillus</i> aspartic proteinase; <i>Aspergillus awamori</i> acid proteinase; <i>Aspergillus</i> carboxyl proteinase; (see also Comments); carboxyl proteinase; <i>Aspergillus kawachii</i> aspartic proteinase; <i>Aspergillus saitoi</i> acid proteinase; pepsin-type aspartic proteinase; <i>Aspergillus niger</i> acid proteinase; sumizyme AP; proctase P; denapsin; denapsin XP 271; proctase; and Acid Fungal Protease ¹² . |

3.2 Amino Acid Sequence

The amino acid sequence of the AFP enzyme and nucleotide sequence of the gene encoding it is known and is shown in Appendix 1.

4. MANUFACTURING PROCESS

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This section describes the manufacturing process for the AFP enzyme which follows standard industry practice.²²⁻²⁴ For a diagram of the manufacturing process, see Appendix 2. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme

preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for this AFP concentrate are standard ingredients used in the enzyme industry.²²⁻²⁴ All the raw materials conform to the specifications of the Food Chemicals Codex, 6th edition, 2008 ("FCC"),¹⁴ except for those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoam used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoam in the production process is $\leq 0.15\%$.

4.2 Fermentation Process

The AFP enzyme is manufactured by submerged straight-batch or fed-batch pure culture fermentation of the genetically modified strain of *T. reesei* 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 conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

A new lyophilized stock culture vial of the *T. reesei* production organism described in Section 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed, and main fermentor) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units (CFU) of bacteria or fungi other than the production strain are present at levels $>10^3$ CFU/ml.

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If a fermentation batch is determined to be contaminated, it will be rejected if deemed appropriate. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

The recovery process is a multi-step operation, which starts immediately after the fermentation process and consists of both concentration and formulation processes.

4.3.1 Concentration process

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

1. Primary separation –centrifugation or filtration;
2. Concentration – ultrafiltration;
3. Formulation; and
4. Polish filtration.

4.3.2 Formulation and standardization process

The ultrafiltered concentrate is stabilized with 50% glycerol, 2.4- 3.4% sodium sulphate, and 0.0 –0.3 % sodium benzoate. The remaining 43 to 45% is water.

4.3.3 Quality control of finished product

The final AFP liquid concentrate from *T. reesei* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA)¹⁵ in 2006 and the Food Chemical Codex 6th edition (FCC)¹⁴. These specifications are described in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The liquid concentrate is stabilized with the formulation ingredients listed below and tested to demonstrate that it meets the specifications.

The formulation of the enzyme preparation is as follows:

- >2.8% active protein
- 50% Glycerol
- 2.4- 3.4% Sodium Sulphate
- ≤0.3 % Sodium Benzoate
- 43.5-45% H₂O.

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5.2 Specifications

Acid Fungal Protease meets the purity specifications for enzyme preparations set forth in the FCC 6th edition¹⁴. In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA in the Compendium of Food Additive Specifications¹⁵.

6. APPLICATION

6.1 Mode of Action

Acid Fungal Protease catalyses hydrolysis of proteins with broad specificity. It generally favors hydrophobic residues in P1 and P1', but also accepts Lys in P1, which leads to activation of trypsinogen. It does not clot milk.

6.2 Use Levels

Acid Fungal Protease will be added to the steeping process for carbohydrate processing at 0.009 mg/g corn; to apple juice at 1 mg/g; and used in potable alcohol production at 0.160 mg/ ml pure alcohol.

Corn Steeping:

Currently the corn wet milling industry employs a steeping process that uses SO₂ and warm water to soften and hydrate the corn kernels as well as break disulfide cross-links that are present in the corn. The steeping process allows for a better separation of the starch from the main fractions that include endosperm, germ, fiber and pericarp. The starch is removed from the grain through a number of separation steps to give a pure starch stream that can be further processed into a number of sweetener products.

The current process consists of large cylindrical steeping tanks that contain warm water that is maintained around 30-40° C. The water flow through the steeping tanks is countercurrent where the corn that has been steeped the longest receives the "freshest" water. The current steep process has a minimal contact time of 24 hours and the longer the steep time, the better the separation of the starch from the granule.

The water is acidified by bubbling SO₂ in through the water to a saturation level of around 4000 ppm, which then reacts with water to form sulfurous acid. It has been shown that an acidic environment is conducive to the steeping of corn. The dissolved SO₂ also acts as an antibacterial agent and it is believed to be effective in preventing/controlling microbial contamination throughout the downstream processing of the starch stream. It should be noted that although SO₂ is considered an antimicrobial agent it does not prevent lactobacillus fermentations in the steeping process, and it has actually been shown that this bacterial fermentation is beneficial in the steeping process.

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The spent steep water can be concentrated and sprayed on the corn fiber to make a product referred to as corn gluten feed, which is sold and fed to animals. It can also be sold as nutrient for a number of other fermentation processes.

The steeping process employing our new AFP enzyme has been coined as “e-milling” and offers an alternative to using high levels of SO₂ as the main additive to promote the steeping of corn. This new process can complete the corn steeping process in 6-10 hours as compared with the current process with a minimal of 24 hours. This would be a huge advantage to the industry if the demand for the end products was greater than the supply capabilities of the industry, but that is not the case. For plants that already have steep tanks that are in good shape, there is no real advantage. Most of the existing wet milling plants have steep tanks that are constructed out of Cyprus wood. Over time, these tanks begin to rot and eventually need to be replaced. One advantage of the e-milling process is that the number of tanks that need to be constructed is reduced as the throughput is increased by the reduced steeping time. The tanks can be constructed out of stainless steel instead of wood, which also increases the life of the steeping tanks.

Another advantage of the process is the reduction in the level of SO₂ from the current level of 4000 ppm to 200 ppm. The use of SO₂ in industry has been under attack due to concerns around allergic reactions to exposed workers. In some plants, due to this allergic concern, they have started burning elemental sulfur to reduce the potential exposure of workers to SO₂. It is believed that the SO₂ will not be entirely replaced due to the fact that it appears to keep the plant contamination free.

Overall, this new e-milling process offers reduced capital outlay for plants that are in need of new steep tanks and for those that are concerned about workers' exposure to SO₂. The major hurdle though will be to implement an enzyme system that is economical and can compete with the use of SO₂ as this is a relatively cheap, readily available, bulk chemical. Therefore, it is anticipated that some manufacturers will convert to this system, but not all.

Potable Alcohol:

The AFP will be used in the fermentation step to increase the level of free amino acid nitrogen in the fermentation to help sustain yeast vitality.

The enzyme has a secondary effect of reducing foaming due to protein. This is particularly important when using cereals with high protein content such as wheat and sorghum.

Juice Processing:

AFP will be used to dehaze apple juice and degum membranes in orange juice factories. Typical dose levels are 0.1% (1mg/g) for 1 hour @ 50°C.

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Membrane Degumming:

AFP will be used to degum membranes during orange juice manufacture. The membranes will be rinsed after degumming. Therefore AFP will not be present in the orange juice and this use was not included in the risk assessment.

6.3 Enzyme Residues in the Final Foods

As discussed above, the enzyme will be removed in all applications and will not be present in the final food products.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food.¹ If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume.⁴ Pariza and Foster² 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 non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *T. reesei* meets these criteria for non-toxicity and non-pathogenicity.

7.1.1 Safety of the host

T. reesei was first isolated from nature in 1944. The original isolate, QM 6a, and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. In the 1980s, it was suggested by Bissett that *T. reesei* be placed into synonymy with *T. longibrachiatum*¹³. Later however, evidence emerged indicating that the two species are not identical¹⁶. The proposal by Kuhls *et al.*¹⁰ that *T. reesei* was a clonal derivative of *Hypocrea jecorina* is being accepted by more and more people in the science community, and the US National Center for Biotechnology Information (NCBI) refers to *T. reesei* as the anamorph of *H. jecorina* and no longer includes it in the genus *Trichoderma*. Therefore, the names *Trichoderma reesei*, *Trichoderma longibrachiatum*, and *Hypocrea jecorina* may appear in different documents, but they refer to essentially the same microorganism species.

A review of the literature search on the organism (1972 - 2009) uncovered no reports that implicate *T. reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult humans and animals. The species is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U. S.

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Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees. BSL1 microorganisms are not known to cause diseases in healthy adult humans.

Brückner and Graf reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound that exhibited antibiotic activity¹⁷. Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains¹⁸. However, peptaibols' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those in enzyme manufacturing. Strain QM 9414 and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by the FCC.

