SERINE PROTEASE (TRYPSIN) FROM *FUSARIUM OXYSPORUM* EXPRESSED IN *FUSARIUM VENENATUM*

Chemical and Technical Assessment

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1. Summary

This Chemical and Technical Assessment (CTA) summarizes data and information on the serine protease with trypsin specificity from *Fusarium oxysporum* expressed in *Fusarium venenatum* enzyme preparation submitted to JECFA by Novozymes A/S in a dossier dated November 25, 2011 (Novozymes, 2011)[a‡] . In this CTA, the expression 'serine protease (trypsin)' is used when referring to the serine protease with trypsin specificity enzyme and its amino acid sequence, whereas the expression 'serine protease (trypsin) enzyme preparation' is used when referring to the final enzyme preparation. This document also discusses published information relevant to serine protease (trypsin), the *F. oxysporum* production organism, and the *F. venenatum* organism that is the source for the serine protease (trypsin) gene.

Serine protease (trypsin) catalyses the hydrolysis of peptide bonds in a protein, primarily at the carboxyl side of lysine (Lys-X) or arginine (Arg-X), when X is not proline. The enzyme preparation is to be used as a food additive in the manufacture of partly or extensively hydrolyzed proteins of vegetable and animal origin. The serine protease (trypsin) enzyme preparation is intended for use as a food additive, in the manufacture of partially or extensively hydrolyzed proteins for applications in food and beverages, for protein-fortification, and for emulsification or flavour enhancement. The enzyme preparation is expected to be inactivated during food processing.

Prior to the introduction of the serine protease (trypsin) gene, the *F. venenatum* host strain was rendered incapable of producing toxins including trichothecene toxins and intermediates including diacetoxyscirpenol (DAS) by the deletion of the *tri5* gene encoding the trichodien synthetase and replacing it with the acetamidase (*amdS*) gene from *A. nidulans*[1]. The modified host strain was then transformed with an amplifiable plasmid DNA fragment harbouring the serine protease (trypsin) gene from *F. oxysporum*. The individual transformed colonies were spore-purified and a high yielding transformant was selected for enzyme production. The recombinant production strain was free of antibiotic resistance genes and mycotoxins. The final production strain was genetically stable and does not contain any residual recombinant DNA.

The serine protease (trypsin) is an extracellular enzyme that is secreted by the production strain to the fermentation broth. The fermentation broth is subsequently purified and concentrated to obtain serine protease (trypsin) within the desired activity range. The serine protease (trypsin) enzyme is then formulated with sodium benzoate, and potassium sorbate, glycerol and sorbitol to

[a]The serine protease (trypsin) enzyme preparation is marketed as Novozym 12001 iZyme B by Novozymes A/S

achieve desired activity and stability. The final serine protease (trypsin) enzyme preparation contains 4% Total Organic Solids (TOS), 50% water, 45% glycerol, 0.1% calcium chloride, and 0.2% each of sodium benzoate and potassium sorbate. The serine protease (trypsin) enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

The potential for serine protease (trypsin) enzyme to be a food allergen was examined by comparing the amino acid sequence with the sequences of known allergens in the Structural Database of Allergenic Proteins (SDAP) and Allermatch allergen databases using internationally accepted search criteria. Based on the results obtained the oral intake of the serine protease (trypsin) is not anticipated to pose a risk of allergenicity.

2. Description

Brownish liquid at room temperature

3. Method of Manufacture

3.1 Fusarium venenatum

F. venenatum is a fungus belonging to the class of hyphomycetales[2]. It is a saprophyte found in the soil and is not considered as human pathogens[3]. Also, *F. venenatum* is not classified as a Class 2 organism or higher per NIH guidelines[4]. *F. venenatum* is considered as a safe host organism for expression of various enzymes for use in human food applications[5] [6] [7].The host strain, WTY842-1-11 is a tri5-deleted derivative of an isolate of *F. venenatum* Nirenberg sp[8]. nov, named A3/5. The A3/5 strain was isolated in 1968 from a soil sample in the United Kingdom. It was initially identified as *Fusarium graminearum* Schwabe and deposited in several international culture depositories including the American Type Culture Collection (ATCC, Manassas, VA) [9] where the strain was designated as ATCC 20334. The A3/5 strain was subsequently reclassified as *F. venenatum* based on morphological, molecular, and mycotoxin data[10].

