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BETA-GLUCANASE ENZYME PREPARATION FROM *STREPTOMYCES VIOLACEORUBER* EXPRESSING A GENE ENCODING GLUCANASE FROM *S. VIOLACEORUBER*

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1. Summary

This Chemical and Technical Assessment summarizes data and information on the beta-glucanase enzyme preparation from *Streptomyces violaceoruber* expressing a beta-glucanase gene from the same species (beta-glucanase enzyme preparation) that was submitted to JECFA. This document also discusses published information relevant to the safety of beta-glucanase enzyme, including the *S. violaceoruber* production organism and details related to the manufacturing, specifications, use and use levels of the enzyme in food. This document uses the expression "beta-glucanase" to refer to the modified enzyme and its amino acid sequence, and the expression "beta-glucanase enzyme preparation" to refer to the products formulated for commercial use.

Beta-glucanase catalyses the hydrolysis of the (1→3)-β-D-glucosidic linkages in (1→3)-beta-D-glucans to produce corresponding beta-glucans and glucose. The beta-glucanase is used in the manufacture of foods rich in glucans such as yeast extracts; it is intended for use at levels up to maximum level of 151 mg of powdered beta-glucanase TOS/kg raw material (mg TOS/kg), and 202 mg of liquid beta-glucanase TOS/kg raw material.

The *S. violaceoruber* production organism is also referred to as *S. lividans* or *S. coelicolor*. It has been shown to be non-pathogenic and non-toxigenic (Korn-Wendish and Kutzner 1992; Bergey's Manual, 1994). It occurs in nature as a component of soil (Duangmal *et al.*, 2005), and has a history in the production of enzymes intended for use in food processing (Pariza and Johnson, 2001).

S. violaceoruber strains deposited at public type culture collections have been designated as Safety Level 1. The production strain, *S. violaceoruber* pGlu, was obtained by transforming a plasmid from *S. violaceoruber* ATCC No. 35287 into the host organism, *S. violaceoruber* 1326.

The beta-glucanase enzyme preparation is manufactured by controlled fermentation of *S. violaceoruber* pGlu in accordance with Good Manufacturing Practices (GMP). The beta-glucanase enzyme produced is subsequently recovered and concentrated using multiple filtration techniques. The enzyme is standardised into a liquid preparation with glycerol. Alternately, the liquid filtrate is filtered and freeze dried, followed by standardization with sodium chloride to a powdered enzyme preparation. The beta- glucanase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006).

Beta-glucanase is not known to be allergenic when used in food processing. The sponsor made examination of the potential for this enzyme to be a food allergen by comparing its amino acid sequence to sequences of known allergens contained within the AllergenOnline and Allermatch databases using internationally accepted search criteria. No meaningful identity with known allergens

was observed. Based on the results obtained, oral intake of beta-glucanase is not anticipated to pose any allergenicity concern.

2. Description

Brown powder or brown liquid.

3. Method of manufacture

3.1 S. violaceoruber

S. violaceoruber belongs to the genus *Streptomyces*, which is the type genus of the family Streptomycetaceae. There are more than 570 different *Streptomyces* species reported, and there are 39 families and 130 genera that have been identified by 16S rRNA sequence analysis (Ventura *et al.*, 2007). Bacteria belonging to this genus are mainly found in soil but are also occasionally isolated from manure and other sources. *Streptomyces* are Gram-positive bacteria with high proportion of G + C in their DNA. *S. violaceoruber* occurs in nature as a component of soil (Duangmal *et al.*, 2005). It is also referred to as *S. lividans* or *S. coelicolor.*

The taxonomic classification of this microorganism is as follows:

Kingdom:	Bacteria
Phylum:	Actinobacteria
Class:	Actinobacteria
Order:	Streptomycetales
Family:	Streptomycetaceae
Genus:	*Streptomyces*
Species:	*Streptomyces violaceoruber*

S. violaceoruber is a known source organism for production of enzymes intended for use in food processing (Pariza and Johnson, 2001). In Europe, *S. violaceoruber* is not included on the list of pathogens Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work or on the list of pathogens in Belgium (EC, 2000; Belgian Biosafety Server, 2008). Strains of *S. violaceoruber* that have been deposited in public type culture collections, such as American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), have been designated as Safety Level 1. *S. violaceoruber* is globally regarded as a safe microorganism (EC, 2000; Belgian Biosafety Server, 2008). A search of the scientific literature identified publications reporting other *Streptomyces* species, such as *S. somaliensis, S. madurae, and S. sudanensis*, as pathogenic (Boiron *et al.*, 1998; Khatri *et al.*, 2002; Dieng *et al.,* 2003, 2005; Quintana *et al.*, 2008), but no such report of pathogenicity of *S. violaceoruber* were identified in the scientific literature.