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen et al.¹⁹, Blumenthal²⁰ and Olempska-Beer et al.²¹. The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases in the Pariza and Johnson paper¹ and various strains have been approved to produce commercial enzyme products internationally, for example, in Canada (Food and Drugs Act Division 16, Table V, Food Additives That May Be Used As Enzymes), the United States (FDA 1999), France, Australia/New Zealand, China (MOH 1996), JECFA²⁹ and Japan.

The production organism of the AFP enzyme preparation, the subject of this submission, is *T. reesei* strain NSP24 #22-1. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to enhance AFP production levels. RL-P37, a commercial production strain, is derived, as a result of several classical mutagenesis steps, from the well-known wild-type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a. Genencor has used strain RL-P37 for production of cellulases for over fifteen years and has developed many production strains from it using recombinant DNA techniques. The strain has been determined to be non-pathogenic and non-toxicogenic through an acute intraperitoneal study in rats. All of the food/feed grade products produced by this lineage were determined to be safe for their intended uses and are the subject of numerous GRAS determinations. A GRAS Notice was filed for one of the products from this strain lineage and FDA issued a no questions letter (see GRAS Notice 230³⁰).

From the information reviewed, it is concluded that the organism *T. reesei* provides no specific risks to human health and is safe to use as the production organism of Acid Fungal Protease.

It is concluded that the strain is non-pathogenic and non-toxicogenic.

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7.1.2 Safety of the donor source

In this case, the donor organism is the parent strain of the host strain, *T. reesei* QM6a, therefore the safety assessment is the same as for the host.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of AFP is conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration and formulation, resulting in a liquid Acid Fungal Protease enzyme preparation. The process is conducted in accordance with current food good manufacturing practice (cGMP) as set forth in 21 CFR Part 110. The resultant product meets the purity specifications for enzyme preparations of the Food Chemicals Codex, 6th Edition¹⁴ and the general specifications for enzyme preparations used in food processing proposed by WHO/JECFA¹⁵.

7.3 Safety of Acid Fungal Protease

7.3.1 Allergenicity

According to Pariza and Foster², there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a 2006 investigation of possible oral allergenicity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded “that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy²⁵.”

Despite this lack of general concern, the potential that AFP could be a food allergen was assessed by comparison with sequences of known allergens and is summarized here. The *T. reesei* Aspergillopepsin I (AFP) mature peptide sequence was subjected to FASTA alignment of the sequence with known allergens using the SDAP database (http://fermi.utmb.edu/SDAP/sdap_who.html)³¹ containing 887 allergens (listed in http://fermi.utmb.edu/cgi-bin/SDAP/sdap_01), which reveals significant (48%; E score = 4.8e-70) homology with aspergillopepsin from *A. fumigatus*, also referred to as Asp f 10, a respiratory allergen³².

Guidelines developed by the Codex Alimentarius Commission (2003)²⁶ recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids; these

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guidelines further state that a search for stepwise contiguous identical amino acid segment searches may be performed to identify sequences that may represent linear epitopes, but that these techniques need to be scientifically validated. This cautionary statement relates to the fact that ILSI/IFBC had previously advocated the use of matches of at least 8 contiguous identical amino acids³³ and that the 2001 Joint FAO/WHO Expert Consultation³⁴ suggested moving from 8 to 6 identical amino acid segments in contiguous searches. Following the FAO³⁴ guidelines but taking the comments in Codex (2003)²⁶ into account, two additional searches against the SDAP database were performed.

A search for 80-amino acid stretches with greater than 35% identity to known allergens confirmed multiple stretches throughout the peptide sequence with over 35% identity with Asp f 10, with maximum sequence identity across 80 amino acids of 64%.

A secondary search for exact matches of short stretches (≥ 6 amino acids) of sequence that could serve as potential IgE binding sites indicated the existence of one 14-amino acid match, one 8-amino acid match, one 7-amino acid match, and one 6-amino acid match, all with Asp f 10³⁵, as indicated in the sequence below in bold red color.

LPTEGQKTASVEVQYNKNYVPHGPTALFKAKRKYGAPISDNLKSLVAARQAKQALAKRQTGSA
PNHPSDSADSEYITSVSI GTPAQVLP**LD****F****D****T****G****S****S****D****L****W****V****F****S****S****E****T**PKSSATGHAIYTPSKSSTSK
KVSGASWSIS**Y****G****D****G****S****S**SSGDVYTDKVTIGGFSVNTQGVESATRVSTEFVQDTVISGLVGLAFD
SGNQVRPHQKTWFSNAASSLAEPFLTADLRHGQNGSYNFGYIDTSVAKGPVAYTPVDNSQGF
WEFTASGYSVGGGKLNRRNSID**G****I****A****D****T****G****T****L****L****L****L****L****D****D**NVVDAYYANVQSAQYDNQQEGVVFDCE
DLPSFSFGVGSSTITIPGDLLNLTPLEEGSSTCFGGGLQSSSGIGINIFGDVALKAALVVFDLG
NERLGWAQK

Any match with 6 contiguous amino acids is not conclusive as it is likely to be a false positive^{27,36-38} as also recognized by the Codex (2003)²⁶ guidelines and others (e.g., Taylor³⁹ and Goodman⁴⁰). A match with 8 or more amino acids provides more certainty^{27,36-38}. Note that the first two matching stretches in *T. reesei* AFP are separated by only 1 amino acid, thus forming a stretch of 16 amino acids with only one mismatch. Along with the 14-amino acid match, the sequence appears to contain 2 non-random matches with Asp f 10.

Asp f 10 is not a food allergen, (Allergen Nomenclature, International Union of Immunological Societies (IUIS; www.allergen.org). Although most inhalation or skin allergens are not active via the oral route, cross-reactivity cannot be excluded. Of note is that the within the final 14-amino acid contiguous match with Asp f 10 there is also a 6-amino acid match with a potential soybean allergen (colored in purple in the sequence), Gly m Bd28K⁴¹, although this potential soybean allergen is not listed in the IUIS database as a food allergen.

However, regardless of the size of the contiguous stretch, a match of the subject protein sequence with any random short stretch of amino acids of a known allergenic protein does not mean the subject protein has the IgE binding epitope(s) contained within that allergenic protein.

7.3.3 Safety Studies

All safety studies were conducted in accordance with internationally accepted guidelines (OECD) and are in compliance with the principles of Good Laboratory Practices (“GLP”) according to the FDA/OECD.

As mentioned in Section 7.3.2, Genencor has conducted two safety studies on the *T. reesei* organism itself, one on a recombinant strain derived from RL-P37, modified to overexpress an endoglucanase enzyme, and another on strain A83, also derived from RL-P37 through mutation and selection (see Appendix 3 for summary of the safety studies).

Genencor has conducted six studies on the AFP enzyme preparation, including 1) ocular irritation; 2) skin irritation; 3) acute oral toxicity; 4) 91-day, repeated dose feeding study (gavage) in rats; 5) a Chromosomal Aberration Assay with AFP using Human Peripheral Lymphocytes, and 6) a Bacterial Reverse Mutation Assay. Summaries are included in Appendix 3. Characterization of the tox lot is given in Appendix 4.

Results:

7.3.3.1 Ocular Irritation in the Rabbit, Scantox No. 60627, 2006

In the initial study, slight conjunctivitis was observed at the 1-hour observation period with clearing by 24 hours. In the confirmatory assay, no irritation was observed.

The primary eye irritation score was 0.0.

7.3.3.2 Acute dermal irritation study in rabbits (sequential approach). Scantox No. 60628, 2006

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No reactions were noted at any test site in both preliminary and confirmatory assays.

The mean score for skin edema and erythema was 0.0. According to the Directive of the Commission 93/21/EEC of April 27, 1993, AFP is not a skin irritant.

7.3.3.3 Acute oral toxicity in rats, Scantox No. 60626, 2006

No mortality was recorded in this study. There were no treatment related effects noted throughout the 14-day observation period.

Using the GHS classification system (December 2001), AFP can be classified as non-hazardous (oral LD₅₀ > 2000 mg/kg bw).

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7.3.3.4 A 13-week Oral (Gavage) Toxicity Study in Rats, LAB Scantox No. 60623, 2006

There were no treatment-related deaths in this study. No clinical signs were seen that could be considered to be treatment related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, body weights, body weight gains, hematology, clinical chemistry, and ophthalmologic examinations. At study termination, in the males, the absolute and relative liver weights were statistically significantly increased and the relative testes weight was statistically significantly decreased compared to the concurrent control group. However, there were no treatment-related macroscopic and histopathologic changes. In the functional observation battery testing, there were no statistically significant changes noted in treated groups.

Although statistically significant variations in liver and testes weights were noted in high dose males, these weight variations were still within the 95% confidence interval of historical control data collected at LAB Scantox. Further, in the absence of corresponding histopathologic changes, these weight variations were considered to be of no toxicological significance. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested (31.25 mg total protein/kg bw/day or 35.81 mg TOS/kg bw/day).

