AB Enzymes GmbH – Feldbergstrasse 78 , D-64293 Darmstadt

July 18, 2018

RE: GRAS Notification – Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) AB Enzymes GmbH hereby claims that Serine Endopeptidase (IUBM 3.4.21.65) from a Genetically Modified *Trichoderma reesei* produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:
Proposed 21C.F.R. § 170.36 (c)(i) *The name and address of notifier.*
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt, Germany

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance:*
Serine Endopeptidase (IUBM 3.4.21.65) from a Genetically Modified *Trichoderma reesei*.

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use:*
The Serine Endopeptidase enzyme is to be used as a processing aid for partial or extensive hydrolysis of proteins from both animal and vegetable sources.

Proposed 21C.F.R. § 170.36 (c)(iv) *Basis for GRAS determination:*
This GRAS determination is based upon scientific procedures.

Proposed 21C.F.R. § 170.36 (c)(v) *Availability of information:*
A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times (customary business hours) at a specific address set out in the notice or will be sent to FDA upon request (electronic format or on paper).

§170.225(c)(8) - FOIA (Freedom of Information Act):
Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

AB Enzymes GmbH – Feldbergstrasse 78 , D-64293 Darmstadt

§170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to AB Enzymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

July 18, 2018

Candice Cryne
Regulatory Affairs Manager

Date

AB Enzymes GmbH – Feldbergstrasse 78 , D-64293 Darmstadt

GRAS Notification of a Serine Endopeptidase from a Genetically Modified *Trichoderma reesei*

AB ENZYMES GmbH

July 17, 2018

Contents

1 PART 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATIONS

§170.225(c)(1) – Submission of GRAS notice:
AB Enzymes GmbH hereby claims that Serine Endopeptidase (IUBM 3.4.21.65) from a Genetically Modified *Trichoderma reesei* produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

§170.225(c)(2) -The name and address of the notifier:
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt, Germany

§170.225(c)(3) – Appropriately descriptive term:
Serine Endopeptidase (IUBM 3.4.21.65) from a Genetically Modified *Trichoderma reesei*

§170.225(b) – Trade secret or confidential:
This notification does not contain any trade secret or confidential information.

§170.225(c)(4) – Intended conditions of use:
The Serine Endopeptidase enzyme is to be used as a processing aid for partial or extensive hydrolysis of proteins from both animal and vegetable sources.

§170.225(c)(5) -Statutory basis for GRAS conclusion:
This GRAS determination is based upon scientific procedures.

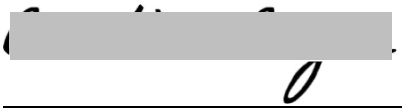
§170.225(c)(6) – Premarket approval:
The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of the intended use.

Proposed 21C.F.R. § 170.36 (c)(v) *Availability of information:*
A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times (customary business hours) at a specific address set out in the notice or will be sent to FDA upon request (electronic format or on paper).

§170.225(c)(8) - FOIA (Freedom of Information Act):
Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to AB Enzymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.



July 17, 2018

Candice Cryne

Regulatory Affairs Manager

2 PART 2 §170.230 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 Identity of the notified substance

The dossier concerns a **Serine Endopeptidase from a genetically modified *Trichoderma reesei*.**

2.1.1 Common name of the enzyme

Name of the enzyme protein: Serine Endopeptidase/serine protease

Synonyms: Serine (S8) peptidase, Thermomycolin, Thermomycolase

2.1.2 Classification of the enzyme

IUBMB #	3.4.21.65
Production Strain	*Trichoderma reesei* RF8963

EC 3. is for Hydrolases;

EC 3.4. is for Peptidases (acting on peptide bonds);

EC 3.4.21. is for Serine endopeptidases;

EC3.4.21.65 is for Thermomycolin.

2.2 Identity of the Source

2.2.1 Recipient Strain

The recipient strain used for the genetic modification is *Trichoderma reesei* mutant strain which is derived from *Trichoderma reesei* RF4847, a classical mutant originating from QM6a. This strain

has been shown to be genetically stable for industrial production.

The *T. reesei* parental strain RF4847 was characterized by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands as *Trichoderma reesei*. It was identified based on the sequences of Internal Transcribed Spacer 1 and 2 and the 5.8S gene and Translation Elongation Factor 1a. *T. reesei* RF4847 was deposited as a CBS culture (safe deposit) as CBS 114041.

Therefore, the recipient can be described as followed:

Genus:	*Trichoderma*
Species:	*Trichoderma reesei*
Subspecies (if appropriate):	not applicable
Commercial name:	Not applicable. The organism is not sold as such.

2.2.2 Donor:

The serine endopeptidase gene described in this application derives from *Malbranchea cinnamomea* ALKO4122. *Malbranchea* species are naturally found from soil and have worldwide distribution. *M. cinnamomea* is a thermophilic fungus and a biosafety level 1 microbe. The genome of *M. cinnamomea* strain FCH 10.5 has been recently published (Granchi et al. 2017).

Genus:	*Malbranchea*
Species:	*Malbranchea cinnamomea*
Subspecies (if appropriate):	not applicable
Commercial name:	Not applicable. The organism is not sold as such.

2.3 Genetic modification

Trichoderma reesei strain RF8963 was constructed for production of *Malbranchea cinnamomea* serine endopeptidase. In constructing the strain RF8963, the expression cassette (serine endopeptidase gene under the control of the *T. reesei* promoter) was introduced into the genome of the *Trichoderma reesei* recipient strain.

The transformation of the recipient *T. reesei* strain with the expression cassette was performed as described in Penttilä et al. (1987) with the modifications described in Karhunen et al. (1993). The transformants were selected according to their ability to grow on acetamidase plates (*amdS* marker gene).

The plasmid vector (pUC19) was only used in constructing the expression cassette but was not introduced into the *T. reesei* recipient strain in fungal transformation.

Expression cassette:

- ***Malbranchea cinnamomea* serine endopeptidase gene:** the serine endopeptidase gene encodes an alkaline S8 serine endopeptidase ("Thermomycolin").
- ***Synthetic amdS* gene and promoter:** synthetic *amdS* gene encodes the amino acid sequence of the *Aspergillus nidulans* acetamidase (Kelly and Hynes 1985; Hynes et al. 1983). The introns have been removed from the original *amdS* gene and selection of restriction sites has been removed by codon changes, to ease the cloning steps. The synthetic *amdS* gene is expressed from the native *A. nidulans amdS* promoter. The native *amdS* terminator is used as a transcription terminator. The encoded acetamidase enables the strain to grow on plates containing acetamide as a sole nitrogen source. This characteristic has been used for selecting the *T. reesei* transformants. Acetamidase has been widely used as a selection marker in fungal transformations for more than 30 years without any disadvantage.
- ***Trichoderma reesei* promoter and terminator:** the *Malbranchea cinnamomea* serine endopeptidase gene is fused to *T. reesei* native promoter. For transcription terminator the native *T. reesei* terminator is used.
- ***Linker*** : synthetic DNA sequence is included directly after the coding and stop sequences of the protease gene. This linker contains *Bam*HI and *Pst*I restriction sites of which the *BamHI* was used in the construction of the expression plasmid.

2.3.1 Stability of the Transformed Genetic Sequence

In practice, the fermentation process always starts from identical replicas of the *T. reesei* RF8963 seed ampoule. Production preserves from the "Master Cell Bank" are used to start the fermentation process.

A Master Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The MCB is prepared from a selected strain. A MCB ampoule is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the MCB ampoule. The accepted MCB ampoule is used as seed material for the inoculum.

Potential changes in the genome of the production strain or rearrangements of the expression cassette(s) could theoretically occur during the production process. Analysis via Southern blot based analysis performed from different independent batches of pilot scale fermentation of RF8963 revealed that the strain stays genetically stable and the expression cassette(s) is (are) stable over necessary time needed for industrial fermentation process of RF8963 production strain.

Additionally, testimony to the stability of the strain is also given by comparable levels of protease activity in many fermentation batches performed for the RF8963 *T. reesei* strain. The activity measurements from parallel fermentations showed that the productivity of the strain remains similar.

The data of the analysis of enzyme activities from preparations from different fermentation batches of the recombinant *T. reesei* RF8963 strain is presented in Appendix #1.

2.3.2 Structure and amount of vector and/or nucleic acid remaining in the GMM

Trichoderma reesei RF8963 strain does not harbor any vector DNA. The expression cassettes used for transformations were cleaved from the pUC19 vector plasmids by restriction enzyme digestions followed by isolation of the expression cassettes from agarose gel.

A Southern blot hybridization experiment using plasmid with the pUC19 vector backbone as a labeled probe and genomic DNA of the production host RF8963 was performed to confirm that no vector DNA is included in the genome of RF8963. It produced negative result (no hybridization), demonstrating that no part of the plasmid vector removed to generate the linear

transforming DNA fragments was introduced into the *Trichoderma* production host.

2.3.3 Demonstration of the absence of the GMM in the product

The down-stream process following the fermentation includes unit operations to separate the production strain. The procedures are executed by trained staff according to documented standard operating procedures complying with the requirements of the quality system.

The RF8963 production strain is recovered from the fermentation broth by a widely used process that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch, using an internal Roal method. This method has been validated in-house. The sensitivity of the method is 1 cfu/20 ml in liquid and 1 cfu/0,2 gram in dried semifinals.

2.3.4 Inactivation of the GMM and evaluation of the presence of remaining physically intact cells

The RF8963 enzyme preparation is free from detectable, viable production organism (Appendix #1). As the absence of the production strain is confirmed for every production batch, no additional information regarding the inactivation of the GMM cells is required.

2.3.5 Information on the possible presence of recombinant DNA

RF8963 enzyme preparation is produced by an aerobic submerged microbial fermentation using a genetically modified *T. reesei* strain. All viable cells of the production strain, RF8963, are removed during the down-stream processing: the fermentation broth is filtered with pressure filters and subsequent sheet filters, concentrated with ultra-filtration, and optionally followed by sheet filtration(s).

After this the final product does not contain any detectable number of fungal colony forming units or recombinant DNA. Three separate food enzyme samples (concentrates from industrial scale production and pilot scale fermentations) were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. No recombinant DNA (recDNA) of the production strain was shown to be present above the detection limits (Appendix #1).

2.3.6 Absence of Antibiotic Genes and Toxic Compounds

As noted above, the transformed DNA does not contain any antibiotic resistance genes. Further, the production of known mycotoxins according to the specifications elaborated by the General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper *(Food and Agriculture Organization of the United Nations 2006)* has been also tested from the fermentation products. Adherence to specifications of microbial counts is routinely analysed. Three production batches produced by the production strain *T. reesei* RF8963 (concentrates) were analyzed and no antibiotic or toxic compounds were detected (Appendix #1*)*.

2.4 ENZYME PRODUCTION PROCESS

2.4.1 Overview

The food enzyme is produced by ROAL Oy[1] by submerged fermentation of *Trichoderma reesei* RF8963 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

The enzyme preparation described herein is produced by controlled fed-batch submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. Finally, measures are taken to comply with cGMPs and HACCP. The manufacturing flow-chart is presented in Appendix #2.

It should be noted that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in a clear majority of cases, the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

[1] See footnote 1

2.4.2 Fermentation

The production of food enzymes from microbial sources follows the process involving fermentation as described below. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. The main fermentation steps are:

- Inoculum
- Seed fermentation
- Main fermentation

2.4.3 Raw materials

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for ROAL OY. The safety is further confirmed by toxicology studies. The raw materials conform to either specifications set out in the Food Chemical Codex, 10th edition, 2016 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. The maximum use levels of antifoam and flocculants are ≤0.15% and ≤1.5% respectively.

2.4.4 Materials used in the fermentation process (inoculum, seed and main fermentation)

- Potable water
- A carbon source
- A nitrogen source
- Salts and minerals
- pH adjustment agents
- Foam control agents

2.4.5 Inoculum

A suspension of a pure culture of RF8963 is aseptically transferred to shake flasks containing fermentation medium.

When a sufficient amount of biomass is obtained the shake flasks cultures are combined to be

used to inoculate the seed fermentor.

2.4.6 Seed fermentation

The inoculum is aseptically transferred to a pilot fermentor and then to the seed fermentor. The fermentations are run at a constant temperature and a fixed pH. At the end of the seed fermentation, the inoculum is aseptically transferred to the main fermentor.

2.4.7 Main fermentation

The fermentation in the main fermentor is run as normal submerged fed-batch fermentation. The content of the seed fermentor is aseptically transferred to the main fermentor containing fermentation medium.

In order to control the growth of the production organism and the enzyme production, the feed-rate of this medium is based upon a predetermined profile or on deviation from defined set points.

The fermentation process is continued for a predetermined time or until laboratory test data show that the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When these conditions are met, the fermentation is completed.

2.4.8 Recovery

The purpose of the recovery process is:

- to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein,
- to concentrate the desired enzyme protein and to improve the ratio enzyme activity/Total Organic Substance (TOS).

During fermentation, the enzyme protein is excreted by the producing microorganism into the fermentation medium. During recovery, the enzyme-containing fermentation medium is separated from the biomass.

This Section first describes the materials used during recovery (downstream processing), followed by a description of the different recovery process steps:

- Pre-treatment
- Primary solid/ liquid separation
- Concentration
- Polish and germ filtration

The nature, number and sequence of the different types of unit operations described below may vary, depending on the specific enzyme production plant.

2.4.9 Materials

Materials used, if necessary, during recovery of the food enzyme include:

- Flocculants
- Filter aids
- pH adjustment agents

Potable water can also be used in addition to the above-mentioned materials during recovery.

2.4.10 Pre-Treatment

Flocculants and/or filter aids are added to the fermentation broth, in order to get clear filtrates, and to facilitate the primary solid/liquid separation. Typical amount of filter aids is 2.5 %.

2.4.11 Primary solid/liquid separation

The purpose of the primary separation is to remove the solids from the enzyme containing fermentation medium. The primary separation is performed at a defined pH and a specific temperature range to minimize loss of enzyme activity.

The separation process may vary, depending on the specific enzyme production plant. This can be achieved by different operations like centrifugation or filtration.

2.4.12 Concentration

The liquid containing the enzyme protein needs to be concentrated in order to achieve the desired enzyme activity and/or to increase the ratio enzyme activity/TOS before formulation.

Temperature and pH are controlled during the concentration step, which is performed until the desired concentration has been obtained. The filtrate containing the enzyme protein is collected for further recovery and formulation.

2.4.13 Polish and germ filtration

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied at various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

2.4.14 Formulation and Packaging

Subsequently, the food enzyme is formulated. The resulting product is defined as a 'food enzyme preparation'.

The protease enzyme preparations from *T. reesei* RF8963 are sold mainly as liquid preparations. For all kinds of food enzyme preparations, the food enzyme is adjusted to a declared activity, standardized and preserved with food ingredients or food additives (food grade quality).

The food enzyme preparation is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations, and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations.

2.4.15 General Production Controls and Specifications

To comply with cGMPs and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account and controlled during production as described below:

Identity and purity of the producing microorganism:

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore, it is essential that the identity and purity of the microorganism is controlled.

Production of the required enzyme protein is based on a well-defined Master (MCB) and Working Cell Bank (WCB). The MCB contains the original deposit of the production strain. The WCB is a collection of ampoules containing a pure culture prepared from an isolate of the production strain in MCB. The cell line history, propagation, preservation and the production of a Working Cell Bank is monitored and controlled. A WCB is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

Microbiological hygiene:

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Microbial contamination can result to decreased growth of the production organism, and consequently, in a low yield of the desired enzyme protein, resulting in a rejected product.

Measures utilized by ROAL OY to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

- Hygienic design of equipment:
    - all equipment is designed, constructed and used to prevent contamination by foreign micro-organisms
- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the production area and equipment: all fermentor, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place), where hot caustic soda and nitric acid are used as

cleaning agents. After cleaning, the vessels are inspected manually; all valves and connections not in use for the fermentation are sealed by steam at more than 120°C; critical parts of down-stream equipment are sanitized with disinfectants approved for food industry

- Sterilization of all fermentation media:
    - all the media are sterilized with steam injection in fermentors or media tanks
- Use of sterile air for aeration of the fermentors:
    - Air and ammonia water are sterilized with filtration (by passing a sterile filter).
- Hygienic processing:
    - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor
    - Maintaining a positive pressure in the fermentor
- Germ filtration

In parallel, hygienic conditions in production are furthermore ensured by:

- Training of staff:
    - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- Pest control
- Inspection and release by independent quality organization according to version-controlled specifications
- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production take place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

Chemical contaminants:

It is also important that the raw materials used during fermentation are of good quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

In addition to these control measures in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high-quality product (cGMPs). The whole process is controlled with a computer control system which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

Absence of significant microbial contamination is analyzed by microscopy or plate counts before inoculation of the seed and main fermentations and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring of fermentation parameters may include:

- pH
- Temperature
- Aeration conditions

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent

process.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):

This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

2.4.16 Formulation and Packaging

Subsequently, the food enzyme is formulated. The resulting product is defined as a 'food enzyme preparation'. Serine protease enzyme preparation from *Trichoderma reesei* is sold as liquid preparations.

For all kinds of food enzyme preparations, the food enzyme is adjusted to the desired activity and is standardized and preserved with food-grade ingredients or additives.

2.4.17 Stability of the enzyme during storage and prior to use

Food enzymes are formulated into various enzyme preparations to obtain standardized and stable products. The stability thus depends on the type of formulation, not on the food enzyme as such.

The date of minimum durability or use-by-date is indicated on the label of the food enzyme preparation. If necessary, special conditions of storage and/or use will also be mentioned on the label.

2.5 Composition and specifications

2.5.1 Characteristics of the enzyme preparation

The characteristics of the enzyme preparation are:

Property	Requirement	
Activity	min.	100.000 BPU/g

Appearance	Light brown liquid
Density	1.0 – 1.1 g/ml

2.5.2 Formulation of a typical enzyme preparation

Composition	
Constituent	**%**
Protease	26.3
Sodium benzoate	0.35
Glycerol	30.0
Sorbitol	30.0
Tap water	13.35

2.5.3 Molecular mass and amino acid sequence of the enzyme

The mature peptidase protein subject for this dossier consists of 281 amino acid residues with a calculated molecular mass of 28.5 kDa.

2.5.4 Purity and identity specifications of the enzyme preparation

It is proposed that the food enzyme serine protease should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006):

Lead:	Not more than 5 mg/kg
Salmonella sp.:	Absent in 25 g of sample
Total coliforms:	Not more than 30 per gram
Escherichia coli:	Absent in 25 g of sample
Antimicrobial activity:	Not detected
Mycotoxins:	No significant levels[2]

[2] See JECFA specifications, ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf, page 64: Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

The proof that the food enzyme complies with these specifications is shown by the analyses on 3 different batches (see Appendix #1) and summarised below:

2.5.5 Composition of the enzyme preparation

Batch Number				
Ash (%)	0.27	< 0.1	0.12	*0.16*
Water (%)	84.8	80.5	83.2	*82.8*
Protein (%)	13.3	16.4	14.1	*14.6*
TOS (%)	14.9	19.5	16.7	*17.0*
Activity (BPU/g concentrate)	344,000	462,000	415,000	*407,000*
Activity/mg TOS	**2,309**	**2,369**	**2,485**	***2,394***

TOS values were calculated using the following formula: % TOS = 100 % - (% Ash + % Moisture + % Diluents) as recommended by JECFA. The 3 samples do not contain any diluents.