S. violaceoruber is a recognised source for food enzyme preparations, such as phospholipase A2 and beta-glucanase, that have Generally Recognized as Safe (GRAS) status for use in food in the U.S. (U.S. FDA, 2004, 2007, 2015).

3.2 S. violaceoruber production strain

The host *S. violaceoruber* 1326 strain was obtained from the John Innes Center. It is classified as a Group 1 biological agent that is unlikely to cause disease in humans (EC, 2000) and is not on the list of microbiological hazards (ANSES – Legifrance, 2006). It complies with the Organisation for Economic Co-operation and Development's GILSP (Good Industrial Large-Scale Practice Microorganisms) criteria

(OECD, 1992) and the criteria on the safety of food enzymes set by Pariza & Foster (1983). The host organism, *S. violaceoruber* 1326, was confirmed to be non-toxigenic and non-pathogenic by the French Haut Conseil des Biotechnologies (HCB).

The production strain, *S. violaceoruber* pGlu, was obtained by transforming the host *S. violaceoruber* 1326 strain with the plasmid containing the beta-glucanase encoding gene from *S. violaceoruber* ATCC 35287. The pIJ702 plasmid included a promoter sequence, ssmp-pro, obtained from *S. cinnamoneus* TH 2, the structural gene of beta-glucanase, glu, obtained from *S. violaceoruber* NBRC 15146 (or *S. coelicolor* A3), a terminator sequence, pld-ter, obtained from *S. cinnamoneus* NRBC 12852, and a selectable marker. The tyrosinase genes (melC1, melC2) of pIJ702 were removed to improve yield of the beta-glucanase. The stability of the introduced gene sequence was assessed by the production strain performance over several generations by measuring the enzyme activity for each generation. The final enzyme preparations were tested for absence of antibiotic resistance gene by PCR. The production strain has been deposited at National Institute of Technology and Evaluation in Japan.

3.3 Fermentation, recovery, and formulation

Beta-glucanase is produced by controlled submerged fermentation of a pure culture of *S. violaceoruber* pGlu. The manufacture of the beta-glucanase enzyme preparation consists of three steps: fermentation (pre, seed and main fermentation), recovery, and formulation. Control measures are in place for physical and chemical quality control during fermentation. Samples are tested for identity, viability and microbial purity at the completion of each primary seed lot. All raw materials used in the manufacture of beta-glucanase enzyme preparation are food-grade.

Following fermentation, the culture broth containing the enzyme is separated from the biomass that consists of the production organism, other microbials, and spent fermentation medium, by sedimentation and several filtration steps (vacuum drum and germ filtration). The liquid filtrate containing the enzyme is formulated with water and sorbitol, and potassium sorbate and sodium chloride are added as preservation agents. The liquid filtrate is formulated with water, sorbitol, potassium sorbate, and sodium chloride to obtain the liquid beta-glucanase enzyme preparation. Alternatively, the liquid filtrate is freeze dried and formulated with sodium chloride to a powdered beta-glucanase enzyme preparation. The entire process is performed in accordance with current Good Manufacturing Practices using raw materials of food grade quality. The final enzyme preparation contains no major food allergens from the fermentation medium, is tested to be free from the production organism and any antibiotic activity.

4. Identity and Characterization

4.1 Beta-Glucanase

Beta-glucanase catalyses the hydrolysis of (1→3)-β-D-glucosidic linkages in (1→3)-β-D-glucans. It is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB) as follows:

Accepted name: glucan endo-1,3-β-D-glucosidase

Other name(s): endo-1,3-β-glucanase; laminarinase; laminaranase; oligo-1,3-glucosidase; endo-1,3-β-glucanase; callase; β-1,3-glucanase; kitalase; 1,3-β-D-glucan 3-glucanohydrolase; endo-(1,3)-β-D-glucanase; (1→3)-β-glucan 3-glucanohydrolase; endo-1,3-β-D-glucanase; endo-1,3-β-glucosidase; 1,3-β-D-glucan glucanohydrolase

Reaction: hydrolysis of (1→3)-β-D-glucosidic linkages in (1→3)-β-D-glucans
Systematic name: 3-β-D-glucan glucanohydrolase
EC No.: 3.2.1.39
CAS No.: 9025-37-0

Beta-glucanase produced by *S. violaceoruber* is not known to have any subsidiary or secondary enzymatic activities. The primary sequence of beta-glucanase has been determined to consist of 453 amino acids; its molecular weight by calculation from the determined amino acid sequence is 42.7 kDa.