7.3.3.5 *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes, Scantox No. 60625, 2006

In the preliminary dose-range assay, cytotoxicity was observed at 5000 µg/ml in both non-activated and activated cultures. Based on these results, the following doses were used:
First main test, without S-9 mix: 625, 1250 and 2500 µg/ml
First main test, with S-9 mix: 1250, 2500 and 5000 µg/ml
Second main test, without S-9 mix: 313, 625 and 1250 µg/ml
Second main test, with S-9 mix: 1250, 2500 and 5000 µg/ml

In the absence of S-9 mix, AFP caused reductions in mean mitotic index of 52 and 53% at the highest concentration tested in the first and second main test, respectively, and this level of toxicity meets the requirements of OECD 473 guideline for the highest concentration to be scored for aberrations (> 50% reduction in mitotic index). In the presence of S-9 mix, the 5000 µg/ml dose level is the maximum required by OECD guideline.

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with AFP concentrate both in the presence and absence of metabolic activation. Significant increases in aberrant metaphases were demonstrated with the positive controls.

Under the conditions of this test, AFP concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation.

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7.3.3.6 Bacterial Reverse Mutation Assay – Ames assay, Scantox No. 60624, 2006

In the preliminary toxicity test, AFP was not toxic to the bacteria at any dose level tested. Therefore, the highest dose level selected for the main assays was 5000 µg/plate, which is the maximum required by OECD guideline.

In the first main test, AFP concentrate was not toxic to tester strains at any dose level, either in the presence or absence of S-9 mix. In the second main test, a statistically significant reduction in the number of revertant colonies was observed in strain TA 1535 with S-9 mix, but this effect was not dose related.

Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

Although a statistically significant reduction in the number of revertant colonies was noted in TA 1535 with S-9 mix, this is not considered to be biologically significant in the absence of a dose-related effect. Some variations in revertant colonies were noted but the variations were not reproducible between the three replicate plates and none of these variations meet the positive criteria recognized by regulatory agencies worldwide. Under the conditions of this assay, AFP has not shown any evidence of mutagenic activity in the Ames assay.

7.4 Safety Assessment

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats (Scantox No. 60623), a NOAEL was established at 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day). The study was conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice and was designed based on OECD guideline No. 408. As human exposure to AFP is through oral ingestion, selection of this NOAEL is thus appropriate.

7.4.2 Human Exposure Assessment

Estimation of daily consumption of AFP from its potential uses in three major commodities (consumption data based on USDA-NASS Agricultural Statistics 2005; www.usda.gov/nass/pubs/agr05/)

7.4.2.1 Corn sweeteners

- a. Annual consumption: 79.2 lbs or 35.95 kg (1 lb = 0.454 kg)
- b. Daily consumption: 98.4 g or 1.51 g/kg bw (avg human = 65 kg)
- c. Dose of AFP (mg of protein) used/corn: 0.009 mg/g corn
- d. Daily consumption of AFP: 0.009 mg X 1.51 g/kg bw = 0.014mg/kg bw

7.4.2.2 Noncitrus juice (i.e., Apple juice)

- a. Annual consumption: 66.8 lbs or 30.32 kg

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- b. Daily consumption: 83 grams or 1.28 g/kg bw (avg human = 65 kg)
- c. Dose of AFP used/juice 0.1% or 1 mg/g
- d. Daily consumption of AFP 1 mg X 1.28 g/kg bw = 1.28 mg/kg bw

7.4.2.3 Potable Alcohol (pure)

- a. Annual consumption: 10 liters
- b. Daily consumption of pure alcohol: 27.4 ml or 0.42 ml/kg bw
- c. Dose of AFP used: 0.160 mg/ ml pure alcohol
- d. Daily consumption of AFP 0.160 mg X 0.42 ml/kg bw = 0.067 mg/kg bw

Maximum intake of AFP from all commodities (cumulative)

$$0.014 \text{ mg/kg bw} + 1.28 \text{ mg/kg bw} + 0.067 \text{ mg/kg bw} = 1.37 \text{ mg/kg bw/day}$$

The maximum daily consumption of AFP from corn sweeteners, potable alcohol, and noncitrus juice is 1.37 mg/kg bw/day under the scenario that (1) all above commodities are treated with AFP and (2) 100% of AFP remains in the product after processing. In reality, it is expected that residues of the enzyme processing aid in the final products would be negligible after processing. It is also assumed that when used to degum membranes during orange juice processing, the enzyme is washed away and therefore not present at all in the juice.

For risk analysis purposes a 10% residue is used to represent a worst-case scenario.

Maximum Daily Intake of AFP = 0.137 mg/kg bw

Based on the results from the 90-day oral (gavage) feeding study cited above

$$\text{Margin of safety} = \frac{\text{No observed adverse effect level}}{\text{Daily exposure}}$$

$$\text{Margin of safety} = \frac{31.25 \text{ mg/kg bw/day}}{0.137 \text{ mg/kg bw/day}} = 230$$

7.4.3 Conclusion

The safety of AFP as a food processing aid in corn syrup, noncitrus juice and potable alcohol is assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. AFP is not an eye and skin irritant, is not mutagenic in the Ames assay and is not clastogenic in mammalian system (human peripheral lymphocytes). Daily administration of AFP for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at greater than 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day).

Based on a worst-case scenario that a person is consuming corn syrup, noncitrus juice and potable alcohol treated with AFP (i.e., cumulative risk), this NOAEL still offers at least a 230X fold margin of safety.

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8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *Trichoderma reesei*, enzyme preparations derived there from, including cellulase, beta-glucanase, xylanase and transglucosidase enzyme preparations, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA, as well as Genencor's own unpublished safety studies, support such a conclusion.

Trichoderma reesei is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production.

Using the decision tree of Pariza and Johnson¹ the enzyme preparation was determined to be acceptable for the proposed uses.

The safety studies conducted by Genencor established a NOAEL at greater than 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day). Based on a worst-case scenario that a person is consuming corn syrup, noncitrus juice and potable alcohol treated with AFP (i.e., cumulative risk), this NOAEL still offers at least a 230X fold margin of safety.

Based on the available data from the literature and generated by Genencor, the company has concluded that aspergillopepsin I from *Trichoderma reesei* strain 3243 is safe and suitable for use in corn steeping, production of apple juice, and potable alcohol. In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, was reviewed by a GRAS panel consisting of Dr. Michael Pariza, Dr. Joseph Borzelleca and Dr. Herbert Blumenthal, who concurred with the company's conclusion that the product is GRAS (see Appendix 6) for its intended uses.

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10. LIST OF APPENDICES

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- Appendix 2 - The Manufacturing process
- Appendix 3 – *T. reesei* Strain Lineage and Summary of Safety Studies
- Appendix 4 - Composition of Tox Lot
- Appendix 5 – The Pariza/Johnson Decision Tree
- Appendix 6 – GRAS Panel Report

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Appendix 1 – The amino acid sequence of Acid Fungal Protease

The *T. reesei* Aspergillopepsin I (AFP) sequence is given below in FASTA format, with the secretion signal underlined.