3.2 Fusarium oxysporum

The donor organism for the serine protease (trypsin) gene is *Fusarium oxysporum*. *F. oxysporum* belongs to the section Elegans of the genus *Fusarium* in the class of Hyphomycetes. The specific strain is *F. oxysporum* Schlechtendahl:Fries, DSM 2672. *F. oxysporum* is not regarded as primary human pathogen. It is a common plant pathogen and has been shown to be related to opportunistic mycosis in humans [11] [12] **Error! Bookmark not defined.** *F. oxysporum* has mainly been associated with eye infections in humans and specific strains of this species have been reported to produce various secondary metabolites, including fusaric acid, monoliformine and zearalenone. The pathogenic potential of the donor strain is not relevant to safety since the DNA fragments in the genetic construct are limited only to the serine protease coding sequence. The introduced DNA does not code for any known toxic substances.

3.3 F. venenatum Production Strain

The serine protease (trypsin) enzyme preparation is produced by fermentation of a non-pathogenic and non-toxigenic, trichodien synthetase (tri5)-deficient *F. venenatum* strain encoding a serine protease (trypsin) gene from *F. oxysporum.* The production was developed by first rendering the host, WTY842-1-11, derived from *F. venenatum* strain A3/5, incapable of producing toxins including trichothecene and DAS within the trichothecene pathway by the deletion of the tri5 gene encoding the trichodien synthetase and replacing it with the acetamidase (amdS) gene from *A. nidulans*[13]. The deletion of the tri5 gene was confirmed by Southern hybridization. The modified host strain was then transformed with an amplifiable plasmid DNA fragment harbouring the serine protease with trypsin specificity gene from *F. oxysporum.* The donor of the serine protease (Trypsin) gene and the terminator sequences were from *F. oxysporum* while the promoter sequence was obtained from *F. venenatum.* The plasmid DNA harbouring the serine (trypsin) gene from *F. oxysporum* was incubated with protoplasts of WTY842-1-11 *F. venenatum* strain, and a high yielding transformant was selected for enzyme production. The inserted DNA was tested for stability and integration into the production organism via Southern blotting. The absence of antibiotic resistance genes due to genetic modifications performed was also confirmed and the production strain was tested for absence of mycotoxin production of toxicological concern.

While there are some strains of *F. venenatum* known for their ability to produce mycotoxins within the group of trichothecenes, the production strain discussed in the present dossier, under the given manufacturing conditions, is incapable of producing any toxins. Both wild type and A 3/5 derived *F. venenatum* were analyzed to confirm the non-production of Fumonisin B1, a mycotoxin. Batch lots of serine protease (trypsin) were also tested to confirm the absence of production of aflatoxin B1, Ochratoxin A, Sterigmatocystin, T-2 toxin, Zearalenone, and DAS under enzyme production conditions.

The genetic modifications performed to obtain the construct for improved serine protease enzyme yields are well-characterized, use well-known plasmids in the construction of the vectors, and the introduced DNA does not encode for, or express any known toxic substances. The production organism compiles with the Organization for Economic Cooperation and Development (OECD) criteria for Good Industrial Large Scale Practice (GILSP) micro-organisms[14].

3.4 Fermentation, Recovery and Formulation

The three steps in the manufacture of serine protease (trypsin) enzyme preparation are fermentation, purification and formulation. The serine protease (trypsin) enzyme preparation is prepared in accordance with Good Food Manufacturing Practices. The fermentation medium consists of food-grade raw materials that provide adequate supply of carbon and nitrogen (carbohydrates, proteins, minerals, and vitamins) and compounds used for pH control. The fermentation process is conducted under sterile conditions to prevent contamination with foreign microorganisms. Each fermentation lot is tested before inoculation, at regular intervals during

cultivation and before transfer/harvest. Growth characteristics are observed macroscopically and microscopically. Contaminated lots are rejected. Fermentation parameters that are monitored include enzyme activity and pH. Serine protease (trypsin) is secreted into the fermentation broth. Upon completion of fermentation, the enzyme is subjected to a series of purification steps to separate the cell mass from the broth. Pre-treatment with flocculants are used to facilitate this separation. This is followed by concentration via ultrafiltration and/or evaporation to remove residual production strain and insoluble substrate components from the fermentation step, under controlled pH and temperature conditions. The enzyme concentrate is then preserved by the addition of glycerol, sodium benzoate and potassium sorbate.

Serine protease (trypsin) enzyme preparation is a liquid product. All raw materials used in its manufacture are food grade. Equipment used for the manufacture of serine protease (trypsin) enzyme are constructed to prevent contamination and carefully cleaned between fermentation runs.

