# SERINE PROTEASE (CHYMOTRYPSIN) FROM *NOCARDIOPSIS PRASINA* *EXPRESSED IN BACILLUS LICHENIFORMIS*

**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 chymotrypsin specificity from *Nocardiopsis prasina* expressed in *Bacillus Licheniformis* enzyme preparation submitted to the Joint FAO/WHO Expert Committee on Food Additives (JECFA) by Novozymes A/S in a dossier dated November 25, 2011 (Novozymes, 2011)[a]. In this CTA, the expression 'serine protease (chymotrypsin)' is used when referring to the serine protease with chymotrypsin specificity enzyme and its amino acid sequence, whereas the expression 'serine protease (chymotrypsin) enzyme preparation' is used when referring to the enzyme preparation. This document also discusses published information relevant to serine protease (chymotrypsin), the *B. licheniformis* production organism, and the *N. prasina* organism that is the source for the serine protease (chymotrypsin) gene.

Serine protease (chymotrypsin) catalyses the hydrolysis of peptide bonds in a protein, preferably at the carboxyl end of Tyr (Tyr-X), Phe (Phe-X), Trp (Trp-X), when X is not proline. It also catalyses the hydrolysis of peptide bonds at the carboxyl end of other amino acids, primarily Met and Leu, albeit at a slower rate. It is intended for use in the production of hydrolysed animal and vegetable proteins including casein, whey, soy isolate, soy concentrate, wheat gluten and corn gluten. These hydrolysed proteins will be used for various applications as ingredients in food, protein-fortified food, and beverages. The serine protease (chymotrypsin) enzyme preparation is expected to be inactivated during food processing.

Prior to the introduction of the serine protease (chymotrypsin) gene, the *B. licheniformis* host strain was genetically modified through deletion of the genes responsible for sporulation, and two endoproteases. The modified host strain was then transformed with an amplifiable DNA cassette containing the serine protease (chymotrypsin) gene from *N. prasina*. The strain containing multiple copies of serine protease (chymotrypsin) gene was selected for production. The recombinant production strain was free of any markers including antibiotic resistance genes. The final production strain, SJ8373, was genetically stable and did not contain the plasmid DNA used in the transformation of the host strain.

The serine protease (chymotrypsin) 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 (chymotrypsin) within the desired activity range. The serine protease (chymotrypsin) enzyme is then formulated with sodium benzoate, and potassium sorbate.

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

Glycerol and sorbitol are added to stabilize the enzyme concentrate. The final serine protease (chymotrypsin) enzyme preparation contains 7.7% Total Organic Solids (TOS), 52% water, 20% sorbitol, 20% glycerol, 0.2% sodium benzoate and 0.1% potassium sorbate. The serine protease (chymotrypsin) enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

Serine protease (chymotrypsin) enzyme is not known to be allergenic when used in food processing. Nevertheless, the potential for the 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) using internationally accepted search criteria. Based on the results obtained oral intake of the enzyme is not anticipated to pose any allergenicity concern.

## *2. Description*

Brown liquid

## *3. Method of Manufacture*

### *3.1 Bacillus licheniformis*

*B. licheniformis* belongs to the class of firmibacteria as confirmed by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, 28.8.95. Also, *B. licheniformis* is classified as a Risk Group 1 organism according to National Institute of Health (NIH) guidelines for Research Involving Recombinant DNA Molecule[1]. It has also been granted a Qualified Presumption of Safety status by the European Food Safety Authority[2]. The qualification for Gram-positive sporulating bacteria such as *B. licheniformis* has been recently updated to "absence of toxigenic potential"[2]. The safety of *B. licheniformis* has been evaluated as a safe host for the production of harmless industrial products[3] [4]. This nonpathogenic and nontoxigenic strain lineage has been used previously in the production of several food grade enzymes in the U. S[5]. In 2003, JECFA evaluated α-amylase from *B. licheniformis* and established an acceptable daily intake of "not specified"[6].

### *Nocardiopsis prasina*

The donor organism for the serine protease (chymotrypsin) gene is *Nocardiopsis prasina*.

### *3.3 B. licheniformis Production Strain*

The production strain is a sporulation-deficient non-pathogenic and non-toxigenic *B. licheniformis*, expressing 4 open reading frames (ORFs) encoding the serine protease (chymotrypsin) from *N. prasina*. The 4 ORFs are part of two expression cassettes; each cassette containing 2 ORFs in tandem. One expression cassette with 2 ORFs is inserted into the *amyL* region, and the other expression cassette with 2 ORFs is inserted into the *xylA* region of *B.*

*licheniformis* chromosome. The recombinant production strain is free of any markers including antibiotic resistance genes.

The production strain of *B. licheniformis* was developed by introducing expression cassettes into the host strain, TH6, which is marker-free. The introduced expression cassette consists of a chimeric promoter, a chimeric open reading frame (ORF) and a terminator. The chimeric promoter is composed of three promoter elements from three different donors (Pamyl 4199, PamyQ(sc) and PcryIIIA_stab). The chimeric ORF is composed of synthetic DNA sequences encoding *B. clausii* signal peptide sequence that is fused in-frame to synthetic DNA sequences encoding the *N. prasina* serine protease (chymotrypsin) pro and mature regions. The terminator is from *B. licheniformis* alpha-amylase gene.

The inserted DNA is stably integrated into the production organism and is genetically stable during fermentation. This was verified by Southern blotting of three fermentation lots against DNA samples from the master cell bank. Absence of antibiotic resistance genes due to genetic modifications performed was confirmed via testing.