Other enzymatic activities: the food enzyme is standardized on serine endopeptidase activity. Apart from it, the production organism *Trichoderma reesei* produces other endogenous *Trichoderma* proteins, e.g. xylanases. However, they are present in a small amount and those enzyme activities are already present in the human diet and are not relevant from a safety point of view.

Therefore, there are no relevant side activities from an application and/or safety point of view.

2.6 Enzymatic Activity

The main activity of the enzyme preparation is serine protease.

The serine endopeptidase, thermomycolin, catalyzes the hydrolysis of peptide bonds in proteins with preferential cleavage at Alanine, Tyrosine, and Phenylalanine in small molecule substrates.

Thermomycolin belongs to the peptidase family S8 (see: https://www.ebi.ac.uk/merops/cgi-

bin/famsum?family=S8) which have been identified in many other sources, including microorganism and animals.

The substrates for the enzyme are polypeptide protein chains which can be found in living organisms and therefore occur naturally in nature and are a natural part of the human diet.

Reaction products: as a result of the catalytic activity of the serine endo-peptidase, smaller proteins and peptides of variable lengths are formed.

The method to analyse the activity of the enzyme is company specific, and using casein as a substrate, is capable of quantifying serine endopeptidase as defined by its IUBMB classification. The enzyme activity is usually reported in BPU/g.

2.6.1 Side activities of the enzyme protein which might cause adverse effects

Food enzymes are biological concentrates containing – apart from the desired enzyme protein (expressing the activity intended to perform a technological purpose in a certain food process, also called 'main enzyme activity') - also some other substances. This is the reason why JECFA developed the TOS concept for food enzymes and why it is important that the source of a food enzyme is safe.

These other substances may include various enzyme activities (defined as 'side activities') derived from the producing microorganism. Like all living cells, microorganisms produce a variety of enzymes responsible for the hundreds of metabolic processes that sustain their life. As microorganisms do not possess a digestive system, many enzymes are excreted to digest the material on which the microorganisms grow. Most of these enzymes are hydrolases that digest carbohydrates, proteins and lipids (fats). These are the very same activities that play a role in the production of fermented food and in the digestion of food by – amongst others – the intestinal micro flora in the human body. In addition, if a food raw material contains a certain substrate (e.g. carbohydrate, protein or lipid), then, by nature, it also contains the very same enzymatic activities that break down such a substrate; e.g. to avoid its accumulation. Consequently, the

presence in food of such enzyme activities and of the potential reaction products is not new and should not be of any safety concern. In addition, it is generally accepted that the enzyme proteins themselves do not pose any safety concern either.

Apart from serine endopeptidase, the food enzyme also contains other enzymatic side activities in small amount which are naturally and typically produced by the production organism *Trichoderma reesei,* mainly xylanases.

As far as AB Enzymes is aware, the protease described in this dossier does not possess any enzymatic side activities which might cause adverse effects.

2.7 Allergenicity

As some enzymes manufactured for use in food have been reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities, serine protease may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the protease residues in food seems remote. To address allergenicity by ingestion, it may be taken into account that:

- The allergenic potential of enzymes was studied by Bindslev-Jensen et al. (2006) and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.
- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Daurvin et al. 1998). The overall conclusion was that – as opposed to exposure by inhalation – there are no scientific indications that the small amounts of enzymes in food can

sensitize or induce allergy reactions in consumers.

- Enzymes when used as digestive (Abad et al. 2010) aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more).

Thus, there are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers.

Additional considerations supporting the assumptions that the ingestion of an enzyme protein is not a concern for food allergy should also be considered:

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of enzyme proteins used in food being homologous to known food allergens[3].
- The food enzyme is used in small amounts during food processing resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al. 2008).
- In the case where proteins are denatured which is the case for this enzyme due to the food process conditions, the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta 2002; Valenta and Kraft 2002; Takai et al. 1997; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).
- In addition, residual enzyme proteins still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less

[3] The only enzyme protein used in food an known to have a weak allergenic potential is egg lysozyme

likely to be allergenic (Food and Agriculture Organization of the United Nations January/2001; Goodman et al. 2008).

- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

2.7.1 Allergenicity Search

To specifically evaluate the risk that the serine endopeptidase enzyme would cross react with known allergens and induce a reaction in an already sensitized individual, sequence homology testing to known allergens was performed. This test used an 80-amino acid (aa) sliding window search as well as conventional FASTA alignment (overall homology), with the threshold of 35% homology as recommended in the most recent literature (Food and Agriculture Organization of the United Nations January/2001; Ladics et al. 2007; Goodman and Tetteh 2011).

A sequence homology comparison test was then performed using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 17, January 18, 2017), which contains the amino acid sequences of known and putative allergenic proteins.

Altogether 25 hits with sequence identity above the set 35 % to the query sequence were obtained. All the hits were to serine endopeptidases which was an expected result as all serine proteases share some degree of amino acid identity. Six of the hit sequences were described as allergens in the database, Tri m 2 (two different database hits, the other was a partial sequence), Tri r 2, Pen n 13, Pen n 18 and Asp fl 1. The allergenic potential of the RF8963 protease was further evaluated by searching and analyzing the exact match peptides in the RF8963 protease sequences and the hit sequences, by comparing these peptide sequences to the B cell epitopes predicted *in silico* from the mature RF8963 protease sequence and by analyzing the location of the cleavage sites of the major proteases in the digestive track on the RF8963 protease and the

above peptides. Basing on the results from the analysis it could be concluded that it is unlikely that the RF8963 protease would pose a risk of food allergenicity.

Furthermore, the final enzyme preparation does not contain any major food allergen from the fermentation media.

Conclusion:

Based on the results obtained from the bioinformatics approach to estimate potential allergenicity on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data, and based on the fact that the enzyme is typically denatured during the food manufacturing process and that any residual enzyme still present in the final food will be subject to digestion in the gastro-intestinal system, it is not likely that serine endopeptidase produced by *Trichoderma reesei* RF8963 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

2.8 Technological purpose and mechanism of action of the enzyme in food

Like any other enzyme, the serine endopeptidase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

The **substrates** for the enzyme are polypeptide protein chains which can be found in all living organisms and therefore occur naturally in nature and are a natural part of the human diet.

The **function** of the serine endopeptidase is to catalyse the hydrolysis of peptide bonds in proteins with preferential cleavage at Alanine, Tyrosine, and Phenylalanine in small molecule

substrates.

Reaction products: as a result of the catalytic activity of the serine endo-peptidase, smaller proteins and peptides of variable lengths are formed. Being the result of protein catabolism, these reaction products also naturally occur in all living organisms. Consequently, also the reaction product(s) occur(s) naturally in foods and adverse effects on nutrients are not to be expected.

Like most of the enzymes, the serine endopeptidase performs its technological function during food processing. The serine endopeptidase from *Trichoderma reesei* RF8963, object of this dossier, can theoretically be used as processing aid for partial or extensive hydrolysis of proteins from both animal and vegetable sources (e.g. casein, whey, gluten, and proteins from meat, fish, corn, soy, rice, peas, lentils etc.) but this specific enzyme **is intended to be used in protein processing, mainly in fish and meat processing.**

The enzyme is added during the manufacturing process and does not perform any technological function in the final foods. The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing, lack of water activity, wrong pH, etc. In some cases (e.g. after alcohol distillation), the enzyme may no longer be present in the final food.

In protein processing, the serine endopeptidase performs its technological function during the food manufacturing to ease and optimize the hydrolysis process. The serine endopeptidase is denatured by high temperatures used in the processes.

Consequently, it can be concluded that the serine endopeptidase does not exert any

(unintentional) enzymatic activity in the final foods.

Enzymes including serine endopeptidases have a long history of use in food (Pariza and Johnson 2001). In 2010, Nielsen reported that enzymatic processing of proteins using selected serine endopeptidases to hydrolyse specific peptide bonds have been used for many years (since the 1970s) to produce peptides with improved functional properties. Microbial serine endopeptidase enzyme preparations have been evaluated worldwide and multiple national (US GRAS, DK, France) and international (JECFA) bodies have specifically approved the usage of those enzymes in various applications, which together with the extensive use for decades in a number of EU countries justifies the technological need of this serine endopeptidase in these food processes.

Below, the benefits of the use of industrial serine endopeptidase in these processes are described. The beneficial effects are of value to the food chain because they lead to better and/or more consistent product quality. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste.

PROTEIN PROCESSING

Protein hydrolysates are produced by hydrolysis of proteins or protein containing raw materials from different origins, e.g.:

- Vegetable (derived) raw materials, such as soy, wheat, maize, etc
- Animal (derived) raw materials, such as milk and milk derived products (whey proteins, caseins), meat, fish, collagen, gelatin.

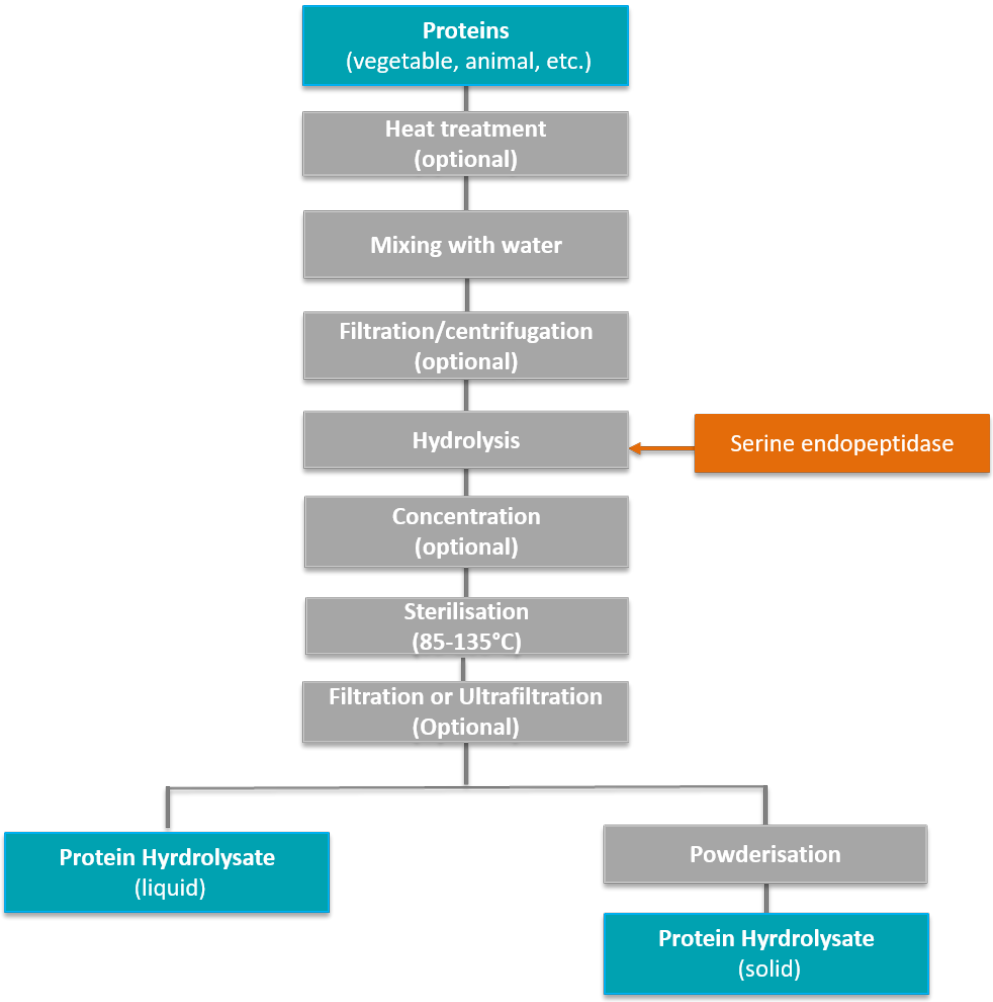
The main intention of the use of the serine endopeptidase in Protein processing is to facilitate protein hydrolysis.

Traditionally, protein hydrolysis was carried out using e.g. hydrochloric acid or by boiling meat and fish pieces. From the 1960s, proteases came into use because of their effective hydrolysis activity, leading to increased yield and enhanced flavours. In the beginning, a limited number of proteases were used (Criswell L. G. et al. 1964), but currently a bigger range of proteases (animal, plant, fungal, bacterial origin and alkaline, acid, neutral, heat-resistant etc.) including serine endopeptidase are used. Enzymatic hydrolysis has been utilized for over four decades.

The serine endopeptidase subject of this dossier has a preferential cleavage at Alanine, Tyrosine, and Phenylalanine. It can be used for partial or extensive hydrolysis of animal and vegetable proteins such as meat and fish, casein, whey, gluten, and proteins from soy, corn, rice, peas, lentils, etc.

The resulting peptides are used as ingredients in a variety of food products. The applicability of use as food ingredients are often determined by the functional properties of the processed proteins which to a large extent are governed by their molecular size and their distribution of hydrophobic amino acids.

The typical protein process in which the serine endopeptidase may be applied is illustrated in the process flow scheme given below:



The main intention of the use of Serine endopeptidase is to **facilitate protein hydrolysis**. However, depending on the specific application, the enzymatic hydrolysis of proteins can result in some benefits on the final foods where the hydrolysed proteins will be used:

- Facilitate the production of peptides with better functional properties such as solubility, emulsification, gelling and foaming (Whitehurst and van Oort 2010)
- Increase the protein content in certain foods with nutritional purposes,
- Improve digestibility and reduce allergenic potential: Milk proteins such as casein and whey protein are significant sources of nutrition to growing infant and young children. However, babies and children might have difficulty fully digesting the proteins because

of their immature digestive systems. They might also face problems due to the allergenicity of the proteins. By converting the proteins into peptides, absorption is improved and antigenicity (the potential of the proteins to cause an allergic response from the immune system) is reduced (Takase et al. 1979). Enzymatic methods have been utilized for over three decades (USA patent US 8.449,938 B2).

The fate of the enzyme during protein processing: The enzyme protein is inactivated by heat in a specific inactivation step or in a sterilisation/pasteurization process or further drying steps.

Specific examples of the use of this serine endopeptidase, in fish and meat processing, are provided below.

FISH PROCESSING

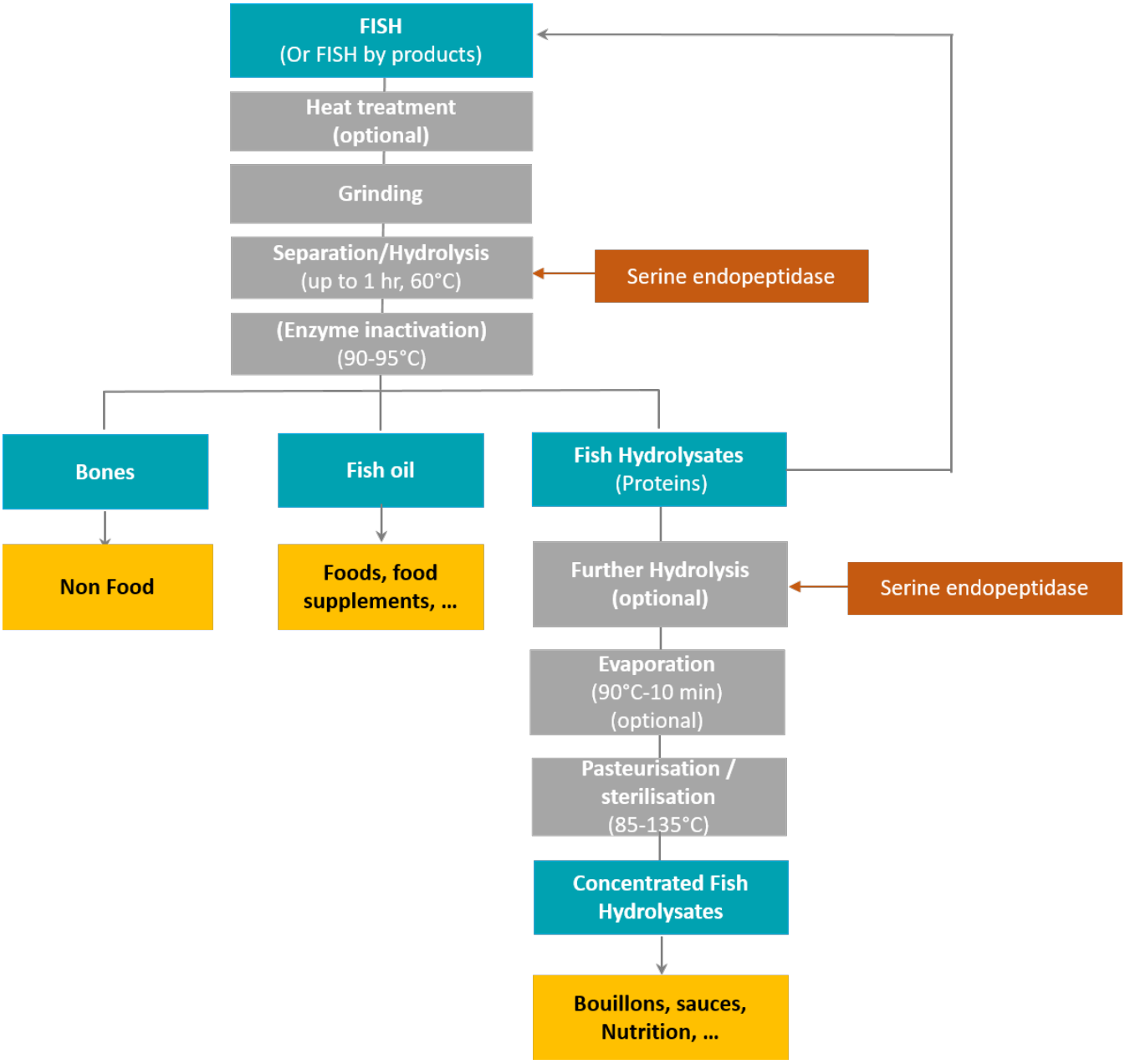
Serine endoprotease can be used in fish processing to help during the primary hydrolysis, ie separating the protein fraction (fish hydrolysates) from the bones and oil fraction.

Production of more concentrated fish hydrolysates requires the application of proteolytic enzymes in order to reduce the viscosity due to the high protein content. High viscosity complicates the process by:

- Reducing the heat transfer resulting in a decreased rate of evaporation (reduced evaporation efficiency);
- Increasing the possibility of scale formation of the equipment, resulting in more frequent cleaning.

The serine endopeptidase is added into the tank and left there during a couple of hours (depending on the temperature conditions in the tank) to get the time to react before evaporation takes place.