4. Characterization

4.1 Serine Protease (Trypsin)

Serine protease (trypsin) is described by the International Union of Biochemistry and Molecular Biology (IUBMB)[15] as follows:

Common Name: Serine Protease

IUB No.: EC 3.4.21.4

Reaction: Endoprotease with a preferential cleavage of Arg, Lys residue in proteins

Other name(s): α-trypsin; β-trypsin; cocoonase; parenzyme; parenzymol; tryptar; trypure; pseudotrypsin; tryptase; tripcellim; sperm receptor hydrolase

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The sequence of the mature serine protease (trypsin) enzyme as deduced from the DNA sequence was shown to contain 224 amino acid residues.

The activity of serine protease (trypsin) is determined by the rate of enzyme hydrolysis of the substrate chromophoric substrate Ac-Arg-*p*-nitro-anilide. The liberated *p*-nitroaniline produces an increase in absorption at 405 nm, directly proportional to the enzyme activity. Serine protease (trypsin) activity is calculated in Kilo Microbial Trypsin Unit (KMTU) activity units per gram of enzyme preparation. 1 KMTU is defined as the amount of serine protease that catalyzes 1 µmol *p*-nitroaniline per minute Suc-Ala-Ala-Pro-Phe-pNA at pH 9 and 37° C.

The sequence of the serine protease (trypsin) from *F. oxysporum* was compared with the sequences of known allergens in two different databases, namely, Allermatch (http://www.allermatch.org/) and SDAP (http://fermi.utmb.edu). The comparisons produced multiple matches showing a 35% or greater identity in a sliding window of 80 amino acids between *F. oxysporum* serine protease (trypsin) and several allergenic proteins (Blo t 3, Der f 3, Der f 6, Der p 3, Der p 9, Eur m 3). These allergens are not identified as food allergens in the WHO-IUIS list. Additionally, multiple matches of 6 contiguous amino acids were also identified between *F. oxysporum* serine protease (trypsin) and many allergenic proteins from the WHO-IUIS list that are not identified as food allergens. Because all these allergenic proteins are serine proteases (trypsin, collagenolytic serine protease, chymotrypsin), some degree of amino acid sequence identity is expected. A further comparison of these sequences (6 contiguous amino acid sequences as well as the sequences producing 35% or greater identity in a sliding window of 80 amino acids) in the general protein database revealed that they are also widely distributed in various trypsin, trypsin-like proteases, chymotrypsin, chymotrypsin-like proteases, other serine proteases, and in non-trypsin and non-allergenic proteins in prokaryotes as well as lower and higher eukaryotes; some are also present in human trypsin and other human proteins. For example, many of the 6 contiguous amino acid sequences of *F. oxysporum* serine protease (trypsin) that are shared by the allergenic proteins Blo t 3, Bom p 4, Der f 3, Der p 3, Der p 9, and Eur m 3, Tyr p 3 are present in human trypsins. This suggests that these sequences are not likely to be part of any allergenic epitopes. Therefore, the serine protease (trypsin) from *F. oxysporum* does not appear to have the characteristics of a potential food allergen, and is not likely to produce an allergenic or sensitization response upon oral consumption.

4.2 Serine protease (trypsin) enzyme preparation

Serine protease (trypsin) enzyme preparation is a brownish colored liquid with a specific gravity of 1.057 g/ml. The serine protease (trypsin) enzyme preparation contains the active serine protease (trypsin) as well as other components that stabilize the enzyme and prevent microbial growth.

The serine protease (trypsin) enzyme preparation is available in a liquid formulation. The product contains 4% enzyme total organic solids (TOS). TOS consists of the enzyme of interest and residues of organic materials, such as proteins, peptides, and carbohydrates, derived from the production organism and the manufacturing process. The TOS content is calculated according to the following equation:

$$TOS\ (\%) = 100 - (A + W + D)$$

where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients[16].

The serine protease (trypsin) enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing[17]. It does not contain

significant levels of secondary enzyme activities and is free from the production strain and transformable DNA.

The other ingredients of this preparation include 45% water, 50% glycerol, 0.5% Calcium Chloride, and 0.2% each of Potassium sorbate and Sodium benzoate.

5. Functional Use:

Serine protease (trypsin) enzyme preparation is used as a food additive in the manufacture of partially or extensively hydrolyzed proteins for applications in food and beverages, for protein-fortification and for emulsification or flavour enhancement. The recommended level of serine protease (trypsin) enzyme preparation is 12 g per kilogram of protein in the product. This corresponds to 480 mg TOS/kg processed protein.

6. Reactions and Fate in Food

The serine protease (trypsin) enzyme preparation is active during the processing of certain foods, but the activity of the enzyme is terminated by high temperatures. Therefore no active enzyme would be expected to be present in the final food. No reaction products are formed during the production or storage of the enzyme treated foods.

7. References

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