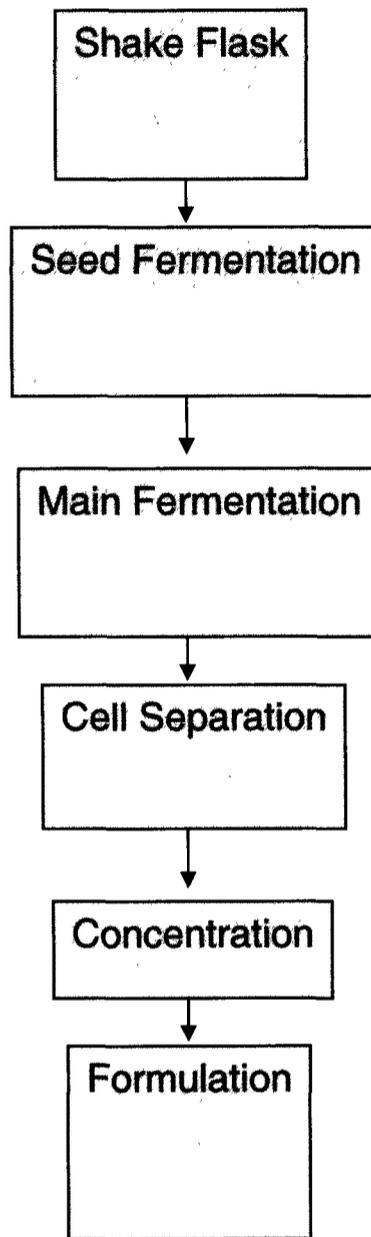
```
MOTFGAFLVSEFLAASGLAAALLPTEGQKTASVEVQYNKKNYVPHGPTALFKAKRKYGAPI  
SDNLKSLVA  
ARQAKQALAKRQTGSAPNHPSDSADSEYITSVSIGTPAQVLPDLDFTGSSDLWVFSSETPKSSATGHA  
IYTPSKSSTSKKVSGASWSISYGDGSSSSGDVYTDKVTIGGFSVNTQGVESATRVSTEFVQDTVISGL  
VGLAFDSGNQVRPHPQKTWFSNAASSLAEPFTADLRHGQNGSYNFGYIDTSVAKGPVAYTPVDNSQG  
FWEFTASGYSVGGGKLNRRNSIDGIADTGTTLLLLDDNVVDAYYANVQSAQYDNQQEGVVFDCEDE  
LPS  
FSFGVGSSTITIPGDLLNLTPLEEGSSTCFGGLQSSSGIGINIFGDVALKAALVVFDLGNERLGWAQK
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Appendix 2- Diagram of the Manufacturing Process



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Appendix 3 – *T. reesei* Strain Lineage and Summary of Safety Studies

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Toxicology Test Summaries

The safety of the six enzyme preparations derived from the six recombinant production strains were assessed in a number of toxicology tests as shown in the table below. The table also includes the toxicology tests for the non-recombinant strain *T. reesei* A83 and product derived from it.

| PRODUCTION ORGANISM | ENZYME PREPARATION | TOXICOLOGY TEST | RESULT |
|---|----------------------------|---|---------------------------------|
| I. <i>T. reesei</i> A83 (traditionally modified) | <i>T. reesei</i> cellulase | Pathogenicity study, rats | Non-pathogenic, non-toxicogenic |
| | | 91-day subchronic study, rats | No adverse effect detected |
| | | Bacterial reverse mutation assay | Not mutagenic |
| | | <i>In vitro</i> chromosome assay, human lymphocytes | Not clastogenic |
| II. <i>T. reesei</i> (homologous rDNA) | High pI Xylanase | 91-day subchronic study, rats | No adverse effects detected |
| | | Bacterial reverse mutation assay | Not mutagenic |
| | | <i>In vitro</i> chromosome assay, Chinese hamster ovary cells | Not clastogenic |
| III. <i>T. reesei</i> (homologous rDNA) | Low pI Xylanase | 91-day subchronic study, rats | No adverse effects detected |
| | | Bacterial reverse mutation assay | Not mutagenic |
| | | <i>In vitro</i> chromosome assay, human lymphocytes | Not clastogenic |
| IV. <i>T. reesei</i> (homologous rDNA) | Endoglucanase III | 28-day subacute study, rats | No adverse effects detected |
| | | Bacterial reverse mutation assay | Not mutagenic |

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| PRODUCTION ORGANISM | ENZYME PREPARATION | TOXICOLOGY TEST | RESULT |
|--|-------------------------------|--|---|
| V. <i>T. reesei</i> (homologous rDNA) | Endoglucanase I | 14-day subacute study, rats | No adverse effects detected |
| | | Pathogenicity study, rats | Non pathogenic |
| | | 91-day subchronic study, rats | No adverse effects detected NOAEL = 1000 mg/kg/d |
| | | <i>In vitro</i> chromosome assay, human lymphocytes | Not clastogenic |
| VI. <i>T. reesei</i> (homologous rDNA) | Xylanase (protein engineered) | 91-day subchronic study, rats | No adverse effects detected |
| | | Bacterial reverse mutation assay | Not mutagenic |
| | | <i>In vitro</i> chromosome assay, human lymphocytes | Not clastogenic |
| VII. <i>T. reesei</i> (homologous rDNA) | Glucoamylase | Acute dermal irritation study in rabbits (sequential approach) | Not a skin irritant |
| | | Bacterial reverse mutation assay | Not mutagenic |
| | | <i>In vitro</i> chromosome assay, human lymphocytes | Not clastogenic |
| | | 91-day subchronic study, rats | No adverse effects detected |

All enzyme preparations were found to be non-toxic, non-mutagenic and not clastogenic.

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I. Cellulase from *T. reesei* A83 (traditionally modified)

A. Pathogenicity study in rats

This study was conducted in compliance with the Good Laboratory Practice Standards as published by the OECD Guidelines, the US FDA and the US EPA. In this investigation, *Trichoderma reesei* strain A83 was administered as a single intraperitoneal dose of 2.2×10^7 cfu to 15 male and 15 female Sprague Dawley rats. Five additional animals per sex were used as the untreated control group. Groups of animals were killed on Days 4, 8, 15, 22 and 32 for microbial enumeration and necropsy observations (BioReliance, 1998).

No deaths occurred and no adverse clinical observations were noted. No treatment related differences in body weights or body weight changes were observed at any time point, when compared to controls. No abnormal findings were noted at necropsy. By Day 15, *T. reesei* was detected in only the spleen and liver of both males and females and, by Day 22, in only the spleen of both male and female rats. Total clearance of the test microbe from the spleen was achieved by Day 32. Under the conditions of this investigation, *T. reesei* strain A83 was not found to be toxic or pathogenic in rats.

B. 91-day subchronic feeding study in rats

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of 20 male and female CD rats were fed with 0 (control), 1, 2, or 5% w/w of the test material in the diet for 13 consecutive weeks (IRDC, 1990).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* cellulase. Based upon these findings, it was concluded that the treatment of male and female rats with cellulase from *T. reesei* did not result in toxicity up to and including a dose level of 5% w/w in the diet. A NOEL (No Observed Effect Level) was established at 5% in the diet corresponding to 3.35 and 4.05 grams of *T. reesei* cellulase/kg body weight/day for male and female rats, respectively.

C. Bacterial reverse mutation assay (Ames assay)

The test material, a cellulase from *T. reesei* A83 was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvrA* in the presence and absence of metabolic activation (BioReliance, 2002). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, eight dose levels ranging from 2.5 to 5,000 $\mu\text{g}/\text{plate}$ were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels ranging from 75 to 5,000 $\mu\text{g}/\text{plate}$ were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this assay, *T. reesei* cellulase was not a mutagen.

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D. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

The test material, a cellulase from *T. reesei* A83 was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation (BioReliance, 2002). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels of 625, 1250, 2500 and 5000 µg/ml were selected for the chromosome aberration assay. In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of this investigation, cellulase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

II. **High pI Xylanase from *T. reesei* (homologous rDNA)**

A. 91-day subchronic feeding study in rats

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of 10 male and female CD rats were fed with 0 (control), 10000, 20000, or 50000 ppm (expressed in terms of total organic solids) of the test material in the diet for 13 consecutive weeks (Pharmaco LSR, 1995).

Two high dose rats (1 male and 1 female) died but the deaths were not attributed to be treatment related. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* High pI xylanase. Based upon these findings, it was concluded that the treatment of male and female rats with High pI xylanase from *T. reesei* did not result in toxicity up to and including a dose level of 50000 ppm (expressed in terms of TOS) in the diet. A NOEL (No Observed Effect Level) was established at 50000 ppm in the diet.

B. Bacterial reverse mutation assay (Ames assay)

The test material, high pI xylanase from *T. reesei* was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvrA* in the presence and absence of metabolic activation (Corning Hazleton, 1995). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, various dose levels ranging from 0 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In

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the confirmatory assay, dose levels up to 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this assay, high pI xylanase from *T. reesei* was not a mutagen.

C. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

The test material, high pI xylanase from *T. reesei* A83 was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation (Corning Hazleton, 1995). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to various concentrations of the test material ranging from 0.5 to 5000 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 5000 µg/ml were selected for the chromosome aberration assay. In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of this investigation, high pI xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

III. **Low pI Xylanase from *T. reesei* (homologous rDNA)**

A. 91-day subchronic oral study in rats

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of male and female rats were treated orally with 0 (control), 750, 1500 or 3000 mg/kg/day of the test material for 13 consecutive weeks (BioResearch, 1997).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with Low pI xylanase from *T. reesei*. Based upon these findings, it was concluded that the treatment of male and female rats with low pI xylanase from *T. reesei* did not result in toxicity up to and including a dose level of 3000 mg/kg/day. A NOEL (No Observed Effect Level) was established at 3000 mg/kg/day for male and female rats, respectively.

B. Bacterial reverse mutation assay (Ames assay)

The test material, low pI xylanase from *T. reesei*, was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2

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uvrA in the presence and absence of metabolic activation (BioReliance, 1997). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, eight dose levels ranging from 0 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels up to 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this assay, low pI xylanase from *T. reesei* was not a mutagen up to the maximum recommended dose level of 5,000 µg/plate in both the presence and absence of metabolic activation.

C. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