The process flow chart is presented below and shows the conditions under which the food enzyme is used:



The benefits of the protein hydrolysis with the help of the serine endopeptidase in fish processing can be summarised as follows:

- By improving liquid/solid separation process and leading to higher yield of soluble proteins and peptides, the use of the enzyme helps to improve the quality of the final products and maximize the value of the trimmings
- Mild process conditions compared to alternative protein hydrolysis processes (as alkaline or acid hydrolysis or heat treatment)
- Viscosity reduction, leading to improvement of the overall processing of fish products
    - Improved evaporation rate of the fish hydrolysate, leading to:
        - A higher solids proportion, reduced energy consumption in drying the final fraction,
        - Reduced scaling of equipment and therefore reduced cleaning cycles.

The main intention of the use of the serine endopeptidase is to facilitate protein hydrolysis for better processing as described above. It is true that depending on the specific application, the enzymatic hydrolysis of proteins can in theory result in some benefits on the final foods where the hydrolysed proteins will be used. However, this is not the purpose of the use of this enzyme.

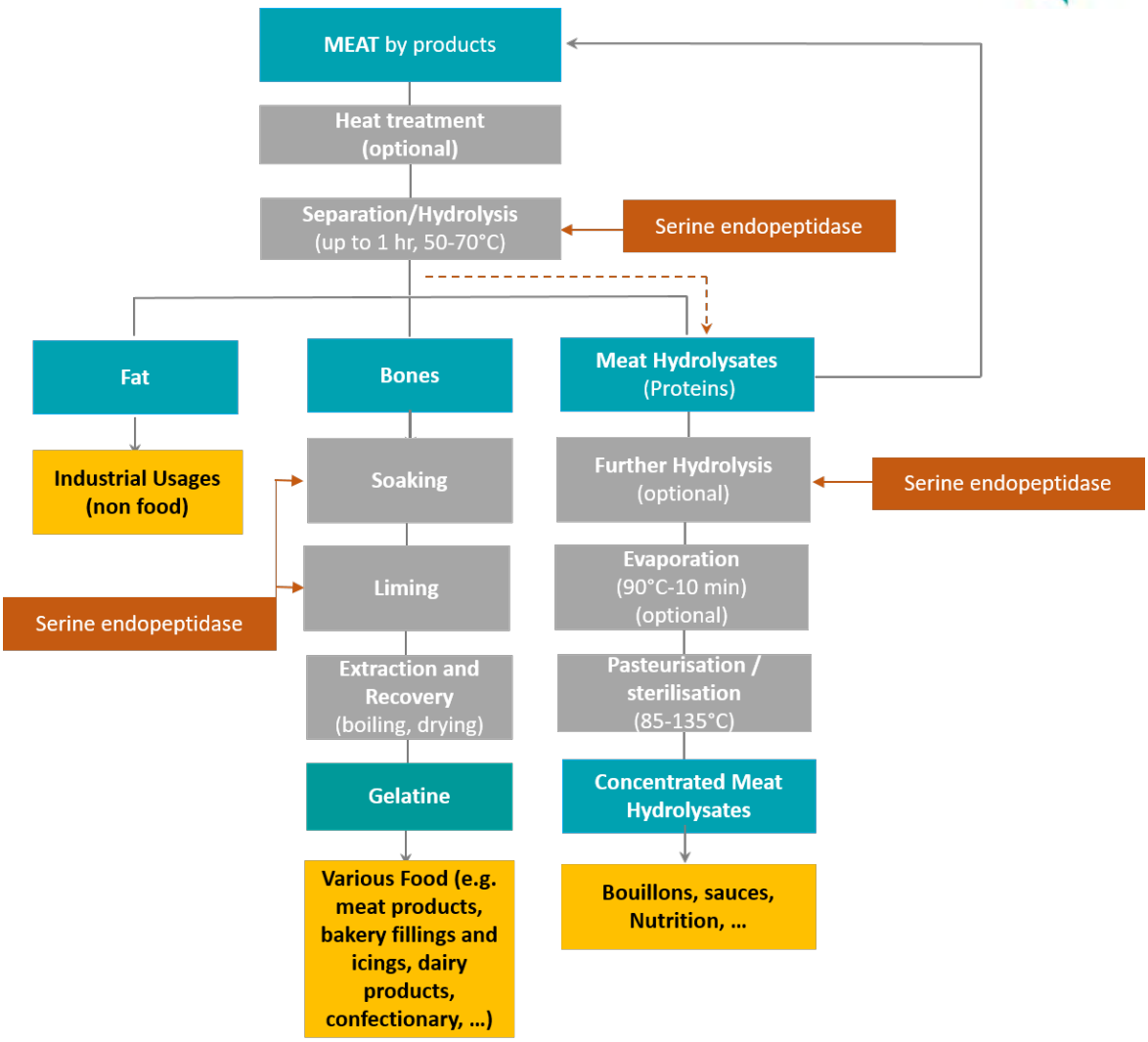
The fate of the enzyme protein during the fish processing: The serine endopeptidase performs it technological function during the process. When the serine endopeptidase is added to the fish by products, it performs its technical function there and all the enzyme is carried over into the fish hydrolysates fraction. None of the enzyme remains with the bones or the fish oil fractions. Further down the process, the serine endopeptidase can be added in the tank to help further concentrate the hydrolysate fraction, and thus it performs its technological function before the evaporation step, when temperatures are above 85°C. Therefore, the enzyme is

inactivated during the evaporation or by heat during the subsequent pasteurisation or sterilisation steps. The pasteurised fraction can be further dryed and powderised with high temperatures.

MEAT PROCESSING

In meat processing, the serine endopeptidase is similarly used to facilitate the protein hydrolysis in the treatment of meat by products (e.g. bone cleaning, gelatin production…) and the production of meat hydrolysates (meat or bone stocks, hydrolysed animal proteins-HAP).

The process flow chart is presented in the next page and shows the conditions under which the food enzyme is used.



The benefits of the protein hydrolysis with the help of the serine endopeptidase in meat processing are similar to those described above for fish processing.

In addition, for gelatin production, the benefits are:

- Significant cost reduction and capacity increase through reduction in soaking and liming times
- Increased gelatin yield
- Improved raw material quality (cleaner bones)

The fate of the enzyme protein during meat processing: The serine endopeptidase performs

it technological function during the process. When the serine endopeptidase is added to the meat by-products, it performs its technical function there and all the enzyme is carried over into the meat hydrolysates fraction. None of the enzyme remains with the bones or the fat fractions. Further down the process, the serine endopeptidase can be added to help further concentrate the hydrolysate fraction, and thus it performs its technological function before the evaporation step, when temperatures are above 85°C. Therefore, the enzyme is inactivated during the evaporation or by heat during the subsequent pasteurisation or sterilisation steps. The pasteurised fraction can be further dryed and powderised with high temperatures. When used in gelatin production, the enzyme is denatured during gelatin extraction (when temperatures increase up to boiling) and further downstream processes involving drying step.

To summarize, the use of serine endopeptidase in **protein processing** ensures a maximum compatibility with modern industrial processes.

The use of peptidases in such applications has been specifically approved in France, which - together with the extensive use for decades in a number of EU countries and in the rest of the world - demonstrates the technological need of such food enzymes in food processes.

2.9 Use Levels

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer must be determined case by case, based on the desired effect and process conditions.

Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune his process

and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and protease is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

The dosage of a food enzyme depends on the activity of the enzyme protein present in the final food enzyme preparation (i.e. the formulated food enzyme). However, the activity Units as such do not give an indication of the amount of food enzyme actually added.

Microbial food enzymes contain, apart from the enzyme protein in question, also some substances derived from the producing microorganism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS). From a safety point of view, the dosage on basis of TOS is more relevant. It must also be noted that the methods of analysis and the expression of the Units are company specific. Consequently, in contrast to when the amount is expressed in TOS the activity Units of a certain enzyme cannot be compared when coming from different companies. Because of these reasons, the use levels are expressed in TOS in the Table below.

The Table below shows the range of recommended use levels for each application where the serine endopeptidase from *Trichoderma reesei* RF8963 may be used, are shown in the table below:

Food Application	**Raw material (RM)**	**Suggested recommended use levels (mg TOS/kg RM)**
Protein processing	Proteins	10

2.10 Fate in food

It is not the food enzyme itself, but the result of the enzymatic conversion that determines the

effect in the food or food ingredient (including raw materials). This effect remains, irrespective of whether the food enzyme is still present or removed from the final food.

Protease performs its technological function during food processing. In some cases, the enzyme may no longer be present in the final food. In other cases, where the enzyme protein is still present in the final food, it does not perform any technological function in the final food, just like the endogenous protease present in food.

To be able to perform a technological function in the final food, many conditions have to be fulfilled at the same time:

- the enzyme protein must be in its 'native' (non-denatured) form, AND
- the substrate must still be present, AND
- the enzyme must be free to move (able to reach the substrate), AND
- conditions like pH, temperature and water content must be favourable

In protein processing, the serine endopeptidase performs its technological function during the food manufacturing to ease and optimise the hydrolysis process. The serine endopeptidase is denatured by high temperatures used in the processes.

Consequently, it can be concluded that the serine endopeptidase does not exert any (unintentional) enzymatic activity in the final foods.

3 PART 3 § 170.325 - DIETARY EXPOSURE

The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen 1966; Douglass et al. 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Average consumption over the course of a lifetime/kg body weight/day 0.025	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	0.0125	0.025

For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food or beverage is obtained per kg raw material (see below the table) and it is assumed that all the TOS will end up in the final product.

Applications		Raw material (RM)	Suggested recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Suggested level in final food (mg TOS/kg food)
LIQUID FOODS	Protein processing	Proteins from various origins	10	Drinks supplemented with proteins	0.17	1.7
SOLID FOODS	Protein processing	Proteins from various origins	10	Proteins hdrolysates used in e.g. soups, bouillons, dressings, fish meals, meat products, …	0.16	1.6
				Gelatin used in various foods (e.g. confectionary, meat products, dairy products…)	0.05	0.5

* **Assumptions behind ratios of raw material to final food**:

Protein processing:

Dietary proteins are found in variable proportions in different foods resulting in variability of dietary protein intake within and between populations. Data from Health, United States[4], 2016 shows that the mean protein intake for men and women is 16.1% and 15.6% respectively. The assumption used for calculation of dietary exposure is an average intake of 16% of the total diet. The corresponding RM/FF ratio will therefore be ***0.16 kg protein / kg final food.***

Protein processing can also lead to the ***production of gelatin****. Gelatin is extracted from bone collagen (ossein). Collagen represents approximately 30 % of the bones weight and contains approximately. 65% gelatin. Thus, from 1 kg bone, 200 g gelatin could theoretically be extracted. Gelatin is then used in various foods especially in dairy products (up to 1%), meat products (up to 5%), confectionary and gelatin desserts (up to 9%).[5] Based*

[4] https://www.cdc.gov/nchs/data/hus/hus16.pdf#056
[5] Gelatin Manual, 2012

upon the most considerable applications (confectionary), the corresponding RM/FF ratio is ***0.05 kg meat products/bones per kg final food****.*

The Total TMDI can be calculated on basis of the maximal values found in food (solid) and beverage multiplied by the average consumption of food and beverage/kg body weight/day.

The Total TMDI will consequently be calculated as follows:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
1.6 x 0.0125 = 0.02	1.7 x 0.025 = 0.0425	**0.0625**

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above-mentioned foodstuffs use the specific protease enzyme from *T. reesei* RF8963;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food and in beverages, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS.
- Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease because of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al. 1997).

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL (see **Section 6**) by the Total Theoretical Maximal Daily Intake (TMDI). Total TMDI of the food enzyme is 0.0625 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 1,000 / = 16,000

Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual Margin of Safety in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

Conclusion:

The overall conclusion is that the use of the food enzyme protease from *T. reesei* RF8963 in the production of food is safe. Considering the high safety factor – even when calculated by means of an overestimation of the intake via the Budget method – there is no need to restrict the use of the enzyme in food processing. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.

4 PART 4 §170.240 – SELF-LIMITING LEVELS OF USE

This part is not applicable to this notified substance, see **Section 2.9** for further details regarding use levels.

5 PART 5 § 170.245 – EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

This part is not applicable to this notified substance.

6 PART 6 § 170.250 – GRAS NOTICE - NARRATIVE

The data and information contained in this GRAS notice provides a basis that the notified substance is safe under the conditions of its intended use described herein. In the following sub-sections, the safety of the enzyme, the genetic modification and toxicological studies are presented. The information is generally available and PART 6 § 170.250 does not contain any confidential information. This section provides the basis that the notified substance is generally recognized, among qualified experts, and study data, to be safe under the conditions of its intended use.

All available known information has been reviewed and AB Enzymes GmbH is not aware of any data or information that is, or may appear to be, consistent with our conclusion of the notified substance GRAS status.

6.1 Safety of the production strain

Production strain

The safety of *Trichoderma reesei* as an enzyme producer has been reviewed by Nevalainen et al.; Olempska-Beer et al.; Blumenthal (1994; 2006; 2004). T. *reesei* is regarded as a safe organism for production of industrial enzymes.

The original *T. reesei* isolate, QM6a is the initial parental organism of practically all currently industrially relevant food enzyme production strains, including our strain RF8963. *Trichoderma reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production (Nevalainen et al. 1994; Olempska-Beer et al. 2006*)*. Cellulases, hemicellulases, β-glucanases, pectinases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries.

Food enzymes deriving from *Trichoderma reesei* strains (including recombinant *T. reesei* strains) have been evaluated by JECFA and many countries which regulate the use of food enzymes,

such as the USA, France, Denmark, Australia and Canada, resulting in the approval of the use of food enzymes from *Trichoderma reesei* in the production of various foods, such as baking, brewing, juice production, wine production and the production of dairy products.

T. reesei strains have been cultivated in the production plant of Alko Oy/Roal Oy starting from year 1987 and the parental strain RF4847 described here has been used from year 1995 on.

6.1.1 Pathogenicity and toxigenicity

Trichoderma reesei strains are non-pathogenic for healthy humans and animals (*Nevalainen et al. 1994)*. *Trichoderma reesei* is not present on the list of pathogens in the EU (Directive Council Directive2000/54/EC) and is present in major culture collections worldwide.

Trichoderma reesei is globally regarded as a safe microorganism:

- In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH, 1998) Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[6], if this fungus was to be used in submerged standard industrial fermentation for enzyme production.

As a result, *Trichoderma reesei* can be used under the lowest containment level at large scale, GILSP, as defined by *(OECD, 1992)*.

The genus *Trichoderma* contains filamentous fungi which are frequently found on decaying wood and in soil. Industrial *T. reesei* strains have a long history of safe use and several of the

[6] reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce

Trichoderma based products have been approved for food and feed applications[7]. *T. reesei* is listed as a "Risk Group 1" organism according to German TRBA classification (Federal Institute for Occupational Safety and Health, www.baua.de) and as "Biosafety Level 1" organism by the American Type Culture Collection (www.atcc.org). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals. The DNA based identification methods have shown that *T. reesei* is taxonomically different from the other *Trichoderma* species of the section *Longibrachiatum* (Druzhinina et al. 2005).

Some species belonging to *Trichoderma* genus are able to secrete various types of antibiotics in laboratory cultures. However, strains of *T. reesei* used in industrial applications are proven to be devoid of antibiotic activities (Coenen et al. 1995; Hjortkjaer et al. 1986). The absence of antibiotic activities, according to the specifications recommended by JECFA (Food and Agriculture Organization of the United Nations 2006), was also confirmed for RF8963. The analyzed data are presented in Appendix #1.

Additionally, the original host *T. reesei* RF4847 and the genetically modified strain do not carry any acquired antimicrobial resistance genes.

As documented below, peptidases from various micro-organisms (including genetically modified ones) are widely accepted and *Trichoderma reesei* – whether or not genetically modified[8] - is widely accepted as a safe production organism for a broad range of enzymes.

[7] AMFEP. 2009. Association of Manufacturers and Formulators of Enzyme Products List of enzyme products on markets; http://amfep.drupalgardens.com/sites/amfep.drupalgardens.com/files/Amfep-List-of-Commercial-Enzymes.pdf

[8] Overproduction of chosen enzymes and/or modification of enzyme e.g. cellulase profiles has not been observed to introduce harmful properties for the host organism or its products -animal tests Huuskonen 1990.

Non-exhaustive list of authorised food enzymes (other than peptidases) produced by *Trichoderma reesei*		
Authority	**Food Enzyme**	**Reference**
JECFA	Cellulase Beta-glucanase Glucoamylase	FAS 30-JECFA 39/15 and FAS 22-JECFA 31/31 FAS 22-JECFA 31/25, JECFA monograph gluco amylase
Australia/New Zealand	Cellulase Glucan 1-3 beta-glucosidase Beta-glucanase Hemicellulase complex Gluco-amylase Endo 1,4-beta- xylanase Pectinases	Standard 1.3.3 processing aids
Canada	Cellulase Glucanase Pentosanase Xylanase	B.16.100, Table V
USA[9]	Pectinlyase Transglucosidase (GM) Glucoamylase Phospholipase A Polygalacturonase	GRAS Notice Inventory, GRN 32 GRAS Notice Inventory, GRN 315 GRAS Notice Inventory, GRN 372 GRAS Notice Inventory, GRN 524 GRAS Notice Inventory, GRN 557

[9] GRAS affirmations and GRAS notifications

France	Alpha-amylase (GM) Amyloglucosidase (GM) Beta-glucanase (GM) Xylanase Cellulase Lysophospholipase (GM)	Arrêté du 19 octobre 2006

Non-exhaustive list of authorised serine peptidases from production organisms other than *Trichoderma reesei*		
Authority	**Food enzyme**	**Reference**
Australia/NZ	Aspergillus niger[10],[11], Bacillus amyloliquefaciens[11], Bacillus halodurans[11], Bacillus licheniformis[11], Bacillus subtilis[11]	Standard 1.3.3 processing aids
France	Bacillus subtilis[12], Bacillus licheniformis[12], Aspergillus oryzae[12], Aspergillus wentii[12] Fusarium venenatum (carrying a serine protease[13] gene from F. oxysporum) Bacillus licheniformis (carrying a serine protease[14] gene from Nocardiopsis prasina)	Arrêté du 19 octobre 2006
USA[1]	Fusarium venenatum (carrying a serin protease[13] gene from F. oxysporum) Bacillus licheniformis (carrying a serine protease[14] gene from	GRAS Notice Inventory, GRN 563 GRAS Notice Inventory, GRN 564

[10] Endo-protease
[11] Serine proteinase (3.4.21.14)
[12] Serine peptidase
[13] 3.4.21.4
[14] 3.4.21.1

	Nocardiopsis prasina)	
Canada	Aspergillus niger, Aspergillus oryzae, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis, Fusarium venetum, …	Lists of Permitted Food Additives
JECFA	Fusarium venenatum (carrying a serine protease[13] gene from F. oxysporum) Bacillus licheniformis (carrying a serine protease[14] gene from Nocardiopsis prasina)	JECFA, 2011 JECFA, 2011

Conclusion: Based on the above mentioned available data, it is concluded that the organism *T. reesei*, has a long history of safe use in industrial-scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. As an example, *T. reesei* strains have been cultivated in the production plant of Alko Oy/Roal Oy since 1987. During this time, genetic engineering techniques have been used to improve the industrial production strains of *Trichoderma reesei* and considerable experience on the safe use of recombinant *Trichoderma reesei* strains at industrial scale has accumulated. From above, secondary metabolites are of no safety concern in fermentation products derived from *Trichoderma reesei*. Thus, *Trichoderma reesei* and its derivatives can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for heterologous gene products.