The genetic modifications performed to obtain the construct for improved serine protease (chymotrypsin) enzyme yields are well-characterized, specific, using well-known plasmids in the vector constructs, and the introduced DNA does not result in the production of harmful or 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 (Organization for Economic Cooperation and Development, Safety Consideration for Biotechnology, 1992)[7].

### *3.4 Fermentation, Recovery and Formulation*

The three steps in the manufacture of serine protease (chymotrypsin) enzyme preparation are fermentation, purification and formulation. The serine protease (chymotrypsin) enzyme preparation is prepared in accordance with Good Food Manufacturing Practices. The fermentation medium consists of food-grade raw materials that provide nutrients (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 (chymotrypsin) 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 sodium benzoate and potassium sorbate.

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

## *4 Characterization*

### *4.1 Serine protease (chymotrypsin) enzyme:*

Serine protease (chymotrypsin) is described by the International Union of Biochemistry and Molecular Biology (IUBMB) [8] as follows:

**Common Name:** Serine Protease

**Other name(s):** Chymotrypsins A and B; α-chymar ophth; avazyme; chymar; chymotest; enzeon; quimar; quimotrase; α-chymar; α-chymotrypsin A; α-chymotrypsin

**Reaction:** Endoprotease with a preferential cleavage of Tyr, Trp, Phe, Leu residue in proteins

**IUB Nomenclature:** Chymotrypsin

**EC No.:** 3.4.21.1

**Chemical Abstract Service Number (CAS No.):** 9004-07-3

The sequence of the mature serine protease (chymotrypsin) enzyme as deduced from the DNA sequence was shown to contain 188 amino acid residues.

The activity of serine protease (chymotrypsin) is determined by the rate of enzyme hydrolysis of the substrate Suc-Ala-Ala-Pro-Phe-*p*NA at pH 9 and 37° C. The liberated yellow *p*-nitroaniline produces an increase in absorption at 405 nm, directly proportional to the enzyme activity. The activity of serine protease (chymotrypsin) is calculated in protease units (PROT). 1 PROT is defined as the amount of serine protease (chymotrypsin) that releases 1 µmol *p*-nitroaniline from 1mM substrate per minute at pH 9 and temperature of 37° C.

Serine protease (chymotrypsin) was evaluated for potential allergenicity according to the criteria recommended by FAO/WHO (FAO/WHO, 2001). The provided amino acid sequence of was compared with the amino acid sequences of known allergens. No matches of 6 contiguous amino acid identity were found between the *N. prasina* serine protease (chymotrypsin) and any allergenic proteins in the Structural Database of Allergenic Proteins (SDAP)[9]. Also, no matches of 35% amino acid identity were found between the *N. prasina* serine protease (chymotrypsin) and any allergenic proteins using a window of 80 amino acids. However, a 35% amino acid

identity was found with Pla a 2 (*Platanus acerifoli*a; London plane tree) if the window length was extended beyond 80 amino acids and gaps were introduced. Nevertheless, multiple gap openings and gap extensions needed to obtain this identity suggest that the identity is most likely not biologically meaningful. Therefore, oral intake of the enzyme is not anticipated to pose any allergenicity concern.

### *4.2 Serine protease (chymotrypsin) enzyme preparation*

Serine protease (chymotrypsin) enzyme preparation is a brownish colored liquid with a specific gravity of 1.053 g/ml. The serine protease (chymotrypsin) enzyme preparation contains the active serine protease (chymotrypsin) as well as other components that stabilize the enzyme and prevent microbial growth.

The serine protease (chymotrypsin) enzyme preparation is available in a liquid formulation. The product contains 7.7% enzyme 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[10]. 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 serine protease (chymotrypsin) enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing[11]. 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 52% water, 20% each of sorbitol and glycerol, 0.1% Potassium sorbate and 02% Sodium benzoate. A declared standardized activity of the enzyme preparation is 7500 PROT per gram.

### *5. Functional Use:*

Serine protease (chymotrypsin) enzyme preparation will be used in the production of hydrolyzed vegetable and animal proteins for applications as food and beverage ingredients at levels of GMP. The recommended level of the enzyme preparation is 20g of serine protease (chymotrypsin) enzyme preparation per kilogram of processed protein. This corresponds to 1.53 g TOS/kg processed protein.

## *6. Reactions and Fate in Food*

The serine protease (chymotrypsin) 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*


[1] NIH, 2011. NIH Guidelines for Research Involving Recombinant DNA Molecules. January 2011, http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.pdf

[2] EFSA, 2010, Scientific Opinion on the maintenance of the list of QPS microorganisms intentionally added to food and feed (2010 update). EFSA Journal 2010; 8(12):1944.

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[4] Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D., DiNovi M.J., 2006, Food-processing enzymes from recombinant microorganisms – a review. Regul. Toxicol. Pharmacol. 45, 144-158.

[5] 21 CFR 184.1027, http://www.gpo.gov/fdsys/pkg/CFR-2002-title21-vol3/pdf/CFR-2002-title21-vol3-sec184-1033.pdf

[6] FAO/WHO, 2003a, Sixty-second report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series, No. 922, 2004. (http://www.who.int/ipcs/publications/jecfa/reports/en/index.html)

[7] Organisation for Economic Cooperation and Development, Safety Consideration for Biotechnology, 1992

[8] IUBMB, online edition, International Union of Biochemistry and Molecular Biology. http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/21/1.html

[9] http://fermi.utmb.edu/SDAP/

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