The test material, low pI xylanase from *T. reesei* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation (BioReliance, 1997). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 5000 µg/ml were selected for the chromosome aberration assay. In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted in the test material treated cells. Under the conditions of this investigation, low pI xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

IV. Endoglucanase III from *T. reesei* (homologous rDNA)

A. 28-day oral study in rats

This study was conducted in accordance with OECD Guideline 408 in rats. Endoglucanase III (EG III) was given by gavage to groups of male and female Charles River rats at 0 (deionized water), 40, 200 or 1000 mg/kg/day for 28 consecutive days (Pharmakon, 1995).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* EG III. Based upon these findings, it was concluded that the treatment of male and female rats with EG III did not result in toxicity and a NOEL (No Observed Effect Level) was established at 1000 mg/kg/day.

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B. Bacterial reverse mutation assay (Ames assay)

The test article, EG III, was examined for mutagenic activity in 5 strains of *Salmonella typhimurium* and *Escherichia coli* strain WP2uvrA in both the presence and absence of metabolic activation (Hazleton, 1995). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, various dose levels ranging from 2.5 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels of 0, 333, 667, 1,000, 3,330, or 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this investigation, EG III was not mutagenic in the bacterial reverse mutation assay using *Salmonella* and *E. coli*.

V. Endoglucanase I from *T. reesei* (homologous rDNA)

A. Pathogenicity study in rats

This study was conducted in accordance with the US. EPA Microbial Pesticide Test Guideline OPPTS 885.3200 and the US. EPA Good Laboratory Practice Standards (40CFR.160). In this investigation, the pathogenicity potential of *T. reesei* strain EG1-EP9 was tested in male and female CD rats following an acute intraperitoneal injection of 5.6×10^6 colony forming units (cfu). Groups of animals of both sexes were sacrificed on Days 0, 7, 21, and 35 after injection of the test substance for microbial enumeration. The results were compared to those obtained from heat-killed test substance group, naïve control group and shelf control group (IITRI, 2000).

No deaths occurred and no adverse clinical observations were noted. The major necropsy findings were enlarged spleens noted in treated females on Days 7 and 21 and in treated males on Day 21. On Day 7, a significant decrease in relative liver weight was noted in treated males but was no longer observed on subsequent necropsy days. A statistical difference in body weight was noted in treated males between Days 0-7 but all animals showed complete recovery thereafter. By Day 21, *T. reesei* was detected in the peritoneal lavage fluid and masses of treated male rats and from the liver and peritoneal masses of treated female rats. By Day 35, the test substance was detected only from the peritoneal masses. Complete clearance was estimated to be 67 and 43 days for male and female rats, respectively. Under the conditions of this investigation, *T. reesei* strain EG1-EP9 was not found to be toxic or pathogenic in rats.

B. 14-day oral feeding study in rats

This study was conducted in accordance with OECD Guideline 408 in rats. Endoglucanase I (EGI) was administered orally by gavage to groups of male and female Charles River CrICD[®] BR rats at a dosage volume of 10 ml/kg body weight for 14 days at 0 (deionized water), 40, 200 or 1000 mg/kg/day for 28 consecutive days (IRDC, 1993). There were no adverse effects on any parameter monitored in this study for rats treated with EGI. It was concluded that the treatment of male and female rats with EGI enzyme preparation did not produce evidence of systemic toxicity in any of the parameters that were monitored.

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C. 91-day subchronic oral study in rats

A 13-week oral gavage study with endoglucanase from *T. reesei* was conducted in accordance with OECD Guideline 408 in CD rats. Groups of ten male and females rats each were exposed to 250, 500 or 1000 mg/kg/day of the test material by gavage in a constant volume of 5 ml/kg/day. Control animals received deionized water following the same treatment regimen (MDS Pharma, 2002).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with endoglucanase from *T. reesei*. There were no differences in behavioral test (open field) and functional tests (gripping reflex, startle reflex) conducted at study termination between the control and treated groups. Based upon these findings, it was concluded that the treatment of male and female rats with EGI from *T. reesei* did not result in toxicity up to and including a dose level of 1000 mg/kg/day. A NOEL (No Observed Effect Level) was established at 1,000 mg/kg/day.

D. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes\

Endoglucanase I (EGI) from *T. reesei* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation (BioReliance, 2002). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 $\mu\text{g/ml}$ as well as solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels of 625, 1250, 2500 and 5000 $\mu\text{g/ml}$ were selected for the chromosome aberration assay.

In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. In the non-activated 4 hour exposure group, mitotic inhibition was 10% relative to the solvent control at the 5,000 $\mu\text{g/ml}$ dose level. A mitotic inhibition of 7% was also noted at the 5,000 $\mu\text{g/ml}$ dose level in the activated assay. However, the percentage of HPBL cells with structural and numerical aberrations in both 5,000 $\mu\text{g/ml}$ groups (with and without metabolic activation) was not significantly increased above that of the solvent control. Under the conditions of this investigation, EGI was negative for the induction of structural and numerical chromosome aberrations in both the presence and absence of metabolic activation.

VI. Xylanase from *T. reesei* (homologous rDNA)

A. 91-day subchronic oral study in rats

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A 13-week oral gavage study with xylanase from *T. reesei* was conducted in accordance with OECD Guideline 408 in CD rats. Groups of ten male and females rats each were exposed to 5000, 12500 or 37500 RBB U/kg/day of the test material by gavage in a constant volume of 5 ml/kg/day. Control animals received deionized water following the same treatment regimen (MDS Pharma, 2002).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with xylanase from *T. reesei*. There were no differences in behavioral test (open field) and functional tests (gripping reflex, startle reflex) conducted at study termination between the control and treated groups. Based upon these findings, it was concluded that the treatment of male and female rats with *T. reesei* xylanase did not result in toxicity up to and including a dose level of 37500 RBB U/kg/day. A NOEL (No Observed Effect Level) was established at 37500 RBB U/kg/day.

B. Bacterial reverse mutation assay (Ames assay)

The test material, xylanase *T. reesei* (homologous rDNA) was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvrA* in the presence and absence of metabolic activation (MDS Pharma, 2002). The assay was conducted in accordance with OECD Guideline 471 using the plate incorporation method (first assay) and treat and plate method (second assay).

In the first assay, dose levels ranging from 52 to 5,000 µg/plate were used and no treatment-related positive mutagenic response was observed at any dose level in any of the exposure groups. In the second assay, dose levels ranging from 492 to 5,000 µg/plate were used. No precipitate or cytotoxicity was noted. Random changes in the number of revertants were noted in TA 98, TA 100 and TA 1535. However, in the absence of a dose response relationship, these changes were considered as spontaneous variations. Under the conditions of this assay, xylanase from *T. reesei* was not a mutagen in both the presence and absence of metabolic activation.

C. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

The test material, a xylanase from *T. reesei* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation (MDS Pharma, 2003). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to various concentrations of the test material ranging from 0.0 to 3600 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 3600 µg/ml were selected for the chromosome aberration assay. In the chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4

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hours in the activated test system. All cells were harvested 20 hours after treatment. Two hours prior to harvest, Demecolcine (0.1 $\mu\text{g/ml}$) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of this investigation, xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

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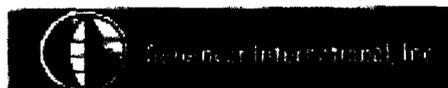
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Appendix 4 – Certificate of Analysis of Tox Lot

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CERTIFICATE OF ANALYSIS

Name of Test Article: ACID FUNGAL PROTEASE
Production/Strain Name: *Trichoderma reesei* AFP 55.3
Production Site: Jamsankoski, Finland

Designation of Lot Tested: AFP501K1
Description: Clear brown liquid

All of the analytical studies listed below were conducted in accordance with GLP regulations and ISO 9002 standards.

Results:

1. **Activity:** 2799 SAPU/g
2. **Total and TCA Protein**
TCA Proteins: 78.6 mg/ml
Total Proteins: 124.7 mg/ml
% Total Organic Solids: 14.29 %
(100% - moisture% - ash%)
3. **Specific gravity:** 1.042 g/ml
4. **pH:** 3.44
5. **Inorganic materials**
% Ash: 0.17 %
% moisture: 85.54 %
6. **Microbial analysis:**
Microbial analysis conducted by GCOR, Rochester, NY