6.1.1 Safety of the genetic modification

The recipient strain used for the genetic modification is a *Trichoderma reesei* mutant strain, which derives from *Trichoderma reesei* strain RF4847/RH7004, a classical mutant originating from QM6a. This strain has been shown to be genetically stable for industrial production.

Trichoderma reesei has a long history (more than 30 years) of safe use in industrial-scale enzyme

production (Nevalainen et al. 1994; Blumenthal 2004). The safety of this organism as an enzyme producer has been reviewed by Nevalainen et al. (1994), Blumenthal (2004), and (Olempska-Beer et al. 2006) and it is concluded that the organism *T. reesei* is non-pathogenic and non-toxigenic and can be considered as a safe organism to be used as a host for production of enzymes for food and feed processing (as well as for other industrial applications) based upon the decision tree (Appendix #3) (Pariza and Johnson 2001; Nielsen 2010).

The gene encoding the heterologous serine endopeptidase produced by RF8963 originates from *Malbranchea cinnamomea*.

Enzymes including serine endopeptidases have a long history of use in food (Pariza and Johnson 2001; Nielsen 2010). Enzymatic processing of proteins using selected serine endopeptidases to hydrolyse specific peptide bonds have been used for many years (since the 1970s) to produce peptides with improved functional properties. Microbial serine endopeptidase enzyme preparations (and serine endopeptidases) have been evaluated worldwide and multiple national (US GRAS, DK, France) and international (JECFA) bodies have concluded that these food enzymes do not constitute a toxicological hazard.

As the serine endopeptidase protein is not toxic our evaluation of the genetically modified *T. reesei* strain is comparable to that of the recipient strain and the enzyme formulations produced are non-pathogenic for healthy humans and animals.

The synthetic acetamidase-encoding *amdS gene* of *Aspergillus nidulans* is used as selectable marker. *A. nidulans* is closely related to *Aspergillus niger* which is used in industrial production of food enzymes. The product of the *amdS* gene, acetamidase (AmdS), can degrade acetamide which enables the strain to grow on media without any other nitrogen sources. The AmdS is not harmful or dangerous; the *amdS* marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 20 years.

The transformed expression cassettes, fully characterized and free from any harmful sequence or any potential hazards, are stably integrated into the fungal genome, and are no more susceptible to any further natural mutations than any other genes in the fungal genome.

No additional growth/mutagenesis cycles have been performed after the RF8963 strain deposition to the culture collection (Master Cell Bank, MCB).

Therefore, it can be concluded that the *Trichoderma reesei* strain RF8963 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food and feed processing.

6.2 DATA FOR RISK ASSESSMENT

6.2.1 Toxicological testing

The following studies were performed:

— *In vitro* Mammalian Cell Gene Mutation Assay in Mouse Lymphoma

— *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes

— 13-week oral toxicity study in rats

Serine endopeptidase that has been tested is a liquid ultra-filtrated concentrate, before its formulation into a food enzyme preparation.

All tests were performed according to the principles of Good Laboratory Practices (GLP) and the current OECD and EU guidelines.

The full reports of the safety studies performed are summarized below.

AMES test and MLA TEST

EFSA guidance from 2009[15] states that two *in vitro* tests would normally be required, including a test for induction of gene mutations in bacteria (Ames test; OECD guideline 471). It is later added that "*If this assay is not applicable, alternatively a test for induction of gene mutations in mammalian cells, preferably the mouse lymphoma tk assay with colony sizing (OECD guideline 476), could be performed*."

In 2014, EFSA published an explanatory note (EFSA supporting publication 2014: EN-579[16]) in which some more recommendations on the performance of bacterial mutagenicity tests to overcome potential problems with histidine in the food enzyme batch (ie "treat and plate assay", instead of the traditionally "plate incorporation assay").

This was discussed with Eurofins, BSL at an early stage as the active serine endopeptidase was expected to generate histidine (as a freely available amino acid) from the available materials (eg

[15] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2011.2379

[16] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2011.EN-579

agar or S9 mix with liver enzymes present). Eurofins – BSL acknowledged the EFSA recommendations of the treat-and-plate method but considered this approach not suitable in this case. Alternatively, it was suggested to run a MLA as recommended by EFSA in the initial EFSA Guidance.

To investigate the tolerability of the substance in the Ames test and to rule out a possible effect of histidine generation by the protease, a minimal screening for histidine release with a bacterial strain (small non-GLP test) was conducted. A dose dependent increase of the revertants was shown, due to a noticeable histidine content in the enzyme preparation that either comes from protease degradation itself (free release of histidine) or liver mix proteins degradation.

Therefore, a Mouse Lymphoma tk assay with colony sizing was conducted, according to OECD 790 Guidelines.

The test, based on OECD Guidelines No. 490 (OECD, 2015), was run at Eurofins BioPharma Product Testing Munich GmbH (Planegg/Munich) – Germany. The study was completed on July 26, 2016.

The test item serine endopeptidase was assessed for its potential to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

The selection of the concentrations used in the main experiment was based on data from the pre-experiment. In the main experiment without metabolic activation 1.8 mg/ml and with metabolic activation 2 mg/ml were selected as the highest concentrations. The experiment without and with metabolic activation was performed as a 4 h short-term exposure assay.

The test item was investigated at the following concentrations:

Without metabolic activation: 0.05, 0.10, 0.25, 0.5, 1.0, 1.4, 1.6 and 1.8 mg/ml;

With metabolic activation: 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2 mg/ml.

No precipitation of the test item was noted in the experiment.

Growth inhibition was observed in experiment I without and with metabolic activation.

In the main experiment without metabolic activation, the relative total growth (RTG) was 14.5% for the highest concentration (1.8 mg/ml) evaluated. The highest concentration evaluated with metabolic activation was 2 mg/ml with a RTG of 43.0%.

In the main experiment, no biologically relevant increase of mutants was found after treatment with the test item (without and with metabolic activation). The Global Evaluation factor (GEF, defined as the mean of the negative/vehicule mutant frequency plus one standard deviation; data gathered from then laboratories) was not exceeded by the induced mutant frequency at any concentration.

No dose-response relationship was observed.

Additionally, in the main experiment, colony sizing showed no clastogenic effects induced by the test item under experimental conditions (without and with metabolic activation).

EMS, MMS and B[a]P were used as positive controls and showed distinct and biologically relevant effects in mutation frequency. Additionally, MMS and B[a]P significantly increased the number of small colonies, thus proving the efficiency of the test system to indicate potential clastogenic effects.

Conclusion: under the experimental conditions reported, the serine endopeptidase produced with *Trichoderma reesei* RF8963, is considered to be non-mutagenic in the in vitro mammalian cell gene mutation assay (thymidine kinase locus) in mouse lymphoma L5178Y cells.

MAMMALIAN MICRONUCLEUS ASSAY IN HUMAN LYMPHOCYTES

To investigate a possible potential of Serine endopeptidase produced with *Trichoderma reesei* for its ability to induce micronuclei in human lymphocytes an *in vitro* micronucleus assay was carried out.

The test, based on OECD Guidelines No. 487 (OECD, 2014), was run at Eurofins BioPharma Product Testing Munich GmbH (Planegg/Munich) – Germany. The study was completed on December 22, 2016.

The following study design was performed:

The protease from *T. reesei* RF8963 was assessed for its potential to induce structural micronuclei in human lymphocytes *in vitro* in two independent experiments. The following study design was performed:

	Without S9 mix		**With S9 mix**
	Experiment I	**Experiment II**	**Experiment I**
Exposure period	4 hrs	44 hrs	4 hrs
Cytochalasin B exposure	40 hrs	43 hrs	40 hrs
Preparation interval	44 hrs	44 hrs	44 hrs
Total culture period*	92 hrs	92 hrs	92 hrs

**exposure started 48h after culture initiation*

The selection of the concentrations was based on data from the pre-experiment.

In the first experiment, 4,000 µg/mL (without metabolic activation) and 5,000 µg/mL (with metabolic activation) of the test item were selected as the highest concentrations. In the long-term experiment, the maximum concentration was 500 µg/mL. The pH of the solution (pH 7.4) was within the physiological range

The following concentrations were evaluated for micronuclei frequencies:

Experiment I with short term exposure (4h):

- Without metabolic activation: 1,000 – 2,500 and 2750 µg/mL
- With metabolic activation: 2,000 – 3,000 and 3,500 µg/mL

Experiment II with long term exposure (44h):

- Without metabolic activation: 25, 100, 300 and 400 µg/mL

No precipitate of the test item was noted in all concentrations groups evaluated in the main experiment.

According to the OECD Guideline 487, the maximum of cytotoxicity should not exceed the limit of 55 ± 5%. Higher levels of cytotoxicity may induce chromosome damage as a secondary effect of cytotoxicity. The other concentrations evaluated should exhibit intermediate and little or no toxicity. However, OECD 487 does not define the limit for discriminating between cytotoxic and non-cytotoxic effects. According to laboratory experience this limit is a value of the relative cell growth of 70% compared to the negative control which corresponds to 30% of cytostasis.

In experiment I without metabolic activation, an increase of the cytostasis above 30 % was noted at the concentrations of 2500 µg/mL (40%) and 2750 µg/mL (60%). With metabolic activation, no increase of the cytostasis above 30 % was noted up to a concentration of 3000 µg/mL. The next higher concentration of 3500 µg/mL induced cytostasis of 65%.

In experiment II without metabolic activation, only the lowest evaluated test item concentration of 25 µg/mL did not induce cytostasis above 30 %. The concentrations 100 µg/mL, 300 µg/mL and 400 µg/mL led to increased cytostasis of 46%, 42% and 56%, respectively.

In experiment I without and with metabolic activation and experiment II without metabolic activation, no biologically relevant increase of the micronucleus frequency was noted after

treatment with the test item compared to the concurrent negative control.

The non-parametric $_x^2$ Test was performed to verify the results in both experiments. No statistically significant enhancement ($p<0.05$) of cells with micronuclei was noted in the dose groups of the test item evaluated in experiment I and II.

The $_x^2$ Test for trend was performed to test whether there is a concentration-related increase in the micronucleated cells frequency in the experimental conditions. No statistically significant increase in the frequency of micronucleated cells under the experimental conditions of the study was observed in experiment I and II.

Ethylmethanesulfonate (EMS, 900 and 1400 µg/mL) and cyclophosphamide (CPA, 15 µg/mL) were used as clastogenic controls. Colchicine (colc, 0.04 and 0.8 µg/mL) was used as aneugenic control. All induced distinct and statistically significant increases of the micronucleus frequency. This demonstrates the validity of the assay.

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item did not induce structural and/or numerical chromosomal damage in human lymphocytes.

Therefore, the serine endopeptidase from *Trichoderma reesei* RF8963 is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity in the *in vitro* Mammalian Cell Micronucleus test.

Further *in vivo* tests were not performed, as there was no *in vitro* mutagenicity detected.

TOXICITY STUDY

The test was performed according to the following guidelines: OECD No. 408 at Eurofins BSL Bioservice (Planegg/Munich) – Germany. The study was completed in November 2017.

The aim of this study was to assess the possible health hazards which could arise from repeated exposure of serine endopeptidase produced with *Trichoderma reesei* via oral administration to rats over a period of 90 days.

The test item was administered daily in graduated doses to 4 groups of test animals, one dose level per group for a treatment period of 90 days. Animals of an additional control group were handled identically as the dose groups but received aqua ad injectionem (sterile water), the vehicle used in this study. The 4 groups comprised of 10 male and 10 female Wistar rats.

The following doses were evaluated:

- Control: 0 mg/kg body weight
- Low Dose: 100 mg/kg body weight
- Medium Dose: 300 mg/kg body weight
- Higher Medium Dose: 600 mg/kg body weight
- High Dose: 1000 mg/kg body weight

The test item formulation was prepared on a daily basis until day 15 and on a weekly basis from day 15 onwards and stored according to the conditions used for validation. The test item was thawed and dissolved in aqua ad injectionem and administered daily during a 90-day treatment period to male and female animals. Dose volumes were adjusted individually on the basis of the body weight most recently measured.

During the period of administration, the animals were observed precisely each day for signs of toxicity. All animals found dead and/or intercurrently euthanised for animal welfare reasons

were subjected to a gross necropsy and the organs preserved for a histopathological examination.

Body weight and food consumption were measured weekly. At the conclusion of the treatment period, all surviving animals were sacrificed and subjected to a detailed gross necropsy, which includes careful examination of the external surface of the body, all orifices and the cranial, thoracic and abdominal cavities and their contents. The wet weight of a subset of tissues was taken and a set of organs/tissues was preserved.

A full histopathological evaluation of the tissues was performed on high dose and control animals. Any gross lesion macroscopically identified will be examined microscopically in all animals.

Summary results:

One of 10 male animals and 2/10 female animals of the HD group were found dead. Female animal no. 97 and male no. 47 died accidentally, but all organs were highly autolytic. Female no. 100 died spontaneously during the course of the study, however, all organs were cannibalized.

One female animal (no. 89) of the HMD group showed a marked alveolar haemorrhage, i.e. a traumatic insult during gavage is likely the cause of death.

Mild clinical signs like moving the bedding, nasal discharge, abnormal breathing and salivation are assumed to be related to local reactions of the test item associated with oral gavaging – but not to systemic toxicity. There were no clinical signs of toxicity.

Serine endopeptidase produced with *Trichoderma reesei* had no adverse effect on body weight and food consumption of female animals. Lower body weights and food consumption were observed throughout the treatment period in male animals of all dose groups – however this was partly already observed before start of treatment. Moreover, significant body weight change and altered food intake were mainly seen in the first treatment week, when compared to controls, and not considerably thereafter. Thus, the effect of Serine endopeptidase produced

with *Trichoderma reesei* on body weights and food consumption of male animals is considered slight and non-adverse.

Serine endopeptidase produced with *Trichoderma reesei* had no effect on haematological and coagulation parameters and parameters of clinical biochemistry determined at the end of the treatment period. Additionally, urinary parameters were unaffected by the test item.

At necropsy at the end of the treatment period there were no gross macroscopic findings present in dosed animals that could be associated with treatment with Serine endopeptidase produced with *Trichoderma reesei*. At none of the dose levels tested were there alterations in organ weights, when compared to controls.

Serine endopeptidase produced with *Trichoderma reesei* did not reveal any pathological indicator for toxicity.

All acceptance criteria for dose formulation analysis were met. All dose formulations tested contained the correct test item concentrations and were homogenous.

Conclusion: On the basis of the present study, the 90-Day Repeated Dose Oral Toxicity study with Serine endopeptidase produced with *Trichoderma reesei* in male and female Wistar rats, with dose levels of 100, 300, 600 and 1000 mg/kg body weight the following conclusions can be made:

Mortality that occurred during the treatment period of this study was assumed to be accidental and not related to Serine endopeptidase produced with *Trichoderma reesei*. In the absence of any signs of toxicity no target tissue was identified and the **dose level of 1,000 mg/kg/d is determined as the NOAEL** in this study.

7 PART 7 §170.255 – LIST OF SUPPORTING DATA AND INFORMATION

This section contains a list of all the data and literature discussed in this dossier to provide a basis that the notified substance is safe under the conditions of its intended use as described in accordance with §170.250 (a)(1). All information presented in this section are publicly available.

Appendices

1. Certificate of Analysis
2. Manufacturing Flow Chart
3. Decision Tree

Publication bibliography

Abad, Ana; Fernández-Molina, Jimena Victoria; Bikandi, Joseba; Ramírez, Andoni; Margareto, Javier; Sendino, Javier et al. (2010): What makes Aspergillus fumigatus a successful pathogen? Genes and molecules involved in invasive aspergillosis. In *Revista iberoamericana de micología* 27 (4), pp. 155–182. DOI: 10.1016/j.riam.2010.10.003.

Bindslev-Jensen, Carsten; Skov, Per Stahl; Roggen, Erwin L.; Hvass, Peter; Brinch, Ditte Sidelmann (2006): Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 44 (11), pp. 1909–1915. DOI: 10.1016/j.fct.2006.06.012.

Blumenthal, Cynthia Z. (2004): Production of toxic metabolites in Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. In *Regulatory toxicology and pharmacology : RTP* 39 (2), pp. 214–228. DOI: 10.1016/j.yrtph.2003.09.002.

Coenen, T. M.; Schoenmakers, A. C.; Verhagen, H. (1995): Safety evaluation of beta-glucanase derived from Trichoderma reesei: summary of toxicological data. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 33 (10), pp. 859–866.

Criswell L. G.; Litchfield J. H.; Vely V. G.; Sachsel G. F. (1964): Studies on Improved Recovery of Protein from Rendering-Plant Raw Materials and Products. In *Food Technology* 1493, pp. 247–251.

Daurvin, T.; Groot, G.; Maurer, K. H.; Rijke, D. de; Ryssov-Nielsen, H.; Simonsen, M.; Sorensen T.B. (1998): Working Group on Consumer Allergy Risk from Enzyme Residues in Food. AMFEP. Copenhagen.

Douglass, J. S.; Barraj, L. M.; Tennant, D. R.; Long, W. R.; Chaisson, C. F. (1997): Evaluation of the budget method for screening food additive intakes. In *Food additives and contaminants* 14 (8), pp. 791–802. DOI: 10.1080/02652039709374590.

Druzhinina, Irina S.; Kopchinskiy, Alexei G.; Komoń, Monika; Bissett, John; Szakacs, George; Kubicek, Christian P. (2005): An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. In *Fungal genetics and biology : FG & B* 42 (10), pp. 813–828. DOI: 10.1016/j.fgb.2005.06.007.

Food and Agriculture Organization of the United Nations (January/2001): Joint FAO/WHO Expert

Consultation on Allergenicity of Foods Derived from Biotechnology,. Rome, Italy.

Food and Agriculture Organization of the United Nations (2006): Compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives : 67th Meeting 2006. Rome: FAO (FAO JECFA monographs, 1817-7077, 3).

Goodman, Richard E.; Tetteh, Afua O. (2011): Suggested improvements for the allergenicity assessment of genetically modified plants used in foods. In *Current allergy and asthma reports* 11 (4), pp. 317–324. DOI: 10.1007/s11882-011-0195-6.

Goodman, Richard E.; Vieths, Stefan; Sampson, Hugh A.; Hill, David; Ebisawa, Motohiro; Taylor, Steve L.; van Ree, Ronald (2008): Allergenicity assessment of genetically modified crops--what makes sense? In *Nature biotechnology* 26 (1), pp. 73–81. DOI: 10.1038/nbt1343.