Beta-glucanase activity is determined spectrophotometrically by measuring the hydrolysis product of a 1,3-β-D-glucan substrate at 490 nm using the phenol-sulfuric acid method; one unit of activity is defined as the quantity of enzyme required to catalyse the formation of one µmol of glucose per minute under the conditions of the assay. The mean activity of β-glucanase from three batches of the liquid and powder enzyme concentrates were 12 897 U/g and 23 041 U/g, respectively.

4.2 Beta-Glucanase Enzyme Preparation

The beta-glucanase enzyme preparation consists of the enzyme and substances from the fermentation process; these constitute proteins, peptides, amino acids, carbohydrates, lipids and salt. The components of fermentation are referred to as Total Organic Solids (TOS).

The TOS content of an enzyme preparation is calculated according to the following equation (NAS/NRC, 1981; FAO/WHO, 2006):

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

where
A is the % ash,
W is the % water and
D is the % diluents and/or other formulation ingredients.

The beta-glucanase enzyme preparation is marketed as a powder or liquid formulation under the trade names DENAZYME GEL-P1 or DENAZYME GEL-L1, respectively.

A representative composition of the powdered beta-glucanase enzyme preparation is provided below:

Enzyme TOS:	5.1 %
Ash:	94.7 %
Water:	0.4 %
Excipient:	Sodium chloride

A representative composition of a commercial liquid formulation of the beta-glucanase enzyme preparation is provided below:

Enzyme TOS:	2.3 %
Ash:	0.06 %
Water:	40.1 %
Excipients*:	55.6 %

*= glycerol, water

The specifications for beta-glucanase powder preparation include activity (>1000 U/g), appearance, lead (≤ 5 mg/kg), arsenic ≤ 4 ppm), mercury ≤ 0.5 ppm, cadmium ≤ 0.5 ppm, ash, coliforms (negative in 1 g),

total viable aerobic count (NMT 10,000 CFU/g), *Salmonella* (negative in 25 g), *E. coli* (negative in 25 g), *Staphylococcus aureus* (negative in 1g), sulphur-reducing anaerobe (<30 CFU/g), antimicrobial activity (absent by test), and loss on drying.

The specifications for beta-glucanase liquid preparation include activity (>400 U/mL), appearance, lead (≤ 5 mg/kg), arsenic ≤ 4 ppm), mercury ≤ 0.5 ppm, cadmium ≤ 0.5 ppm, coliforms (negative in 1g), total viable aerobic count (NMT 10,000 CFU/g), *Salmonella* (negative in 25 g), *E. coli* (negative in 25g), *Staphylococcus aureus* (negative in 1g), sulphur-reducing anaerobe (<30 CFU/g), antimicrobial activity (absent by test), and loss on drying.

Beta-glucanase enzyme preparation complies with the General Specifications for Enzyme Preparations used in Food Processing as established by the 67th meeting of the Joint Expert Committee on Food Additives (FAO/WHO, 2006).

5. Functional Uses

The beta-glucanase enzyme preparation is intended to be used as a processing aid in the manufacture of beer and seasonings from yeast and mushroom extracts. The liquid enzyme preparation is only intended to be used for beer manufacturing, while both the liquid and powder preparations are intended for used in the manufacture of seasonings from yeast and mushroom extracts. The beta-glucanase in all the applications will be inactivated by heat treatment prior to use of the final foods. The beta-glucanase enzyme preparation is used at a maximum level of 151 mg of powdered beta-glucanase TOS/kg raw material, and 202 mg of liquid beta-glucanase TOS/kg raw material.

6. Fate in food

Beta-glucanase is a naturally occurring substance in microorganisms, plants and animal tissues that are commonly ingested by humans. In addition to beta-glucanase, the enzyme preparation will contain proteins, peptides, carbohydrates and salts from the fermentation process that are common to the human diet.

Beta-glucanase enzyme preparation is intended to be used in the manufacture of food ingredients that are intended to be consumed by the general population. While it is assumed that the beta-glucanase is carried over to finals foods, the enzyme is inactivated and denatured during processing by treatment at high temperatures and is not expected to have any technical effect on the final food. If present, enzyme will be digested, as would any other protein occurring in food. Therefore, use of beta-glucanase enzyme preparation in the processing of food categories described will not have a significant effect on the human body.

7. References

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