| <u>Analysis</u> | <u>Results</u> |
|----------------------------|----------------|
| Total viable count | < 1CFU/ml |
| Coliform | < 1CFU/ml |
| E. Coli | negative/25 ml |
| Salmonella | negative/25 ml |
| Staphylococcus aureus | < 1 CFU/ml |
| Production strain | negative |
| Anaerobic sulfite reducers | negative |
| Antibiotic activity assay | negative |

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7. Mycotoxin analysis: Not applicable

8. Heavy metals analysis:
 Conducted at Silliker Laboratories

| <u>Analysis</u> | <u>Results</u> |
|--------------------|----------------|
| Heavy metals as Pb | < 30 ppm |
| Arsenic | < 0.83 ppm |
| Lead | < 0.42 ppm |
| Mercury | < 0.05 ppm |
| Cadmium | < 0.17 ppm |

9. Stability Data

| <u>Sample ID</u> | <u>Dilution</u> | <u>T = 0</u> | <u>T = 5 hours</u> | <u>% of T = 0</u> |
|------------------|-----------------|--------------|--------------------|-------------------|
| AFP501K1 | straight | 2732 | 2809 | 103 |
| AFP501K1 | 1/2 | 2806 | 2897 | 103 |
| AFP501K1 | 1/4 | 2723 | 2863 | 105 |

Refrigerator (4C) Stability

| <u>Sample ID</u> | <u>T = 0</u> | <u>T = 7 days</u> | <u>% of T = 0</u> |
|------------------|--------------|-------------------|-------------------|
| AFP501K1 | 2732 | 2565 | 94 |

Freezer Stability

| <u>Sample ID</u> | <u>T = 0</u> | <u>T = 130 days</u> | <u>% of T = 0</u> |
|------------------|--------------|---------------------|-------------------|
| AFP501K1 | 2732 | 2685 | 98 |

(b) (6) [Redacted]

Christine Rechichi
 Corporate BioAnalytical Representative

3/14/06
 Date

(b) (6) [Redacted]

Aino Kivistinen
 Järvenpää, Finland

14.3.2006
 Date

(b) (6) [Redacted]

Guang Q. Bai
 Study Sponsor Representative

March 14, 2006
 Date

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Appendix 5 -Analysis of Safety Based on Pariza/Johnson Decision Tree

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations (2001). These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that this Acid Fungal Protease enzyme preparation is "ACCEPTED" as safe for its intended use.

- 1. Is the production strain genetically modified?** Yes, go to 2.
- 2. Is the production strain modified using rDNA techniques?** Yes. Go to 3a.
- 3a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?** Yes. Go to 3c. Aspergillopepsin I has been used for years in food processing. The *Trichoderma reesei* aspergillopepsin I is new as an isolate in food processing, but has been present at lower amounts in other enzyme preparations from this host.
- 3c. Is the test article free of transferable antibiotic resistance gene DNA?** Yes, no antibiotic resistance genes were used in the construction. 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, inserted DNA is well characterized and free of unsafe attributes, but complete characterization of the location of all insertions is not possible. Go to 4.
- 4. Is the introduced DNA randomly integrated into the chromosome?** 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?** In general, Yes except as noted in 4. Go to 6;
- 6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes, *T. reesei* safety as a production host and methods of modification are well documented and their safety has been confirmed through toxicology testing; Genencor has conducted toxicology testing on 8 different products derived from this lineage, including this one, and all were determined to be safe for their intended food uses; Accept.

Conclusion: Article is accepted

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Appendix 6 – GRAS Panel Report

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**EXPERT PANEL REPORT OF THE GENERALLY RECOGNIZED AS SAFE (GRAS)
STATUS OF ACID FUNGAL PROTEASE ENZYME PREPARATION FOR USE IN FOODS**

21 JULY 2006

INTRODUCTION

Danisco USA Inc assembled an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, to determine the Generally Recognized As Safe (GRAS) status of Acid Fungal Protease (AFP) Preparation under the intended conditions of use as a protease processing aid in grain processing (corn steeping), alcoholic beverages manufacture, noncitrus juice manufacture (i.e., apple juice), and degumming of membranes during orange juice manufacture, and potentially other similar processes.

The Panel consisted of Professors Joseph F. Borzelleca, Ph.D. (Medical College of Virginia), and Michael W. Pariza (University of Wisconsin, Madison) and Herbert Blumenthal, Ph.D (formerly of the USFDA CFSAN).

Danisco conducted a comprehensive search of the published literature through April 2006 on the production organism, *Trichoderma reesei* (formerly, *T. longibrachiatum*). The Panel, independently and collectively, critically examined a comprehensive package of publicly available scientific information and data compiled and unpublished information prepared by Danisco USA Inc. In addition, the Panel evaluated other information deemed appropriate or necessary, including data and information pertaining to the method of manufacture and product specifications including batch analyses, intended use-levels in specified food products, consumption estimates for all intended uses (EDI), and safety information.

Following independent, critical evaluation of the aforementioned information, the Panel conferred by conference call and unanimously concluded that under the conditions of intended use in traditional foods described herein, AFP is GRAS based on scientific procedures.

A summary of the basis for the Panel's conclusion appears below.

COMPOSITION, MANUFACTURING AND SPECIFICATIONS

AFP Enzyme Preparation contains the protease, aspergillopepsin 1 (CAS No. 9025-49-4 and IUB No. 3.4.23.18), derived from pure culture fermentations of a selected strain of *Trichoderma reesei*.

The manufacturing process involves inoculation of sterilized fermentation media, containing glucose media with *T. reesei* strain NSP24 #22-1. The fermentation process is maintained under controlled pH and temperature conditions until optimum acid protease production is achieved. The acid protease produced is then separated from the biomass via rotary vacuum drum filtration, concentrated via ultrafiltration, formulated with glycerol, sodium sulphate and sodium benzoate and polish filtered. All

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ingredients meet specifications of the Joint Expert Committee for Food Additives (JECFA) or Food Chemicals Codex (FCC), or are considered appropriate for use in foods. Fungal Acid Protease Enzyme Preparation meets the standards for enzyme preparations of JECFA and FCC.

The chemical and microbiological specifications for AFP are presented in Attachment 1 along with analyses of three representative lots demonstrating compliance with established chemical and microbiological specifications.

Stability under various storage conditions has been evaluated. AFP Enzyme Preparation remains stable for extended periods if kept refrigerated.

INTENDED USE AND ESTIMATED INTAKE

Current Protease Use in Foods

AFP Enzyme Preparation from *Aspergillus oryzae* has been Generally Recognized as Safe (GRAS) for use in food production and sold in Japan, the US and elsewhere for many years. This protease has been used in potable alcohol production and several other smaller applications. Other proteases currently in use are derived from other microorganisms, most notably *Aspergillus oryzae*, *A. niger* and *Bacillus subtilis* and are either recognized as GRAS through the GRAS Notice process (see GRSN 89 and 90 for the *Aspergillus* proteases) or are affirmed as GRAS (see 21 CFR § 184.1150 for *B. subtilis* proteases). They have been used in dehazing apple juice and degumming of membranes in orange juice manufacturing, among many other uses. The protease from *T. reesei* has been in commerce as a minor component of other *T. reesei* derived enzymes, but has not been produced at commercially viable levels until the development of this production organism and process.

Proposed New Uses in Foods

The proposed new uses of AFP Enzyme Preparation are as follows:

- Corn steeping,
- Potable alcohol (replacement for *A. oryzae* AFP),
- Dehazing apple juice (replacement for other proteases), and
- Degumming of membranes in orange juice manufacturing (replacement for other proteases).

Application levels

AFP will be added to the steeping process for carbohydrate processing at 0.009 mg/g corn; to apple juice at 0.1% or 1 mg/g; and used in potable alcohol production at 0.160 mg/ ml pure alcohol.

ASSESSMENT OF SAFETY

According to Pariza and Johnson (2001), “the safety of the production strain remains the primary consideration in evaluating enzyme safety, in particular, the *toxigenic potential* of the production strain.” Accordingly, Danisco has given this consideration primary importance. The safety of the enzyme preparation is supported by the safety of the donor organism and the production process. In addition, a series of unpublished toxicology studies support this conclusion.

Safety of the Production Strain

T. reesei was first isolated from nature in 1944. The original isolate, QM 6a, and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. In the 1980s, it was suggested that *T. reesei* be placed into synonymy with *T. longibrachiatum* (Bissett 1984). However, there subsequently appeared evidence that the two species were not identical (Meyer et al. 1992). The proposal by Kuhls et al. (1996) that *T. reesei* was a clonal derivative of *Hypocrea jecorina* is being accepted by more and more people in the scientific community, and the US National Center for (Biotechnology Information (NCBI) refers to *T. reesei* as the anamorph of *H. jecorina* and no longer includes it in the genus *Trichoderma*. Therefore, *Trichoderma reesei*, *Trichoderma longibrachiatum*, and *Hypocrea jecorina* may appear in different documents but they refer to essentially the same microorganism species.