Granchi, Zoraide; van Pelt, Sake; Thanh, Vu Nguyen; Olsson, Lisbeth; Hüttner, Silvia (2017): Genome Sequence of the Thermophilic Biomass-Degrading Fungus Malbranchea cinnamomea FCH 10.5. In *Genome announcements* 5 (33). DOI: 10.1128/genomeA.00779-17.

Hansen, S. C. (1966): Acceptable daily intake of food additives and ceiling on levels of use. In *Food and cosmetics toxicology* 4 (4), pp. 427–432.

Hjortkjaer, R. K.; Bille-Hansen, V.; Hazelden, K. P.; McConville, M.; McGregor, D. B.; Cuthbert, J. A. et al. (1986): Safety evaluation of Celluclast, an acid cellulase derived from Trichoderma reesei. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 24 (1), pp. 55–63.

Huuskonen, H. (1990): Pathogenicity of the Genetically Modified Trichoderma reesei Strain ALKO 2224 in Mice After a Single Intravenous Administration. National Public Health Institute, Department of Environmental Hygiene and Toxicology. Finland.

Hynes, M. J.; Corrick, C. M.; King, J. A. (1983): Isolation of genomic clones containing the amdS gene of Aspergillus nidulans and their use in the analysis of structural and regulatory mutations. In *Molecular and cellular biology* 3 (8), pp. 1430–1439.

Karhunen, T.; Mäntylä, A.; Nevalainen, K. M.; Suominen, P. L. (1993): High frequency one-step gene replacement in Trichoderma reesei. I. Endoglucanase I overproduction. In *Molecular & general genetics : MGG* 241 (5-6), pp. 515–522.

Kelly, J. M.; Hynes, M. J. (1985): Transformation of Aspergillus niger by the amdS gene of Aspergillus nidulans. In *The EMBO journal* 4 (2), pp. 475–479.

Kikuchi, Yuko; Takai, Toshiro; Kuhara, Takatoshi; Ota, Mikiko; Kato, Takeshi; Hatanaka, Hideki et al. (2006): Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. In *Journal of immunology (Baltimore, Md. : 1950)* 177 (3), pp. 1609–1617.

Ladics, Gregory S.; Bannon, Gary A.; Silvanovich, Andre; Cressman, Robert F. (2007): Comparison of conventional FASTA identity searches with the 80 amino acid sliding window FASTA search for the elucidation of potential identities to known allergens. In *Mol. Nutr. Food Res.* 51 (8), pp. 985–998. DOI: 10.1002/mnfr.200600231.

Nakazawa, Takuya; Takai, Toshiro; Hatanaka, Hideki; Mizuuchi, Eri; Nagamune, Teruyuki; Okumura, Ko; Ogawa, Hideoki (2005): Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure. In *FEBS letters* 579 (9), pp. 1988–1994. DOI: 10.1016/j.febslet.2005.01.088.

Nevalainen, H.; Suominen, P.; Taimisto, K. (1994): On the safety of Trichoderma reesei. In *Journal of biotechnology* 37 (3), pp. 193–200.

Nielsen, Per Munk (2010): Enzymes in Protein Modification. In Robert J. Whitehurst, Maarten van Oort (Eds.): Enzymes in food technology. 2nd ed. West Sussex, U.K., Ames, Iowa: Wiley-Blackwell, pp. 292–319.

Olempska-Beer, Zofia S.; Merker, Robert I.; Ditto, Mary D.; DiNovi, Michael J. (2006): Food-processing enzymes from recombinant microorganisms--a review. In *Regulatory toxicology and pharmacology : RTP* 45 (2), pp. 144–158. DOI: 10.1016/j.yrtph.2006.05.001.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (1992): Safety Considerations for Biotechnology. OECD, pp. 1–45.

Pariza, M. W.; Johnson, E. A. (2001): Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. In *Regulatory toxicology and pharmacology : RTP* 33 (2), pp. 173–186. DOI: 10.1006/rtph.2001.1466.

Penttilä, M.; Nevalainen, H.; Rättö, M.; Salminen, E.; Knowles, J. (1987): A versatile transformation

system for the cellulolytic filamentous fungus Trichoderma reesei. In *Gene* 61 (2), pp. 155–164.

Takai, T.; Ichikawa, S.; Yokota, T.; Hatanaka, H.; Inagaki, F.; Okumura, Y. (2000): Unlocking the allergenic structure of the major house dust mite allergen der f 2 by elimination of key intramolecular interactions. In *FEBS letters* 484 (2), pp. 102–107.

Takai, T.; Yokota, T.; Yasue, M.; Nishiyama, C.; Yuuki, T.; Mori, A. et al. (1997): Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. In *Nature biotechnology* 15 (8), pp. 754–758. DOI: 10.1038/nbt0897-754.

Takase, M.; Fukuwatari, Y.; Kawase, K.; Kiyosawa, I.; Ogasa, K.; Suzuki, S.; Kuroume, T. (1979): Antigenicity of Casein Enzymatic Hydrolysate. In *Journal of Dairy Science* 62 (10), pp. 1570–1576. DOI: 10.3168/jds.S0022-0302(79)83463-3.

Valenta, Rudolf (2002): The future of antigen-specific immunotherapy of allergy. In *Nature reviews. Immunology* 2 (6), pp. 446–453. DOI: 10.1038/nri824.

Valenta, Rudolf; Kraft, Dietrich (2002): From allergen structure to new forms of allergen-specific immunotherapy. In *Current opinion in immunology* 14 (6), pp. 718–727.

Whitehurst, Robert J.; van Oort, Maarten (Eds.) (2010): Enzymes in food technology. 2nd ed. West Sussex, U.K., Ames, Iowa: Wiley-Blackwell.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000817	October 5, 2018
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*
   ☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person Candice Cryne		Position or Title Regulatory Affairs Manager	
	Organization *(if applicable)* AB Enzymes GmbH			
	Mailing Address *(number and street)* Feldbergstr. 78			
City Darmstadt		State or Province	Zip Code/Postal Code D-64293	Country Germany
Telephone Number +49(0)6151/3680-100		Fax Number +49(0)6151/3680-120	E-Mail Address candice.cryne@abenzymes.com	

1b. Agent or Attorney *(if applicable)*	Name of Contact Person		Position or Title	
	Organization *(if applicable)*			
	Mailing Address *(number and street)*			
City		State or Province	Zip Code/Postal Code	Country
Telephone Number		**Fax Number**	**E-Mail Address**	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Serine Endopeptidase (IUBM 3.4.21.65) from a Genetically Modified Trichoderma reesei

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

The Serine Endopeptidase enzyme is to be used as a processing aid for partial or extensive hydrolysis of proteins from both animal and vegetable sources.

Recommended Use levels = 10 mg TOS of enzyme preparation/kg RM

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☒ Yes ☒ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Candice Cryne
(name of notifier)

has concluded that the intended use*(s)* of Serine Endopeptidase (IUBM 3.4.21.65) from a Genetically Modified Trichoderma reesei
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. AB Enzymes GmbH
(name of notifier)
agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Feldbergstrasse 78 Darmstadt, 64293 Germany
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
Candice Cryne Digitally signed by Candice Cryne Date: 2018.07.18 12:22:03 -07'00'	Candice Cryne	07/18/2018

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	1_RF8963 composition report.pdf	Submission
	2 - Flow chart of the manufacturing process_with control steps. pdf	Submission
	3_Parzia Johnson Decision Tree.pdf	Submission
	GRAS Notice Protease_7182018	Submission

Viebrock, Lauren

From: Cryne, Candice <Candice.Cryne@abenzymes.com>
Sent: Wednesday, May 29, 2019 12:57 AM
To: Viebrock, Lauren
Subject: RE: [External] Questions regarding GRN 817
Attachments: B_RF8963_protease_allergen_hits_report.docx; MSDS.pdf; Serine Endopeptidase Clarification Response May 27 2019.pdf

Hi Lauren,

I did not hear back from you, so I just am providing to you the report to ensure I don't pass the deadline set.

Please let me know if further clarification is needed.

Best, C

From: Cryne, Candice
Sent: Thursday, May 23, 2019 12:23 PM
To: 'Viebrock, Lauren' <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] Questions regarding GRN 817

Hi Lauren,

The issue is the amino acid sequence, is it possible to provide the written report and black out our amino acid sequence?

Thanks!

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Friday, May 17, 2019 7:06 AM
To: Cryne, Candice <Candice.Cryne@abenzymes.com>
Subject: RE: [External] Questions regarding GRN 817

Hi Candice,

A narrative on how the searches were done and conclusions should be sufficient. Since a GRAS conclusion cannot be based on confidential information, it would be best not to include that information in your response.

Thanks,

Lauren

From: Cryne, Candice <Candice.Cryne@abenzymes.com>
Sent: Thursday, May 16, 2019 11:52 AM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: [External] Questions regarding GRN 817

Hi Lauren,

Thanks for the response and questions.

Regarding the bioinformatics analysis for the allergens, I think it would be more helpful to provide to you our data and the conclusions from our data, however this contains some confidential information relating to the amino acid sequence. In that case, are you able to keep this information confidential?

Thanks!

Candice

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Wednesday, May 15, 2019 1:56 PM
To: Cryne, Candice <Candice.Cryne@abenzymes.com>
Subject: [External] Questions regarding GRN 817

Dear Ms. Cryne,

During our review of GRAS Notice No. 000817, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock]@fda.hhs.gov

Marja Paloheimo
27 April, 2018

In silico analysis of the allergenicity potential of the *T. reesei* RF8963 protease

1. Purpose of the study

The amino acid sequence of the recombinant subtilisin-like serine protease produced by *T. reesei* RF8963 was previously aligned with sequences in the Allergen Online (FARRP) database (see a separate report). Alignments were done with the full-length mature protease sequence and using the 80-mer sliding window approach, as recommended by the European Food Safety Authority (EFSA, 2010). The purpose of the study was to determine whether the protease produced by RF8963 and intended for use in food applications has sequence identity to known allergens.

From the above alignments**, hits to six allergens were obtained which had sequence identity to the query sequence above 35 %** which is the set limit of identity indicative for a possible allergenic cross-reactivity (Table 1). Identical hit sequences were found with both the methods used (alignment of the entire query amino acid sequence and alignment using the sliding 80-mer sliding window to sequences in allergen online database).

As six hits were found in the sequence alignment study, additional analysis was made to further confirm whether there are concerns regarding the RF8963 protease in causing food allergy. In this study the allergenic potential of each of the hit sequence obtained in the Allergen Online database search (Table 1) was studied in more details with the methods described below.

a) A secondary search was made to search for exact matches of short stretches (at least 6 amino acids and at least 8 amino acids) in sequences that could serve as potential IgE binding sites. As the matches with six contiguous amino acids is not regarded conclusive due to high amounts of false positives (EFSA, 2010 and references therein), the analysis concentrated on the sequence stretches having 8 or more identical contiguous amino acids.

b) The potential B cell epitopes were predicted from the RF8963 protease amino acid sequence (http://www.iedb.org).

c) Susceptibility of RF8963 protease to digestion in the digestion track; peptidases, trypsins and endopeptidases (https://web.expasy.org/peptide_cutter/)

2. Hits of the RF8963 protease sequence to sequences of proteins described as allergens

The hits to allergens obtained in the alignments done in the separate study are described in Table 1, including the identity percentage and the length (in amino acids) of the overlap. All the hit sequences derive from Ascomycetes (filamentous fungi).

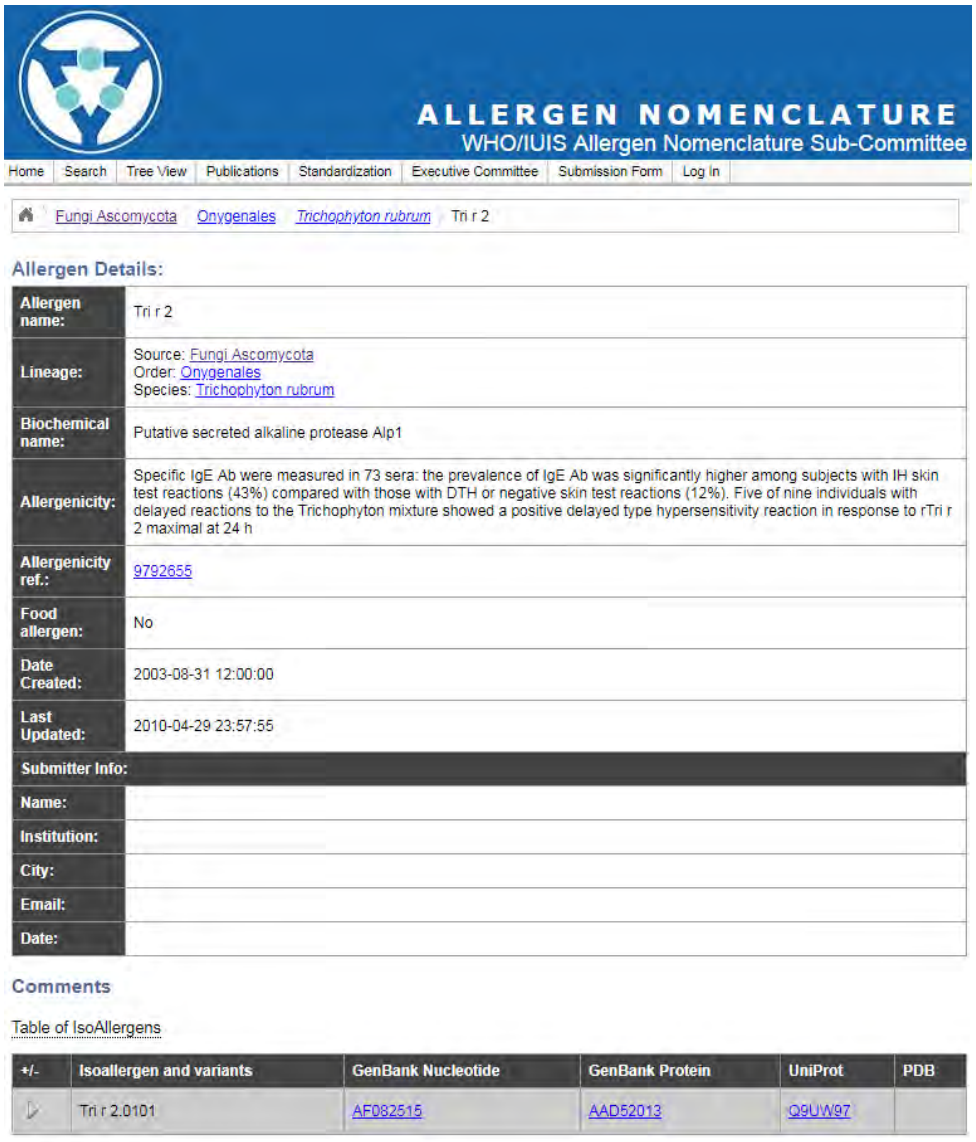
Table 1. Hit sequences obtained from the Allergen Online (FARRP) database for the RF8963 recombinant protease.

Hit sequence (NCBI)	Description	Organism	Identity %	Overlap in identity region (amino acids)
23894240	Tri m2 allergen	*Arthroderma banhamiae*	56.9	269
5813790	Tri r2 allergen	*Trichophyton rubrum*	57.3	286
23894244	Tri m2 allergen	*Arthroderma banhamiae*	57.1	280

6684758	Allergen Pen n 13	*Penicillium chrysogenum*	58.4	279
7963902	Allergen Pen n 18	*Penicillium chrysogenum*	43.0	300
74665726	Allergen Asp fl 1	*Aspergillus flavus*	48.9	282

The alignments of the mature RF8963 protease sequence with the allergen hit sequences and the search results for exact matches of short amino acid stretches are shown for all the hit sequences in the next pages (Figs. 3 – 7).

Of the hit proteins (Table 2), only Tri r2 is included in the WHO/IUIS Allergen list of allergens (www.allergen.ort). In the WHO/IUIS data sheet regarding Tri r2 it is described that **Tri r2 is not a food allergen** (Fig. 1). Thus, it is concluded that the most relevant peptides regarding their allergenic potential are those found from the alignments of the RF8963 protease with Tri m2, Pen n 13, Pen n 18 and Asp fl 1.



ALLERGEN NOMENCLATURE
WHO/IUIS Allergen Nomenclature Sub-Committee

Home | Search | Tree View | Publications | Standardization | Executive Committee | Submission Form | Log In

Fungi Ascomycota | Onygenales | *Trichophyton rubrum* | Tri r 2

Allergen Details:

Allergen name:	Tri r 2
Lineage:	Source: Fungi Ascomycota Order: Onygenales Species: Trichophyton rubrum
Biochemical name:	Putative secreted alkaline protease Alp1
Allergenicity:	Specific IgE Ab were measured in 73 sera: the prevalence of IgE Ab was significantly higher among subjects with IH skin test reactions (43%) compared with those with DTH or negative skin test reactions (12%). Five of nine individuals with delayed reactions to the Trichophyton mixture showed a positive delayed type hypersensitivity reaction in response to rTri r 2 maximal at 24 h
Allergenicity ref.:	9792655
Food allergen:	No
Date Created:	2003-08-31 12:00:00
Last Updated:	2010-04-29 23:57:55
Submitter Info:	
Name:	
Institution:	
City:	
Email:	
Date:	

Comments

Table of IsoAllergens

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▷	Tri r 2.0101	AF082515	AAD52013	Q9UW97	

Fig. 1. Tri r2 is not a food allergen, basing on information from WHO(IUIS) Allergen Nomenclature sub-committee.

Marja Paloheimo
27 April, 2018

Alignment of RF8963 protease with Tri m2

In the alignments, hits to two database entries described as Tri m2 allergens were obtained. Tri m2 sequences, according to the database entries, encode proteinase K like S8 peptidase. In Uniprot GO the molecular function is described as protein having serine-type endopeptidase activity.

An alignment of the two Tri m2 database sequences was performed (Fig. 2). The alignment shows that the other of the hits (23894240) is a partial sequence (as also described in the database). The two Tri m2 entries also have some minor single amino acid differences in their amino acid sequences (Fig. 2). The identity between the sequences in corresponding regions (amino acids 1 – 291 and 103 – 392 in 23894240 and 23894244, respectively) was 95%.

```
Tri m2 (23894240         ---------------------------------------------------
Tri m2 (23894244       1 fitkaipivlaalsavngakileagphaetipnkyivvmkkdvsdeafst

Tri m2 (23894240         ---------------------------------------------------
Tri m2 (23894244      51 httwlsqnlnrrlmrrsgsskamagmqnkyslggifraysgefddamikd

Tri m2 (23894240       1 --nhddvdyiepdfvvrtstngtnltrqenvpswglarvgskkaggttyy
Tri m2 (23894244     101 is.....................................q.......

Tri m2 (23894240      49 ydssagkgvtayiidtgidinhedfggrakwgknfvdkmdedcnghgthv
Tri m2 (23894244     151 ............v.......e.................qr...........

Tri m2 (23894240      99 agtvggtkyglakgvtlvavkvldcdgsgsnsgviegmewamreasgggn
Tri m2 (23894244     201 ............s.s...................r..........-...

Tri m2 (23894240     149 gtakaagkavmnmslggprsqasndaakaisdagifmavaagnenmdaqh
Tri m2 (23894244     250 ........s..................r...e..................

Tri m2 (23894240     199 sspasepsvctvaasteddgkaefsnygavvdvyapgkditslkpggstd
Tri m2 (23894244     300 ...................................................

Tri m2 (23894240     249 tlsgtsmasphvcglgayliglgkqggpglcdtikemaneaiq-------
Tri m2 (23894244     350 ...................................q.......rpgegtt

Tri m2 (23894240         -----
Tri m2 (23894244     400 gkliy
```

Fig. 2. Alignment of the two Tri m2 allergen hit sequences. The four amino acid stretches with 8 or more contiguous amino acids identical to the RF8963 (Fig. 2) are boxed and the five amino acid stretches with 6 or 7 contiguous identical amino acids are underlined.

The full-length Tri m2 sequence 23894244 was used in search of the amino acid stretches of more than 6 contiguous identical amino acids in Tri m2 and RF8963 protease. In the alignment, two stretches having 6 contiguous amino acids, three with 7, one with 8, one with 10 and two with 11 contiguous stretches were found (Fig. 2.). The two Tri m2 sequence entries contained identical amino acids in these sequence stretches (Fig. 2).

```
>>gi|23894244|gid|313|tri m 2 allergen [Arthroderma benhamiae]                (404 aa)
 initn: 1054 init1: 522 opt: 1057  Z-score: 1224.6  bits: 235.0 E(): 4.3e-063
Smith-Waterman score: 1057; 57.1% identity (81.4% similar) in 280 aa overlap (2-276:125-404)

                                                      10        20        30        40
protea                                           ALVTQSNAPSWGLGRISNRQAGIRDYHYDDSAGEGVIVYDV
                                                 :. : :.:::::.:...:::   :.::.:::.:: .: .
gi|238 IFRAYSGEFDDAMIKDISNHDDVDYIEPDFVVRTSTNGTNLTRQENVPSWGLARVGSKQAGGTTYYYDSSAGKGVTAYVI
           90        100       110       120       130       140       150       160

               50        60        70        80        90       100       110       120
protea DTGIDISHPDFEGRAIWGSNHVDRVNQDQNGHGTHVAGTIGGRAYGVAKKATIVAVKVLDAQGSGTISGIIAGLDWSVNH
       :::::: : :: ::: ::.: ::. ..: ::::::::::.::  ::.::....:::::::: .:::. ::.: :..:.. .
gi|238 DTGIDIEHEDFGGRAKWGKNFVDQRDEDCNGHGTHVAGTVGGTKYGLAKSVSLVAVKVLDCDGSGSNSGVIRGMEWAMRE
          170       180       190       200       210       220       230       240

             130           140       150       160       170       180       190
protea ARQNGVTRRAA----LNMSLGGGRSISFNQAAASAVQAGLFVAVAAGNEGQNAGNTSPASEPSVCTVGATSSNDAATSWS
       :  .. : .::    .::::::: :: . :.:: .  .::.:.:::::::..: ..:::::::::::.:.. .:. . .:
gi|238 ASGGNGTAKAAGKSVMNMSLGGPRSQASNDAARAISEAGIFMAVAAGNENMDAQHSSPASEPSVCTVAASTEDDGKAEFS
          250       260       270       280       290       300       310       320

       200       210       220       230       240       250       260       270
protea NYGSVVDVYAPGDAIVSTWPGGGSRSLSGTSMASPHVAGLGAYLIALEGISGGSVCDRIKELAQPVVQ-PGPGTTNRLIY
       :::.:::::::  :.:  :::.. .:::::::::::: ::::::.:   .: ..:: ::.:. ..: :: :::..:::
gi|238 NYGAVVDVYAPGKDITSLKPGGSTDTLSGTSMASPHVCGLGAYLIGLGKQGGPGLCDTIKQMANEAIQRPGEGTTGKLIY
          330       340       350       360       370       380       390       400

        280
protea NGSGR
```

Fig. 3. Alignment of the RF8963 protease with Tri m2. The stretches of 6 or 7 identical amino acids in the sequence are highlighted with yellow and those of 8 – 11 with purple.

Alignment of RF8963 protease with Tri r2

The Tri r2 sequence similarity searches show that it contains all the conserved residues characteristic of the class D subtilase subfamily (41 – 58 % overall sequence identity; Woodfolk *et al*., 1998). As already mentioned, Tri r2 is not a food allergen (Fig. 1).

In the alignment of Tri r2 with RF8963 amino acid sequence, two stretches having 6 contiguous amino acids, four with 7, one with 8, one with 10 and two with 11 contiguous stretches were found (Fig. 4). The long (8 or more contiguous amino acids) hit stretches were identical to those obtained from the Tri m2 alignment (Fig. 3, Table 2). As Tri r2 is not a food allergen, it can be concluded that these hits are not relevant regarding allergenicity. As identical hits were obtained from the alignment of Tri m2 with RF8963 protease, it can be concluded that Tri m2 is not expected to be food allergen, either.

```
>>gi|5813790|gid|313|Tri r 2 allergen [Trichophyton rubrum]                    (412 aa)
 initn: 1129 init1: 517 opt: 1092  Z-score: 1264.9  bits: 242.5 E(): 2.4e-065
Smith-Waterman score: 1092; 57.3% identity (82.2% similar) in 286 aa overlap (2-281:127-412)

                                                      10        20        30        40
protea                                           ALVTQSNAPSWGLGRISNRQAGIRDYHYDDSAGEGVIVYDV
                                                 :. :.:.:::::.:.... :   :.:: :::.:: .: .
gi|581 IFRAYSGEFDDAMIKDISSHDDVDFIEPDFVVRTTTNGTNLTHQDNVPSWGLARVGSKKPGGTTYYYDPSAGKGVTAYII
         90        100       110       120       130       140       150       160

               50        60        70        80        90       100       110       120
protea DTGIDISHPDFEGRAIWGSNHVDRVNQDQNGHGTHVAGTIGGRAYGVAKKATIVAVKVLDAQGSGTISGIIAGLDWSVNH
       :::::::: ::.::: :: : ::. : : ::::::::::.::  ::.:: ...:::::::: .:::. ::.: :..:.. .
gi|581 DTGIDIDHEDFQGRAKWGENFVDQQNTDCNGHGTHVAGTVGGTKYGLAKGVSLVAVKVLDCDGSGSNSGVIKGMEWAMRQ
        170       180       190       200       210       220       230       240

                  130       140       150       160       170       180       190
protea ARQ--NGVTR---RAALNMSLGGGRSISFNQAAASAVQAGLFVAVAAGNEGQNAGNTSPASEPSVCTVGATSSNDAATSW
       :    ::...   ...:::::::: :: . :::: .  .::.:.::::::...: ..:::::::::::.:....:. ...
gi|581 ASGGGNGTAKAAGKSVMNMSLGGPRSEASNQAAKAISDAGIFMAVAAGNENMDAQHSSPASEPSVCTVAASTKDDGKADF
        250       260       270       280       290       300       310       320

        200       210       220       230       240       250       260        270
protea SNYGSVVDVYAPGDAIVSTWPGGGSRSLSGTSMASPHVAGLGAYLIALEGISGGSVCDRIKELAQPVVQ-PGPGTTNRLI
       ::::.:::::::::  :.:  :::.. .:::::::::::: ::::::::   .: ..:: ::..:. :.: :: :::..::
gi|581 SNYGAVVDVYAPGKDITSLKPGGSTDTLSGTSMASPHVCGLGAYLIGLGKQGGPGLCDTIKKMANDVIQSPGEGTTGKLI
        330       340       350       360       370       380       390       400

         280
protea YNGSGR
       :::::.
gi|581 YNGSGK
        410
```

Fig. 4. Alignment of the RF8963 protease with Tri r2. The stretches of 6 or 7 identical amino acids in the sequence are highlighted with yellow and those of 8 – 11 with purple.

Alignment of RF8963 protease with Pen n 13

In Uniprot GO the molecular function of Pen n 13 is described as a protein having serine-type endopeptidase activity. Pen n 13 is an alkaline serine protease that shares more than 39 % identical residues with other kinds of mold allergens (Chow *et al*., 2000).

In the alignment with RF8963 amino acid sequence, three stretches having 6 contiguous amino acids, one with 7, one with 8 and one with 9 contiguous stretches were found (Fig. 5). The identical amino acid stretches were not the same as those hit in Tri m2 and Tri r2 sequences (Figs. 3 and 4, respectively; Table 2).

```
>>gi|6684758|gid|244|allergen Pen n 13 [Penicillium chrysogenum]              (397 aa)
 initn: 1096 init1: 1065 opt: 1108  Z-score: 1283.6  bits: 245.9 E(): 2.2e-066
Smith-Waterman score: 1108; 58.4% identity (86.4% similar) in 279 aa overlap (1-278:116-394)

                                                      10        20        30        40
protea                                              ALVTQSNAPSWGLGRISNRQAGIRDYHYDDSAGEGVIVYD
                                                    : :.:::.:::::.:::....:  .: ::..:::::. :
gi|668 HFEINGLKGYTASFDENTAKDIANDPAVKYIEPDMIVNATANVVQSNVPSWGLARISSKRTGTTSYTYDSTAGEGVVFYG
          80        90       100       110       120       130       140       150

               50        60        70        80        90       100       110       120
protea VDTGIDISHPDFEGRAIWGSNHVDRVNQDQNGHGTHVAGTIGGRAYGVAKKATIVAVKVLDAQGSGTISGIIAGLDWSVN
       ::::::::: :: ::: ::.: ::  : : :::::::.:.: .:  :::::::::.:::::: :.:::: ::.:.:.::.:.
gi|668 VDTGIDISHSDFGGRAKWGTNVVDNDNTDGNGHGTHTASTAAGSKYGVAKKATLVAVKVLGADGSGTNSGVISGMDWAVK
         160       170       180       190       200       210       220       230

              130       140       150       160       170       180       190       200
protea HARQNGVTRRAALNMSLGGGRSISFNQAAASAVQAGLFVAVAAGNEGQNAGNTSPASEPSVCTVGATSSNDAATSWSNYG
        :.. :.. . ..: ::::  : . :.::::.:.:.:.:::::::.::.:.::::   .::.:.:.:.:...:..:.:
gi|668 DAKSRGANGKYVMNTSLGGEFSKAVNDAAANVVKSGIFLSVAAGNEAENASNSSPASAAEACTIAASTSTDGSASFTNFG
         240       250       260       270       280       290       300       310

              210       220       230       240       250       260       270
protea SVVDVYAPGDAIVSTWPGGGSRSLSGTSMASPHVAGLGAYLIALEGISGGSVCDRIKELAQPVVQPGP-GTTNRLIYNGS
       ::::.:::::.:....:::::..:::::::.:::::.:::.::::.:..: :: .::   .. .: ::::.:.:::
gi|668 SVVDLYAPGQSITAAYPGGGSKTLSGTSMAAPHVAGVAAYLMALEGVSAGNACARIVQLATSSISRAPSGTTSKLLYNGI
         320       330       340       350       360       370       380       390

     280
protea GR

gi|668 NV
```

Fig. 5. Alignment of the RF8963 protease with Pen n 13. The stretches of 6 or 7 identical amino acids in the sequence are highlighted with yellow and those of 8 – 11 with purple.

Alignment of RF8963 protease with Pen n 18

In Uniprot GO the molecular function of Pen n 18 is described, like Pen n 13, as a protein having serine-type endopeptidase activity. The deduced amino acid sequence shows extensive similarity with those of vacuolar serine proteases from various fungi (Yu *et al*., 2002)

In the alignment with RF8963 amino acid sequence, four stretches having 6 contiguous amino acids, one with 7 and with 9 contiguous stretches were found (Fig. 6). The 9 amino acids contiguous stretch was overlapping the 11 amino acid stretch found from Tri m2 and Tri r2 searches. As Tri r2 is not a food allergen, it is expected that this 9 amino acid peptide is not relevant regarding food allergenicity.

Yu *et al.* (2002) describe the location of human IgE-binding epitopes of Pen n18. These are marked in Fig. 6. No corresponding identical sequences were found from the RF8963 protease sequence (Fig. 6).

```
>>gi|7963902|gid|243|allergen Pen n 18 [Penicillium chrysogenum]              (494 aa)
 initn: 712 init1: 265 opt: 671  Z-score: 777.2  bits: 152.5 E(): 3.6e-038
Smith-Waterman score: 724; 43.0% identity (69.7% similar) in 300 aa overlap (5-280:142-440)

                                                    10        20            30        40
protea                                          ALVTQSNAPSWGLGRISNRQA----GIRDYHYDDSAGEGVIVYD
                                                ..:::: :::.:::.:..    ..  : : . .:::: .:
gi|796 SLLGYAGHFHEDVIEQIRRHPDVDYIEKDSEVRTMSEGSVEKNAP-WGLARISHRESLSFGNFNKYLYAEEGGEGVDAYV
             110       120       130       140       150       160       170       180

               50        60         70        80        90       100       110
protea VDTGIDISHPDFEGRAIWGSN-HVDRVNQDQNGHGTHVAGTIGGRAYGVAKKATIVAVKVLDAQGSGTISGIIAGLDWSV
       .::: ...: :::::: ::..     ...: :::::: .:::.:. .:::::.. ::::: ..::::.: .. :..:..
gi|796 IDTGANVKHVDFEGRANWGKTIPQGDADEDGNGHGTHCSGTIAGKKFGVAKKANVYAVKVLRSNGSGTMSDVVKGVEWAA
             190       200       210       220       230       240       250       260

     120        130           140       150       160       170       180       190
protea N-HARQNGVTRR----AALNMSLGGGRSISFNQAAASAVQAGLFVAVAAGNEGQNAGNTSPASEPSVCTVGATSSNDAAT
       . : ...    .    .. ::::::: : ... :. .::.::.  :::::.. .: : :::.  .. :::::.  :  .
gi|796 EAHIKKSKKGDKKFKGSVANMSLGGGSSRTLDLAVNAAVDAGIHFAVAAGNDNADACNYSPAAAEKAITVGASTLADERA
             270       280       290       300       310       320       330       340

          200       210         220       230       240         250           260
protea SWSNYGSVVDVYAPGDAIVSTWPGG--GSRSLSGTSMASPHVAGLGAYLIALEGI--SGGSVCD----RIKELAQPVVQP
        .:::::. .:.:::  :.::: :.  .. ..:::::::::::: :: ..:     :. .: :    ..:     :.
gi|796 YFSNYGKCTDIFAPGLNILSTWVGSDHATNTISGTSMASPHIAGLLAYYVSLAPAKDSAYAVADVTPKQLKAALISVATE
             350       360       370       380       390       400       410       420

           270       280
protea G-----PGTT-NRLIYNGSGR
       :     :. : : : .::.:
gi|796 GTLTDIPSDTPNLLAWNGGGSANYTKILADGGYKAHNAETTVEDRIGIIIDSAEKAFHKELGAIYSEIKDAVSV
             430       440       450       460       470       480       490
```

Fig. 6. Alignment of the RF8963 protease with Pen n 18. The stretches of 6 or 7 identical amino acids in the sequence are highlighted with yellow and those of 8 – 11 with purple. The human IgE-binding epitopes of Pen n18 described in Yu *et al*. (2002) are underlined.

Alignment of RF8963 protease with Asp fl 1

In Uniprot GO the molecular function of Asp fl 1 is described, like Pen n 13 and Pen n 18, as a protein having serine-type endopeptidase activity. Asp fl 1 has a degree of identity in a range of 27 to 84 % among related allergens, e.g. those derived from bacterial allergen subtilisin and mold allergen Pen c 1 (Yu *et al*., 1999).

In the alignment of Asp fl 1with RF8963 amino acid sequence, three stretches having 6 contiguous amino acids and one with 7 contiguous stretches were found but no stretches having 8 or more contiguous amino acids were found (Fig. 7).

```
>>gi|74665726|gid|317|Allergen Asp fl 1                                  (403 aa)
 initn: 726 init1: 333 opt: 842  Z-score: 976.0  bits: 189.0 E(): 3e-049
Smith-Waterman score: 842; 48.9% identity (79.1% similar) in 282 aa overlap (2-279:123-402)

                                                      10        20        30        40
protea                                          ALVTQSNAPSWGLGRISNRQAGIRDYHYDDSAGEGVIVYDV
                                                 :.:::.:: :::: ::..     :: :: :::::. .: :
gi|746 YKINKFAAYAGSFDDATIEEIRKNEDVAYVEEDQIYYLDGLTTQKSAP-WGLGSISHKGQQSTDYIYDTSAGEGTYAYVV
            90        100       110       120       130       140       150       160

              50        60        70        80        90       100       110       120
protea DTGIDISHPDFEGRAIWGSNHVDRVNQDQNGHGTHVAGTIGGRAYGVAKKATIVAVKVLDAQGSGTISGIIAGLDWSVNH
       :.:....: .:::::  . : .   . :. ::::::.:::.:..::.::::.:..::::.....:.: : :. :..:..:
gi|746 DSGVNVDHEEFEGRASKAYNAAGGQHVDSIGHGTHVSGTIAGKTYGIAKKASILSVKVFQGESSST-SVILDGFNWAAND
            170       180       190       200       210       220       230       240

            130       140       150       160       170       180       190       200
protea ARQNGVTRRAALNMSLGGGRSISFNQAAASAVQAGLFVAVAAGNEGQNAGNTSPASEPSVCTVGATSSNDAATSWSNYGS
         ..  : .::.::::::: : .::.:. .: . :.. .::::::..::.::::: :.. ::.: ....  .:.::.:.
gi|746 IVSKKRTSKAAINMSLGGGYSKAFNDAVENAFEQGVLSVVAAGNENSDAGQTSPASAPDAITVAAIQKSNNRASFSNFGK
              250       260       270       280       290       300       310       320

            210       220         230       240        250       260        270
protea VVDVYAPGDAIVSTWPGGGS--RSLSGTSMASPHVAGLGAYLIALEGISG-GSVCDRIKELA-QPVVQPGPGTTNRLIYN
       ::::.:::. :.:.: :..:   ..:::::.::..::. :: :::...: ..:  ::.::: . ::.   :. : : ::
gi|746 VVDVFAPGQDILSAWIGSSSATNTISGTSMATPHIVGLSLYLAALENLDGPAAVTKRIEELATKDVVKDVKGSPNLLAYN
            330       340       350       360       370       380       390       400

       280
protea GSGR
       :.
gi|746 GNA
```

Fig. 7. Alignment of the RF8963 protease with Asp fl 1. The stretches of 6 or 7 identical amino acids in the sequence are highlighted with yellow and those of 8 – 11 with purple.

Summary of search results of exact match short stretches in RF8963 protease and the allergen hit sequences

Altogether seven exact match sequences with eight or more contiguous amino acids were found from the alignments of the RF8963 mature protease and the hit sequences from the allergen database (Table 2).