A review of the literature search on the organism (1972-2006) uncovered no reports that implicate *T. reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human and animals. The species is not present on the list of pathogens used by the EU (Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U. S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees. BSL1 microorganisms are not known to cause diseases in healthy adult humans.

Two authors reported the isolation from *T. reesei* strain QM 9414 of a peptaibol compound that exhibited antibiotic activity (Brukner and Graf 1983). Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains (Solfrizzo et al. 1994). However, peptaibols' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those in typical enzyme manufacturing. Strain QM 9414 and its derivatives, have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by the FCC.

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen et al. (1994) and Blumenthal (2004). The organism is considered non-pathogenic for human and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food and feed industries. It is listed as a safe pro-

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duction organism for cellulases in the Pariza and Johnson paper (Pariza and Johnson 2001) and various cellulases from *T. reesei* have been approved as commercial enzyme products internationally, for example, in Canada (Food and Drugs Act Division 16, Table V, Food Additives That May Be Used As Enzymes), the United States (FDA 1999), France, Australia/New Zealand, China (MOH 1996), and Japan.

The production organism of the AFP preparation, the subject of this submission, is *T. reesei* strain NSP24 #22-1. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to enhance AFP production levels. RL-P37, a commercial production strain, is derived, as a result of several classical mutagenesis steps, from the well-known wild-

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type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a.

From the information reviewed, it is concluded that the organism *T. reesei* provides no specific risks to human health and is safe to use as the production organism of aspergillopepsin I.

Thus it is thus concluded that the strain is non-pathogenic and non-toxigenic.

Safety of the Donor Organism

In this case, the donor organism is the parent strain of the host strain, *T. reesei* QM6a, and it is therefore included in the safety assessment of the host strain.

Safety of the Manufacturing Process

The manufacturing process for the production of AFP will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration and formulation, resulting in a liquid aspergillopepsin 1 enzyme preparation. The process, described in Appendix 4, is conducted in accordance with food good manufacturing practice (GMP) as set forth in 21 CFR Part 110. The resultant product meets the general requirements for enzyme preparations of the Food Chemicals Codex, Fifth Edition (FCC V).

Safety of the Protease

AFP Enzyme Preparation from *Aspergillus oryzae* has been Generally Recognized as Safe (GRAS) by self-determination and used in food production in Japan, the US and elsewhere for many years. Other proteases currently in use are derived from other microorganisms, most notably *Aspergillus oryzae*, *A. niger* and *Bacillus subtilis* and are either recognized as GRAS through the GRAS Notice process (see GRSN 89 and 90 for the *Aspergillus* proteases) or are affirmed as GRAS (see 21 CFR § 184.1150). The protease from *T. reesei* has been in commerce as a minor component of other *T. reesei* derived enzymes, but has not been produced at commercially viable levels until the development of this production organism and process. The safety of this AFP has been established using the Pariza and Johnson evaluation scheme (Pariza and Johnson, 2001) and confirmed through unpublished toxicological testing as described below.

Toxicological Studies

Danisco has conducted six studies on the AFP enzyme preparation, including 1) acute oral toxicity; 2) 91-day, repeated dose feeding study (gavage) in rats; 3) a Chromosomal Aberration Assay with AFP using Human Peripheral Lymphocytes; 4) a Bacterial Reverse Mutation Assay 5) ocular irritation; and 6) skin irritation. Results:

1) Acute oral toxicity in rats

No mortality was recorded in this study. There were no treatment related effects noted throughout the 14-day observation period.

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Using the GHS classification system (December 2001), AFP can be classified as non-hazardous (oral LD₅₀ > 2000 mg/kg bw).

2) A 13-week Oral (Gavage) Toxicity Study in Rats

There were no treatment-related deaths in this study. No clinical signs were seen that could be considered to be treatment related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, body weights, body weight gains, hematology, clinical chemistry, and ophthalmologic examinations. At study termination, in the males, the absolute and relative liver weights were statistically significantly increased and the relative testes weight was statistically significantly decreased compared to the concurrent control group. However, there were no treatment-related macroscopic and histopathologic changes. In the functional observation battery testing, there were no statistically significant changes noted in treated groups.

Although statistically significant variations in liver and testes weights were noted in high dose males, these weight variations were still within the 95% confidence interval of historical control data collected at LAB Scantox (on file). Further, in the absence of corresponding histopathologic changes, these weight variations were considered to be of no toxicological significance. Under the conditions of this assay, the NOAEL (No Observed Adverse Effect Level) is established at the highest dose tested (31.25 mg total protein/kg bw/day or 35.81 mg TOS/kg bw/day).

3) *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

In the preliminary dose-range assay, cytotoxicity was observed at 5000 µg/ml in both non-activated and activated cultures. Based on these results, the following doses were used:

First main test, without S-9 mix: 625, 1250 and 2500 µg/ml

First main test, with S-9 mix: 1250, 2500 and 5000 µg/ml

Second main test, without S-9 mix: 313, 625 and 1250 µg/ml

Second main test, with S-9 mix: 1250, 2500 and 5000 µg/ml

In the absence of S-9 mix, AFP caused reductions in mean mitotic index of 52 and 53% at the highest concentration tested in the first and second main test, respectively, and this level of toxicity meets the requirements of OECD 473 guideline for the highest concentration to be scored for aberrations (> 50% reduction in mitotic index). In the presence of S-9 mix, the 5000 µg/ml dose level is the maximum required by OECD guideline.

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with AFP concentrate both in the presence and absence of metabolic activation. Significant increases in aberrant metaphases were demonstrated with the positive controls.

Under the conditions of this test, AFP concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation.

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4) Bacterial Reverse Mutation Assay – Ames assay

In the preliminary toxicity test, AFP was not toxic to the bacteria at any dose level tested. Therefore, the highest dose level selected for the main assays was 5000 µg/plate, which is the maximum required by OECD guideline.

In the first main test, AFP concentrate was not toxic to tester strains at any dose level, either in the presence or absence of S-9 mix. In the second main test, a statistically significant reduction in the number of revertant colonies was observed in strain TA 1535 with S-9 mix, but this effect was not dose related.

Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

Although a statistically significant reduction in the number of revertant colonies was noted in TA 1535 with S-9 mix, this is not considered to be biologically significant in the absence of a dose-related effect. Some variations in revertant colonies were noted but the variations were not reproducible between the three replicate plates and none of these variations meet the positive criteria recognized by regulatory agencies worldwide. Under the conditions of this assay, AFP has not shown any evidence of mutagenic activity in the Ames assay.

5) Ocular Irritation in the Rabbit

In the initial study, slight conjunctivitis was observed at the 1-hour observation period with clearing by 24 hours. In the confirmatory assay, no irritation was observed.

The primary eye irritation score was 0.0.

6) Acute dermal irritation study in rabbits (sequential approach)

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No reactions were noted at any test site in both preliminary and confirmatory assays.

The mean score for skin edema and erythema was 0.0. According to the Directive of the Commission 93/21/EEC of April 27, 1993, AFP is not a skin irritant.

Assessment

The NOAEL

In the 90-day oral (gavage) study in rats (Scantox No. 60623), a NOAEL was established at 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day). The study was conducted

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in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice and was designed based on OECD guideline No. 408. Since human exposure to AFP is through oral ingestion, selection of this NOAEL is thus appropriate.

Human Exposure

As noted above, AFP is expected to be removed during the subsequent production processes for all applications. The enzyme is added to corn steeping at the very beginning of the carbohydrate process to separate the starch for further processing. After that, it goes through several steps of liquefaction, saccharification, etc. so no carryover of the AFP is expected.

Our exposure estimate was based on several conservative assumptions that would encompass any variations in exposure among population subgroups. In our estimation of the daily consumption of AFP, the exposure was based on three major commodities (cumulative exposure): corn sweeteners, non-citrus juice and potable alcohol. The consumption data used was based on the USDA-NASS Agricultural Statistics 2005 and the maximum daily consumption for each commodity was used to calculate the maximum cumulative exposure.

Estimation of daily consumption of AFP was not conducted for women of childbearing age, children or various ethnic groups since there is no reason to believe that these sub-groups are more susceptible to AFP or consume more AFP than an adult. Since there is no information to suggest that AFP is either a developmental toxicant or reproductive toxicant, estimation of risk for women and children would be unwarranted. Based on the potential uses of AFP, only an adult would be exposed to all three commodities and, therefore the maximum daily consumption from an adult would represent the worst-case scenario. The maximum daily consumption of AFP from syrups, potable alcohol, and non-citrus juice is 1.36-mg/kg bw/day under the scenario that (1) all above commodities are treated with AFP and (2) 100% of AFP remains in the product after processing. In reality, it is expected that residues of a processing aid in the final products would be negligible after processing.

Estimation of daily consumption of AFP from its potential uses in three major commodities (consumption data based on USDA-NASS Agricultural Statistics 2005; www.usda.gov/nass/pubs/agr05/).

1. Corn sweeteners

- a. Annual consumption: 79.2 lbs or 35.95 kg (1 lb = 0.454 kg)
- b. Daily consumption: 98.4 g or 1.64 g/kg bw (human = 60 kg)
- c. Dose of AFP (mg of protein) used/corn: 0.009 mg/g corn
- d. Daily consumption of AFP: 0.009 mg X 1.64 g/kg bw = 0.015 mg/kg bw

2. Noncitrus juice (i.e., Apple juice)

- a. Annual consumption: 66.8 lbs or 30.32 kg
- b. Daily consumption: 83 grams or 1.38 g/kg bw (human = 60 kg)
- c. Dose of AFP used/juice 0.1% or 1 mg/g
- d. Daily consumption of AFP 1 mg X 1.38 g/kg bw = 1.38 mg/kg bw

3. Potable Alcohol (pure)

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- a. Annual consumption: 10 liters
- b. Daily consumption of pure alcohol: 27.4 ml or 0.456 ml/kg bw
- c. Dose of AFP used: 0.160 mg/ ml pure alcohol
- c. Daily consumption of AFP 0.160 mg X 0.456 ml/kg bw = 0.073 mg/kg bw

Maximum intake of AFP from all commodities (cumulative)
 0.015 mg/kg bw + 1.38 mg/kg bw + 0.073 mg/kg bw = 1.47 mg/kg bw/day

The maximum daily consumption of AFP from corn sweeteners, potable alcohol, and noncitrus juice is 1.47 mg/kg bw/day under the scenario that (1) all above commodities are treated with AFP and (2) 100% of AFP remains in the product after processing. In reality, it is expected that residues of the enzyme processing aid in the final products would be negligible after processing. It is also assumed that when used to degum membranes during orange juice processing, the enzyme is washed away and therefore not present at all in the juice.

For risk analysis purpose a 10% residue is used to represent a worst-case scenario.

Maximum Daily Intake of AFP = 0.147 mg/kg bw

Based on the results from the 90-day oral (gavage) feeding study cited above

Margin of safety = $\frac{\text{No observed adverse effect level}}{\text{Daily exposure}}$

Margin of safety = $\frac{31.25 \text{ mg/kg bw/day}}{0.147 \text{ mg/kg bw/day}} = 212$

SUMMARY

The safety of AFP as a food processing aid in corn syrup, noncitrus juice and potable alcohol is assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. AFP is not an eye and skin irritant, is not mutagenic in the Ames assay and is not clastogenic in mammalian system (human peripheral lymphocytes). Daily administration of AFP for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day).

Based on a worst-case scenario that a person is consuming corn syrup, noncitrus juice and potable alcohol treated with AFP (i.e., cumulative risk), this NOAEL still offers at least a 212X fold margin of safety.

AFP is derived from a selected non-pathogenic, non-toxigenic strain of *Trichoderma reesei* (formerly *Trichoderma longibrachiatum*), which is genetically modified to over express a native *T. reesei* protease enzyme, aspergillopepsin 1. AFP will replace other proteases currently marketed for the intended uses as well as some of the SO₂ currently used in corn steeping. Other proteases currently in use are derived from other microorganisms, most notably *Aspergillus oryzae*, *A. niger* and *Bacillus sub-*

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tilis. The protease from *T. reesei* has been in commerce as a minor component of other *T. reesei* derived enzymes, but has not been produced at commercially viable levels until the development of this production organism and process.

Initial use of the enzyme product is intended for grain processing (corn steeping), alcoholic beverage manufacture, and noncitrus juice manufacture (i.e., apple juice) and degumming of membranes during orange juice manufacture, but other applications may be of interest as well. In all of these applications, AFP will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food. It is assumed that the enzyme used for degumming of membranes will be washed away after the degumming and will not be present in the orange juice; therefore, this application is not considered in the consumption and human safety assessment. Pursuant to 21 CFR 170.30 (i) that establishes a manufacturer's responsibility to independently establish that a use of a product not stated in an existing GRAS affirmation is GRAS, Danisco independently evaluated the safety of *T. reesei* AFP for such uses.

Given the world-wide use of enzymes in food processing and recent scientific advances, primarily in the fields of molecular biology and protein engineering, guidelines for current and future food safety evaluations of enzyme preparations for use in food have been published (Pariza and Johnson, 2001). These guidelines provide a peer-reviewed decision tree process for the determination of the safety of enzyme preparations used in food.

To assess the safety of the *T. reesei* AFP for use in grain processing (corn steeping), alcoholic beverages manufacture, and noncitrus juice manufacture (i.e., apple juice), Danisco vigorously applied the criteria identified in the guidelines (above) utilizing enzyme safety data, the safe history of use of other enzyme preparations from *T. reesei* and of other proteases in food, the safe history of use of the production organism for the production of other enzymes used in food, and a comprehensive survey of the scientific literature. Based on these sources pursuant to FDA proposed regulation, proposed 21 CFR 170.36, Danisco has determined based on scientific procedures including analysis of publicly available information, that the AFP preparation derived from *T. reesei* is safe and suitable for use in grain processing, alcoholic beverages manufacture, and noncitrus juice manufacture (i.e., apple juice). AFP will be added to the steeping process for carbohydrate processing at 0.009 mg/g corn; to apple juice at 0.1% or 1 mg/g; and used in potable alcohol production at 0.160 mg/ml pure alcohol.

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CONCLUSION

We, the Expert Panel, have independently and collectively, critically evaluated the data and information summarized above and conclude that acid fungal protease (AFP) from *T. reesei* strain NSP24 #22-1 meeting appropriate food-grade specifications and produced and used consistent with current Good Manufacturing Practice (cGMP) is Generally Recognized as Safe (GRAS) based on scientific procedures under the proposed conditions of intended use as a processing aid in foods.

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Scantox Study No. 60623, A 13-week oral (gavage) toxicity study in rats with Acid Fungal Protease (AFP) Concentrate, April 2006.

Scantox Study No. 60624, Ames Test with AFP, 2006.

Scantox Study No. 60625, *In vitro* mammalian chromosome aberration test performed with human lymphocytes, AFP, 2006.

Scantox Study No. 60628, Acute dermal irritation study in the rabbit with AFP, 2006.

Scantox Study No. 60627, Ocular irritation test in the rabbit with AFP, 2006.

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Attachment 1 - Specifications

| Property | Specification | Lot No. | Lot No. | Lot No. |
|---|------------------|----------------|---------------|---------------|
| | | AFP501K1 | 301-05299-189 | 301-06023-020 |
| Protease Activity (SAPU/g)¹ | >1000 SAPU/g | 2799 | 1111 | 1060 |
| PH² | 4.0-5.0 | 3.44 | 3.84 | 3.94 |
| Specific gravity | determine | 1.042 | 1.194 | 1.189 |
| TVC (cfu/g) | < 50,000 | <1 | <1 | 27 |
| Salmonella (per 25g) | Negative | neg | neg | neg |
| E. coli (per 25g) | Negative | neg | neg | neg |
| Coliforms (cfu/g) | < 30 | <1 | <1 | <1 |
| Production strain (cfu/g) | Negative by test | neg | neg | neg |
| Mycotoxin³ | Negative by test | not determined | neg | neg |
| Arsenic | < 3 mg/kg | <0.83ppm | < 3 mg/kg | < 3 mg/kg |
| Cadmium | < 0.5 mg/kg | <0.17ppm | < 0.5 mg/kg | < 0.5 mg/kg |
| Heavy metals as lead | < 30 mg/kg | <30ppm | < 30 mg/kg | < 30 mg/kg |
| Lead | < 5 mg/kg | <0.42ppm | < 5 mg/kg | < 5 mg/kg |
| Mercury | <0.5 mg/kg | <0.05ppm | < 0.5 mg/kg | < 0.5 mg/kg |
| TOS %⁴ | NA | 14.29 | 13.81 | 16.9 |
| Moisture %⁴ | NA | 85.54 | 86.06 | 82.94 |
| Ash %⁴ | NA | 0.17 | 0.13 | 0.16 |

¹ One Spectrophotometric Acid Protease Unit (SAPU/g) is that activity which will liberate 1 micromole of tyrosine equivalents per minute per gram of enzyme product under the conditions of the method.

² pH specification is for final product; pH values given here are for non-formulated concentrate

³ Including Total Aflatoxin, T-2 toxin, Zearalenone, Ochratoxin, Sterigmatocystin

⁴Not product specification, included for comparison to tox lot, AFP501K1; analyzed from non-formulated concentrate

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SUBMISSION END

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