Table 2. The sequences with eight or more contiguous amino acids found from the RF8963 protease and the allergen hit protein sequences. From Asp fl1 and RF8963 sequence comparison only stretches with 6 or 7 contiguous amino acids were found.

No	Peptide	Length (amino acids)	Location in RF8963 protease sequence	Identity in allergen
1	VDTGIDISH	9	41 – 49	Pen n 13
2	NGHGTHVAGT	10	71 – 80	Tri m2, Tri r2
3	YGVAKKAT	8	86 – 93	Pen n 13
4	SPASEPSVCTV	11	174 – 184	Tri m2, Tri r2
5	VVDVYAPG	8	202 – 209	Tri m2, Tri r2
6	LSGTSMASPHV	11	224 – 234	Tri m2, Tri r2
7	SGTSMASPH	9	225 – 233	Pen n 18

Tri r2 is not a food allergen (Fig. 1) and it is thus expected that the four exact match peptides (numbers 2, 4, 5 and 6 in Table 2) from this protein are not relevant regarding food allergenicity. Identical peptides were obtained from the alignment of Tri m2 with the RF8963 mature protease sequence. The 9 amino acids long peptide from Pen n 18 (peptide no 7) is included in the peptide no 6 from Tri r2 (Table 2) and thus this peptide is also not expected to be relevant regarding food allergenicity. In addition, no corresponding identical sequences were found from the RF8963 protease sequence to those described as human IgE-binding epitopes in Pen n 18 (Fig. 6, Yu *et al.,* 2002).

Basing on the above results, it is concluded that the most relevant peptides regarding their allergenic potential are those found from the alignments of the RF8963 protease sequence with Pen n 13 (peptides no 1 and 3 in Table 2). The relevance of these peptides is discussed in connection to the results obtained in the epitope mapping study (see below).

3. Epitope mapping of RF8963 protease

There are various bioinformatics tools available for prediction of the likely antigenic epitopes in proteins. According to EFSA (2010) it is not straightforward how results from the predictions should be viewed upon in risk assessment of allergenicity. However, to exclude the putative allergenicity of RF8963 protease (Fig. 7), *in silico* sequence search using B-cell epitope prediction algorithm was performed.

The tool available at the Immune epitope database and analysis resource site (IEDB; http://www.iedb.org/home_v3.php) was used in the analysis. The IEDB site contains software and a manually curated database of experimentally characterized immune epitopes. The method of Kolaskar and Tongaonkar using linear amino acid sequence as input was used. This method makes use of physicochemical properties of amino acid residues and their frequencies of occurrence in experimentally known segmental epitopes to predict antigenic determinants on proteins. Application of this method to a large number of proteins has proven that it can predict antigenic determinants with high accuracy.

From the analysis of the mature RF8963 protease sequence, altogether 8 predicted peptides having lengths of 8 – 16 amino acids were obtained (Table 3).

Table 3. Predicted B cell antigenic determinants in the amino acid sequence of RF8963 protease. The analysis is based on B cell epitope prediction tool in http://www.iedb.org/home_v3.php.

No.	Start	End	Peptide	
1	34	42	EGVIVYDVD	9
2	91	102	KATIVAVKVLDA	12
3	150	162	ASAVQAGLFVAVA	13
4	178	186	EPSVCTVGA	9
5	200	215	GSVVDVYAPGDAIVST	16
6	231	245	SPHVAGLGAYLIALE	15
7	249	256	GGSVCDRI	8
8	259	267	LAQPVVQPG	9

The locations in the RF8963 protease sequence of the predicted B cell epitope peptides and the hit peptides with 8 or more contiguous amino acids from the alignment of RF8963 protease with the hits from the allergen database (Table 2) are shown in Fig. 8.

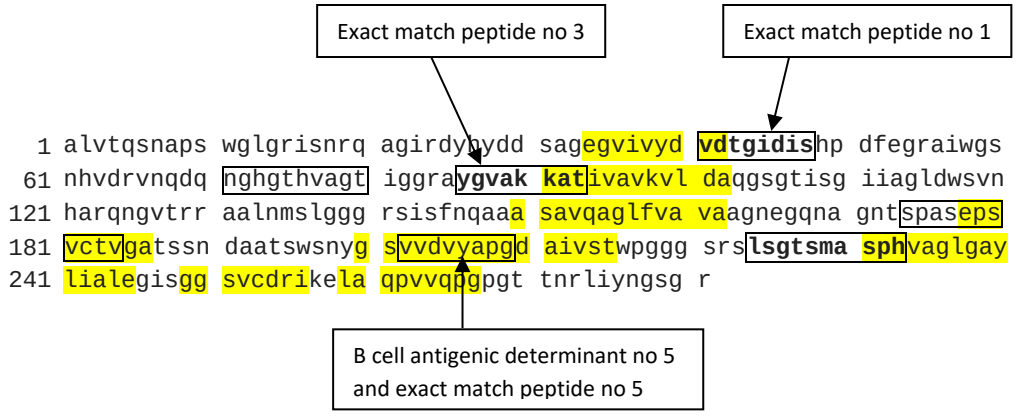


Fig. 7. The location of the B cell antigenic determinants and exact match peptides in the amino acid sequence of mature RF8963 protease. The location of the B cell antigenic determinants are highlighted by yellow. The location of the exact match peptides with 8 or more contiguous amino acids, obtained from the alignment of RF8963 protease with the hits from the allergen database are boxed. The exact match peptides no 1 and 3 deriving from RF8963 and Pen n 13 alignment (no 1 and 3) and no 5, deriving from RF8963 and Pen n 13 and Tri m2/r2 alignments, respectively, are marked with an arrow.

One of the B cell antigenic determinant peptides, no 5 (GSVVDVYAPGDAIVST; Table 3) included the no 5 exact match peptide (VVDVYAPG; Table 2) obtained from the alignment of RF8963 protease with Tri m2 and Tri r2. As Tri r2 is not a food allergen, we expect that this peptide is not relevant regarding food allergy. The location of the exact match peptides (no 1 and 3; Table 2) from the alignment of RF8963 mature protease sequence with Pen n 13 are not identical to the B cell antigenic determinants identified from Pen n 13 by Yu *et al.* (2002). Thus, it can be concluded that these peptide are not relevant regarding food allergenicity, either.

4. Susceptibility of RF8963 protease and exact match contiguous peptides to digestion

The susceptibility of the RF8963 protease, the exact match contiguous peptides (Table 2) and predicted B cell antigenic determinants (Table 3) to proteolytic digestion in the digestive track was evaluated by using the PeptideCutter tool in ExPASy Proteomics Site (https://web.expasy.org/peptide_cutter/). The RF8963 protease has multiple cleavage sites for pepsin, trypsin, chymotrypsin and endopeptidases (Table 4). Many of the protease cleavage sites are located in the exact match peptides (e.g. no 1, 3 and 5; Table 2 and Fig. 7) and B cell antigenic determinant peptides, thus breaking these contiguous amino acid sequence stretches.

Table 4. The number of proteases (pepsins, trypsins, chymotrypsins, endopeptidases) cleaving the RF8963 mature protease sequence basing on PeptideCutter tool results. In addition to the chosen protease groups, additional proteases with different specificities exist which can cleave the RF8963 protease (data not included).

Protease type	Number of cleavage sites in RF8963 mature protease	Position of cleavage sites
Pepsin (pH 1.3)	25	1 2 12 13 52 99 114 115 133 136 137 144 145 156 157 158 223 236 237 240 241 243 244 258 274
Pepsin (pH > 2)	43	1 2 11 12 13 25 27 28 38 39 52 58 85 99 114 115 116 117 133 136 137 144 145 156 157 158 195 196 198 199 205 216 223 236 237 239 240 241 243 244 258 274 276
Trypsin	18	15 19 24 55 65 84 90 91 98 123 129 130 141 222 255 257 273 281
Chymotrypsin (high specificity)	15	11 26 28 39 52 58 86 117 145 158 196 199 206 240 276
Chymotrypsin (low specificity)	36	2 11 13 26 27 28 39 52 58 62 73 76 86 100 115 117 121 133 135 137 145 157 158 196 199 206 224 229 233 237 240 241 244 259 274 276
Endopeptidases (including proline, glutamyl, aspartyl)	21	24 28 29 33 39 41 45 50 52 63 68 100 115 165 177 190 203 209 244 253 257

5. Conclusion

Basing on the results from the analysis done it can be concluded that the RF8963 protease is unlikely posing a risk of food allergenicity.

The exact match peptides in the RF8963 protease sequences and its allergen database alignment hit sequences were analysed and their location was compared to the B cell epitopes predicted *in silico* from the mature RF8963 protease sequence. The putative allergenicity could be excluded. Also, no corresponding sequences to those identified in Yu *et al.* (2002) as human IgE-binding epitopes from Pen n 18 (one of the hit sequences from the allergen database alignment study) were found from the RF8963 protease sequence. In addition, the RF8963 protease contains large number of cleavage sites for the major proteases of the digestive track. Many of these protease cleavage sites are located in the exact match peptides and the predicted B cell antigenic determinant peptides, thus expected to break the contiguous amino acid sequence stretches in the digestive track and thus decreasing the risk of food allergenicity.

References

Chow, L.P., Chiou, S. Hl, Hsiao, M.C., Yu C. J. and Chiang, B.L. 2000. Characterization of Pen n13, a major allergen from the mold *Penicillium notatum*. Biochem. Biophys. Res. Commun. 269: 14 – 20.

EFSA, 2010. Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed by EFSA Panel of Genetically Modified Organisms. EFSA J. 2010; 8(7):1700.

Woodfolk, J.A., Wheatley, L.M., Piyasena, R.V., Benjamin, D.C. and Platts-Mills, T.A. 1998. *Trichophyton* antigens associated with IgE antibodies and delayed type hypersensitivity. Sequence homology to two families of serine proteinases. J. Biol. Chem. 273: 29389 – 29496.

Yu, C.-J., Chiou, S. H., Lai, W. Y., Chiang, B. L. and Chow, L.-P. 1999. Characterization of a novel allergen, a major IgE-ginding protein from *Aspergillus flavus*, as an alkaline serine protease.

Yu, C.-J., Chen, Y.-M., Su, S.-N., Forouhar, F., Lee, S.-H. and Chow, L.-P. 2002. Molecular and immunological characterization and IgE epitope mapping of Pen n18, a major allergen of *Penicillium notatum*. Biochem. J. 363: 707 – 715.

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1. Product identifier

COROLASE® 8000

1.2. Relevant identified uses of the substance or mixture and uses advised against

Use of the substance/mixture

Processing aid

1.3. Details of the supplier of the safety data sheet

Company name:	AB Enzymes GmbH	
Street:	Feldbergstr. 78	
Place:	D-64293 Darmstadt	
Telephone:	+49 (0) 6151-3680-100	Telefax: +49 (0) 6151-3680-120
Internet:	www.abenzymes.com	
Responsible Department:	PES-Ingenieurgesellschaft Benzstr. 2 63768 Hösbach Germany	

1.4. Emergency telephone number:

+49 (0) 700 / 24 112 112 (AECC)

SECTION 2: Hazards identification

2.1. Classification of the substance or mixture

Regulation (EC) No. 1272/2008

Hazard categories:
Serious eye damage/eye irritation: Eye Dam. 1
Respiratory or skin sensitisation: Resp. Sens. 1
Hazardous to the aquatic environment: Aquatic Acute 1
Hazard Statements:
Causes serious eye damage.
May cause allergy or asthma symptoms or breathing difficulties if inhaled.
Very toxic to aquatic life.

2.2. Label elements

Regulation (EC) No. 1272/2008

Hazard components for labelling

protease

Signal word: Danger

Pictograms:

Hazard statements

H318	Causes serious eye damage.
H334	May cause allergy or asthma symptoms or breathing difficulties if inhaled.
H400	Very toxic to aquatic life.

Precautionary statements

P261	Avoid breathing dust/fume/gas/mist/vapours/spray.
P280	Wear protective gloves/protective clothing/eye protection/face protection.

P304+P340	IF INHALED: Remove person to fresh air and keep comfortable for breathing.
P305+P351+P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310	Immediately call a POISON CENTER/doctor.
P342+P311	If experiencing respiratory symptoms: Call a POISON CENTER/doctor.

Labelling of packages where the contents do not exceed 125 ml

Signal word: Danger

Pictograms:

Hazard statements

H318-H334

Precautionary statements

P261-P280-P304+P340-P305+P351+P338-P310-P342+P311

2.3. Other hazards

No information available.

SECTION 3: Composition/information on ingredients

3.2. Mixtures

Hazardous components

CAS No	Chemical name			Quantity
	EC No	Index No	REACH No	
	GHS Classification			
52233-31-5	protease			1 - < 5 %
	822-334-3	647-014-00-9	01-2120772805-45	
	Acute Tox. 4, Skin Irrit. 2, Eye Dam. 1, Resp. Sens. 1, STOT SE 3, Aquatic Acute 1 (M-Factor = 10), Aquatic Chronic 3; H302 H315 H318 H334 H335 H400 H412			

Full text of H and EUH statements: see section 16.

SECTION 4: First aid measures

4.1. Description of first aid measures

After inhalation

Provide fresh air. If breathing is irregular or stopped, administer artificial respiration. Medical treatment necessary.

After contact with skin

After contact with skin, wash immediately with plenty of water and soap. Immediately remove any contaminated clothing, shoes or stockings. In case of skin reactions, consult a physician.

After contact with eyes

Rinse immediately carefully and thoroughly with eye-bath or water. Remove contact lenses, if present and easy to do. Continue rinsing. Consult an ophthalmologist.

After ingestion

Rinse mouth immediately and drink plenty of water. Do NOT induce vomiting. Observe risk of aspiration if vomiting occurs. Never give anything by mouth to an unconscious person or a person with cramps. Get medical advice/attention.

4.2. Most important symptoms and effects, both acute and delayed

Allergic reactions. Respiratory complaints. Risk of serious damage to eyes.

4.3. Indication of any immediate medical attention and special treatment needed

Treat symptomatically.

SECTION 5: Firefighting measures

5.1. Extinguishing media

Suitable extinguishing media

Water, Carbon dioxide, Foam, Powder

Co-ordinate fire-fighting measures to the fire surroundings.

Unsuitable extinguishing media

Full water jet

5.2. Special hazards arising from the substance or mixture

Non-flammable.

5.3. Advice for firefighters

In case of fire: Wear self-contained breathing apparatus. Full protection suit

Additional information

Suppress gases/vapours/mists with water spray jet. Collect contaminated fire extinguishing water separately. Do not allow entering drains or surface water.

SECTION 6: Accidental release measures

6.1. Personal precautions, protective equipment and emergency procedures

Provide adequate ventilation. Do not breathe gas/fumes/vapour/spray. Avoid contact with skin, eyes and clothes. Use personal protection equipment.

6.2. Environmental precautions

Do not allow to enter into surface water or drains.

6.3. Methods and material for containment and cleaning up

Absorb with liquid-binding material (e.g. sand, diatomaceous earth, acid- or universal binding agents). Treat the recovered material as prescribed in the section on waste disposal.

6.4. Reference to other sections

Safe handling: see section 7

Personal protection equipment: see section 8

Disposal: see section 13

SECTION 7: Handling and storage

7.1. Precautions for safe handling

Advice on safe handling

Provide adequate ventilation. Do not breathe gas/fumes/vapour/spray. Avoid contact with skin, eyes and clothes. Use personal protection equipment.

Advice on protection against fire and explosion

No special fire protection measures are necessary.

7.2. Conditions for safe storage, including any incompatibilities

Requirements for storage rooms and vessels

Keep container tightly closed. Keep locked up. Store in a place accessible by authorized persons only. Provide adequate ventilation as well as local exhaustion at critical locations. Store in a cool dry place.

Hints on joint storage

No special measures are necessary.

Further information on storage conditions

Recommended storage temperature: < 10 °C

7.3. Specific end use(s)

Processing aid

SECTION 8: Exposure controls/personal protection

8.1. Control parameters

Exposure limits (EH40)

CAS No	Substance	ppm	mg/m³	fibres/ml	Category	Origin
56-81-5	Glycerol, mist	-	10		TWA (8 h)	WEL

Additional advice on limit values

none

8.2. Exposure controls

Appropriate engineering controls

Provide adequate ventilation as well as local exhaustion at critical locations.

Protective and hygiene measures

Remove contaminated, saturated clothing immediately. Draw up and observe skin protection programme. Wash hands and face before breaks and after work and take a shower if necessary. When using do not eat or drink. Do not breathe gas/fumes/vapour/spray.

Eye/face protection

Suitable eye protection: goggles.

Hand protection

Wear protective gloves.

When handling with chemical substances, protective gloves must be worn with the CE-label including the four control digits. The quality of the protective gloves resistant to chemicals must be chosen as a function of the specific working place concentration and quantity of hazardous substances. For special purposes, it is recommended to check the resistance to chemicals of the protective gloves mentioned above together with the supplier of these gloves.

Skin protection

Wear suitable protective clothing.

Respiratory protection

In case of inadequate ventilation wear respiratory protection.

Environmental exposure controls

Do not allow to enter into surface water or drains.

SECTION 9: Physical and chemical properties

9.1. Information on basic physical and chemical properties

Physical state:	Liquid
Colour:	brown
Odour:	characteristic
pH-Value:	5,4 - 6,4 (100 g/L)

Changes in the physical state

Melting point:	not determined

Initial boiling point and boiling range:	not determined
Flash point:	not determined
Flammability	
Solid:	not applicable
Gas:	not applicable
Explosive properties	
The product is not: Explosive.	
Lower explosion limits:	not determined
Upper explosion limits:	not determined
Ignition temperature:	not determined
Auto-ignition temperature	
Solid:	not applicable
Gas:	not applicable
Decomposition temperature:	not determined
Oxidizing properties	
Not oxidising.	
Vapour pressure:	not determined
Density:	not determined
Water solubility:	not determined
Solubility in other solvents	
not determined	
Partition coefficient:	not determined
Viscosity / dynamic:	not determined
Viscosity / kinematic:	not determined
Vapour density:	not determined
Evaporation rate:	not determined

9.2. Other information

Odour threshold: not determined

SECTION 10: Stability and reactivity

10.1. Reactivity

No hazardous reaction when handled and stored according to provisions.

10.2. Chemical stability

The product is chemically stable under recommended conditions of storage, use and temperature.

10.3. Possibility of hazardous reactions

No known hazardous reactions.

10.4. Conditions to avoid

No information available.

10.5. Incompatible materials

No information available.

10.6. Hazardous decomposition products

No known hazardous decomposition products.

SECTION 11: Toxicological information

11.1. Information on toxicological effects

Acute toxicity

Based on available data, the classification criteria are not met.

CAS No	Chemical name				
	Exposure route	Dose	Species	Source	Method
52233-31-5	protease				
	oral	LD50 1800 mg/kg	Rat	ECHA	OECD 401

Irritation and corrosivity

Causes serious eye damage.

Skin corrosion/irritation: Based on available data, the classification criteria are not met.

Sensitising effects

May cause allergy or asthma symptoms or breathing difficulties if inhaled. (protease)

Carcinogenic/mutagenic/toxic effects for reproduction

Based on available data, the classification criteria are not met.

STOT-single exposure

Based on available data, the classification criteria are not met.

STOT-repeated exposure

Based on available data, the classification criteria are not met.

Aspiration hazard

Based on available data, the classification criteria are not met.

SECTION 12: Ecological information

12.1. Toxicity

Very toxic to aquatic organisms.

CAS No	Chemical name					
	Aquatic toxicity	Dose	[h] | [d]	Species	Source	Method
52233-31-5	protease					
	Acute crustacea toxicity	EC50 0,09 mg/l	48 h	Daphnia magna (Big water flea)	ECHA	OECD 202
	Algea toxicity	NOEC 0,317 mg/l	3 d	Pseudokirchneriella subcapitata	ECHA	OECD 201
	Crustacea toxicity	NOEC 0,56 mg/l	2 d	Daphnia magna (Big water flea)	ECHA	OECD 202

12.2. Persistence and degradability

The product has not been tested.

CAS No	Chemical name			
	Method	Value	d	Source
	Evaluation			
52233-31-5	protease			
	OECD 301B	60 %	7	ECHA
	Readily biodegradable (according to OECD criteria).			

12.3. Bioaccumulative potential

The product has not been tested.

Partition coefficient n-octanol/water

CAS No	Chemical name	Log Pow
52233-31-5	protease	-3,1

12.4. Mobility in soil

The product has not been tested.

12.5. Results of PBT and vPvB assessment

The product has not been tested.

12.6. Other adverse effects

No information available.

Further information

Do not allow to enter into surface water or drains. Do not allow to enter into soil/subsoil.

SECTION 13: Disposal considerations

13.1. Waste treatment methods

Advice on disposal

Do not allow to enter into surface water or drains. Do not allow to enter into soil/subsoil. Dispose of waste according to applicable legislation.

Recommendation Waste code product: 020304

Contaminated packaging

Handle contaminated packages in the same way as the substance itself.

Recommendation Waste code packaging: 150106

SECTION 14: Transport information

Land transport (ADR/RID)

14.1. UN number:	UN 3082
14.2. UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, LIQUID, N.O.S. (protease)
14.3. Transport hazard class(es):	9
14.4. Packing group:	III
Hazard label:	9
Classification code:	M6
Special Provisions:	274 335 375 601
Limited quantity:	5 L
Excepted quantity:	E1
Transport category:	3
Hazard No:	90
Tunnel restriction code:	-

Inland waterways transport (ADN)

14.1. UN number:	UN 3082
14.2. UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, LIQUID, N.O.S. (protease)
14.3. Transport hazard class(es):	9

14.4. Packing group:	III
Hazard label:	9
Classification code:	M6
Special Provisions:	274 335 375 601
Limited quantity:	5 L
Excepted quantity:	E1

Marine transport (IMDG)

14.1. UN number:	UN 3082
14.2. UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, LIQUID, N.O.S. (protease)
14.3. Transport hazard class(es):	9
14.4. Packing group:	III
Hazard label:	9
Special Provisions:	274, 335, 969
Limited quantity:	5 L
Excepted quantity:	E1
EmS:	F-A, S-F

Air transport (ICAO-TI/IATA-DGR)

14.1. UN number:	UN 3082
14.2. UN proper shipping name:	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, LIQUID, N.O.S. (protease)
14.3. Transport hazard class(es):	9
14.4. Packing group:	III
Hazard label:	9
Special Provisions:	A97 A158 A197
Limited quantity Passenger:	30 kg G
Passenger LQ:	Y964
Excepted quantity:	E1
IATA-packing instructions - Passenger:	964
IATA-max. quantity - Passenger:	450 L
IATA-packing instructions - Cargo:	964
IATA-max. quantity - Cargo:	450 L

14.5. Environmental hazards

ENVIRONMENTALLY HAZARDOUS:	yes
Danger releasing substance:	protease

14.6. Special precautions for user

No information available.

14.7. Transport in bulk according to Annex II of Marpol and the IBC Code

not applicable

SECTION 15: Regulatory information

15.1. Safety, health and environmental regulations/legislation specific for the substance or mixture

EU regulatory information

Information according to 2012/18/EU (SEVESO III):	E1 Hazardous to the Aquatic Environment

National regulatory information

Employment restrictions:	Observe restrictions to employment for juvenils according to the 'juvenile work protection guideline' (94/33/EC).
Water contaminating class (D):	3 - highly water contaminating

15.2. Chemical safety assessment

Chemical safety assessments for substances in this mixture were not carried out.

SECTION 16: Other information

Changes

This data sheet contains changes from the previous version in section(s): 2,4,5,9,15,16.

Abbreviations and acronyms

ADR: Accord européen sur le transport des marchandises dangereuses par Route
(European Agreement concerning the International Carriage of Dangerous Goods by Road)
IMDG: International Maritime Code for Dangerous Goods
IATA: International Air Transport Association
GHS: Globally Harmonized System of Classification and Labelling of Chemicals
EINECS: European Inventory of Existing Commercial Chemical Substances
ELINCS: European List of Notified Chemical Substances
CAS: Chemical Abstracts Service
LC50: Lethal concentration, 50%
LD50: Lethal dose, 50%

Classification for mixtures and used evaluation method according to Regulation (EC) No. 1272/2008 [CLP]

Classification	Classification procedure
Eye Dam. 1; H318	Calculation method
Resp. Sens. 1; H334	Calculation method
Aquatic Acute 1; H400	Calculation method

Relevant H and EUH statements (number and full text)

H302	Harmful if swallowed.
H315	Causes skin irritation.
H318	Causes serious eye damage.
H334	May cause allergy or asthma symptoms or breathing difficulties if inhaled.
H335	May cause respiratory irritation.
H400	Very toxic to aquatic life.
H412	Harmful to aquatic life with long lasting effects.

Further Information

The information is based on present level of our knowledge. It does not, however, give assurances of product properties and establishes no contract legal rights. The receiver of our product is singulary responsible for adhering to existing laws and regulations.

(The data for the hazardous ingredients were taken respectively from the last version of the sub-contractor's safety data sheet.)

To: Lauren VieBrock

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety, DFA

Contact Regulatory Affairs
Tel +49(0)6151/3680-100
E-Mail info@abenzymes.com
Darmstadt 2019-05-28

RE: Clarification Request – FDA / FSIS – Corolase 8000, Serine Endopeptidase:

1. **Please provide the CAS No. for the enzyme.**
   A. The CAS No. for the protease enzyme is 52233-31-5

2. **Please provide the strain name for the T. reesei strain used as the recipient strain.**
   A. The strain name is a *Trichoderma reesei* strain, which is a mutant strain derived from Trichoderma reesei RF4847, a classical mutant originating from QM6a. AB Enzymes uses an internal number code name, but that is confidential.

3. **Please confirm that the enzyme is secreted during fermentation.**
   A. The enzyme is secreted during fermentation

4. **The formulation information is provided as a "typical enzyme preparation" on p. 20 (Table 2.5.2) in the notice. Please confirm that this is the formulation for this enzyme preparation**
   A. Yes this is the formulation, the % can change very slightly depending on the enzyme concentrate.

5. **Please provide further clarification on the potential allergenicity of the enzyme.**
   a. On Pg. 25, you state: "Altogether 25 hits with sequence identity above the set 35 % to the query sequence were obtained. All the hits were to serine endopeptidase …. Six of the hit sequences were described as allergens in the database …"

   This statement implies that 19 (25-6) hits were "putative" allergens. Please confirm or provide clarification. In addition, please describe in more detail the relationship between potential association of serine endopeptidases as an allergen (i.e. oral exposure vs. inhalation exposure, etc.) and why this is not a safety concern for consumption of your enzyme.

A. Please see attached allergen search describing why these hits, are not considered "putative" allergens. Since the allergen hits were not considered allergens there is no safety issue with respect to oral consumption of the enzyme. Please see section in 2.7 in the GRAS dossier.

Regarding the relationship between potential association of serine endopeptidase as an allergen – oral vs inhalation:

As the risk of sensitization correlates to the concentration of inhalable enzyme particles, the formulation of the enzyme product and how the enzyme product is handled is very important. Liquid enzyme products can be handled with low release of inhalable enzyme particles. Our product is a liquid and is not prone to dust or particulates that are able to inhaled during handling.

Very detailed guidelines for control of enzyme exposure in production facilities can be found in "The international Association for Soaps, Detergents and Maintenance Products. Guiding principle for the safe handling of enzymes in detergent manufacture" (AISE, 2013) and in (US SDA, 1995). These guidelines were developed for the detergent industry, but the principles stated are generally applicable across all applications. https://www.aise.eu/our-activities/standards-and-industry-guidelines/safe-handling-of-enzymes.aspx

Such as to apply Technical measures; safe handling; cleanliness; personal protective equipment. Please see attached MSDS for Corolase 8000.

Irritation:

Enzyme preparations containing proteolytic enzymes are classified as irritating to eye and skin. Other enzyme classes; e.g. amylases, lipases and cellulases are essentially free from any irritating effects to both eye and skin (Basketter, 2012a https://www.ncbi.nlm.nih.gov/pubmed/22713689)

b. On Pg. 25, you also state:

"The allergenic potential of the RF8963 protease was further evaluated by searching and analyzing the exact match peptides in the RF8963 protease sequences and the hit sequences, by comparing these peptide sequences to the B cell epitopes predicted in silico from the mature RF8963 protease sequence and by analyzing the location of the cleavage sites of the major proteases in the digestive track on the RF8963 protease and the above peptides."

Please provide more information on how this in silico analyses was performed. For example, were the entire RF8963 protease sequence queried against B cell epitope databases, or only certain peptides? Were in silico analyses done only on the predicted protease cleaved peptides? Although there are now many immunoinformatics databases available for use, relatively few are used to predict class-specific (i.e. IgE-specific) B-cell

epitopes. In addition, whether the database/algorism is based on searching for linear or conformational (discontinuous) epitopes has implications on allergens in processed foods. Please provide more detail on your in silico analyses, including the specific database that was used, along with any published literature that are relevant to your rationale for your analyses.

A. Please see attached Allergenicity Report.

Candice Cryne
Regulatory Affairs Manager

Located: Seattle, WA

Feldbergstrasse 78 | 64293 Darmstadt | Germany
T: +49 172 667 1675 | M: +49 172 667 1675
www.abenzymes.com

Analytical report
RF8963
5.4.2018

Objective: **Chemical composition analysis of serine endopeptidase from *Trichoderma reesei* strain RF8963**

Sample:
1. Liquid end fermentation concentrate batch
2. Liquid end fermentation concentrate batch
3. Liquid end fermentation concentrate batch

Table 1. Main and side enzyme activities

Batch			
Protease activity (BPU/g)	415000	462000	344000
Xylanase activity (BXU/g)	3530	20800	10500

BPU: Assay of protease activity, Roal internal method B040 B
BXU: Assay of xylanase activity, Roal internal method B038

Table 2. Antibiotic activity, presence of production strain and microbiological quality

Batch			
Antibiotic activity	not detected	not detected	not detected
Presence of production strain	not detected	not detected	not detected
Escherichia coli (/25 g)	not detected	not detected	not detected
Salmonella (/25 g)	not detected	not detected	not detected
Total coliforms (cfu*/g)	<1	<10	<1
Staphylococcus aureus (/25 g)	not detected	not detected	not detected
Sulphite reducing anaerobes (cfu*/g)	<10	<10	<10

Antibiotic activity: Specifications for Identity and Purity of Certain food Additives, FAO Food and Nutrition Paper 65 (2006), Rome, Vol.4, p. 122.
Production strain: Detection of production strain (Trichoderma reesei, Aspergillus) in enzyme preparations, Roal internal method M001
E. coli: ISO 16649-3:2015, mod.
Salmonella: NMKL 71:1999, mod.
Total coliforms: ISO 4832:2006, mod.
S.aureus: Eur. Pharmac. 6.3.
Sulphite reducing anaerobes: NMKL 56:2008
*cfu: colony forming units

Table 3. Nutritional analysis

Batch			
Protein %	14.1	16.4	13.3
Moisture %	83.2	80.5	84.8
Ash %	0.12	<0.1	0.27
TOS %[1)]	16.7	19.5	14.9

Protein: NMKL 6, Kjeldahl
Moisture: NMKL 23
Ash: NMKL 173
1) Total organic solids TOS%= [100-(moisture % + ash %)]

Table 4. Heavy metals

Batch	[redacted]	[redacted]	[redacted]
Arsenic, As (mg/kg)	<0.5	<0.5	<0.5
Cadmium, Cd (mg/kg)	<0.05	<0.05	<0.05
Lead, Pb (mg/kg)	<0.05	<0.05	<0.05
Mercury, Hg (mg/kg)	<0.05	<0.05	<0.05

Heavy metals: ISO 17294-2:2003

Table 5. Mycotoxins (µg/kg)

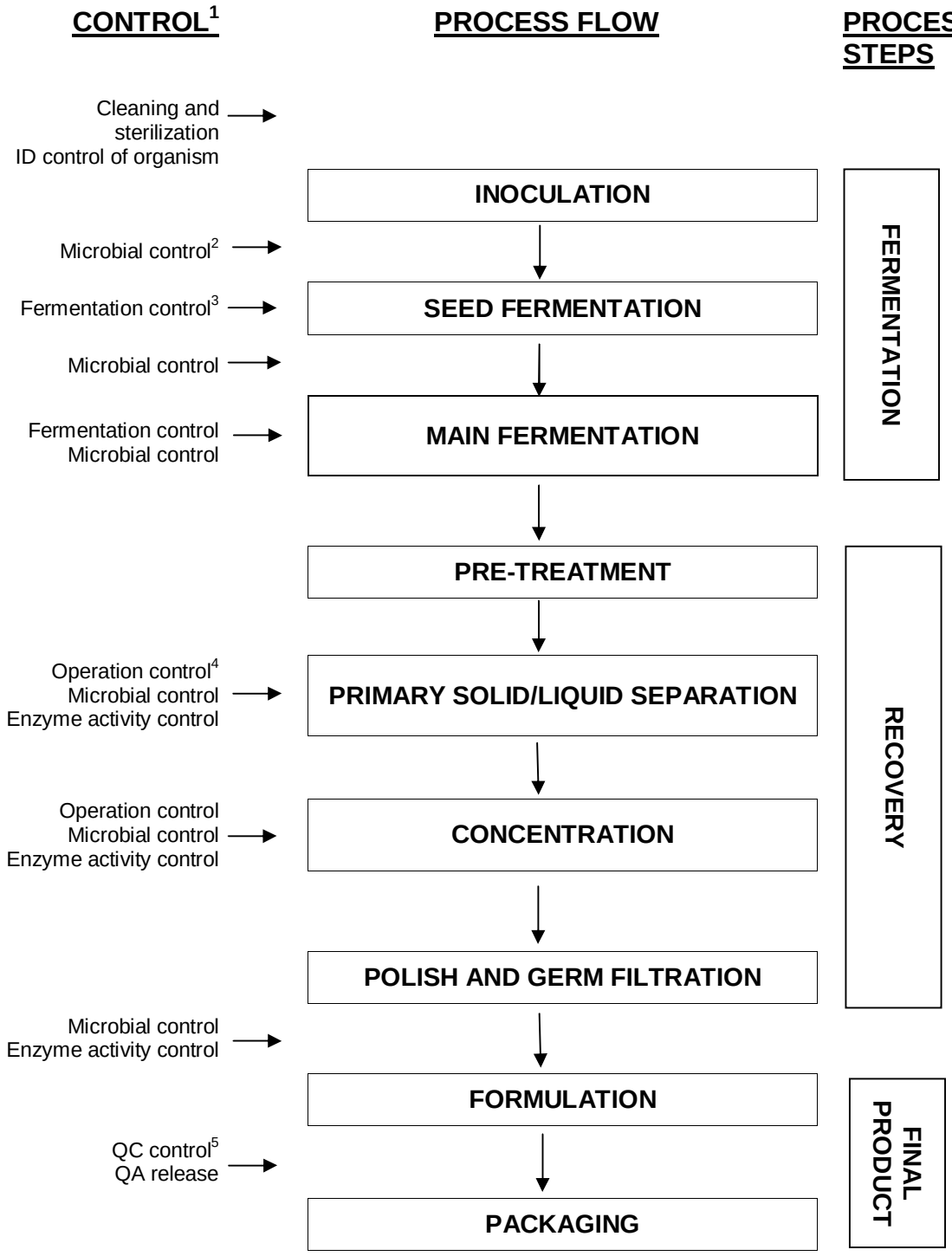
Batch	[redacted]	[redacted]	[redacted]
T2-Toxin	<10	<10	<10
HT-2-Toxin	<10	<10	<10

Wessling J. Agric. Food Chem., 2008, (56) pp. 4968-4975
*Eurofins International method LC-MS/MS

Rajamäki 5.4.2018

[redacted]
Hanna-Riikka Pirttilahti
Quality Management Coordinator
Roal Oy

Production Process of Food Enzymes from Fermentation



[1] The controls shown on the flow chart may vary depending on the production set-up. Controls are conducted at various steps throughout the production process as relevant.

[2] Microbial control: Absence of significant microbial contamination is analyzed by microscope or plate counts

[3] During fermentation parameters like e.g. pH, temperature, oxygen, CO_2, sterile air overflow are monitored / controlled.

[4] Operation control in downstream processes cover monitoring and control of parameters like e.g. pH, temperature

[5] Final QC control will check that product does live up to specifications like e.g. enzyme activity as well as chemical and microbial specification.

ANALYSIS OF SAFETY BASED ON PARIZA/JOHNSON DECISION TREE

Pariza and Johnson have published updated guidelines for the safety assessment of microbial enzyme preparations (2001)[1] from the 1991 IFBC Decision Tree[2]. 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 as outlined in Pariza and Johnson, 2001. The outcome of this inquiry is that protease enzyme preparation from *T. reesei* strain RF8963 expressing the gene encoding serine endopeptidase from *Malbranchea cinnamomea* is "ACCEPTED" as safe for its intended use.

Decision Tree:

1. **Is the production strain genetically modified?**
   Yes go to #2;

2. **Is the production strain modified using rDNA techniques?** Yes go to #3a;

3.

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;

3c. Is the test article free of transferable antibiotic resistance gene DNA? Yes, transferable DNA was not detected in the protease enzyme preparation manufactured using *T. reesei* and production process described herein. Additionally, no antibiotic resistance gene has been integrated. 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. 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**

---

[1] Pariza M.W. and Johnson E.A. Reg. Toxicol. Pharmacol. Vol. **33** (2001) 173-186

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

toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? Yes, there is no concern for pleiotropic effects. Go to #6;

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes, *Trichoderma reesei* has been demonstrated as a safe production host and methods of modification have been well documented. Safety of this organism has been evaluated and confirmed through toxicological testing as described herein.

ACCEPTED