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COLLAGENASE ENZYME PREPARATION FROM *STREPTOMYCES VIOLACEORUBER* EXPRESSING A GENE ENCODING COLLAGENASE FROM *S. VIOLACEORUBER*

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1. ***Summary***

This Chemical and Technical Assessment summarizes data and information on the collagenase enzyme preparation from *Streptomyces violaceoruber* expressing a collagenase gene from the same species (collagenase enzyme preparation) that was submitted to JECFA. This document also discusses published information relevant to the safety of collagenase 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 "collagenase" to refer to the modified enzyme and its amino acid sequence, and the expression "collagenase enzyme preparation" to refer to the product formulated for commercial use.

Collagenase catalyses the hydrolysis of the peptide bonds in collagen to produce collagen fragments. The collagenase enzyme preparation is intended to be used as a processing aid in the manufacture of meat and sausage casings, and collagen hydrolysates used as ingredients in foods, such as those for specific dietary uses (for example, foods for special nutritional purposes, sports foods, health foods) and in dietary supplements. It is intended for use at levels up to maximum level of 1,188 milligrams of Total Organic Solids per kilogram of raw material (mg TOS/kg) as a liquid preparation, and 1,566 mg TOS/kg raw material or 36 mg TOS/kg raw material as powdered preparations.

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* pCol, was obtained by transforming a plasmid from *S. violaceoruber* ATCC No. 35287 into the host organism, *S. violaceoruber* 1326.

The collagenase enzyme preparation is manufactured by controlled fermentation of *S. violaceoruber* pCOL in accordance with Good Manufacturing Practices (GMP). After the main cultivation step the collagenase enzyme is separated from the fermentation medium and subsequently recovered and concentrated using multiple filtration techniques. The enzyme is formulated into a liquid preparation with water and glycerol. Alternately, the liquid filtrate can be further filtered and freeze dried, followed by standardization and formulation with dextrin into two powdered enzyme preparations. The collagenase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006).

Collagenase 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 allergen and Allermatch databases using internationally accepted search criteria. No meaningful identity with known allergens was observed. Based on the results obtained, oral exposure of collagenase 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* pCol, was obtained by transforming the host *S. violaceoruber* 1326 strain with the plasmid containing the collagenase encoding gene from *S. violaceoruber* ATCC 35287. The pIJ702-pCOL 7311 bp plasmid included an expression cassette carrying the collagenase encoding gene, obtained from *S. violaceoruber* NBRC 15146, a promoter sequence, samp-pro, obtained from *S. avermitilis* ATCC 31267 and a terminator sequence, pld-ter, obtained from *S. cinnamoneus* NBRC 12852, and a selectable marker. The tyrosinase genes (melC1, melC2) of pIJ702 were removed to improve yield of the collagenase. 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

Collagenase is produced by controlled fermentation of a pure culture of *S. violaceoruber* pCOL. The manufacture of the collagenase 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 collagenase 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 with ammonium sulphate, followed by several filtration steps. The liquid filtrate is formulated with water and glycerol to obtain the liquid collagenase enzyme preparation. Alternatively, the liquid filtrate is freeze-dried and formulated with dextrin to two powdered collagenase enzyme preparations. The entire process is performed in accordance with current Good Manufacturing Practices using raw materials of food grade quality. The enzyme concentrate was also tested to be free from the production organism.

4. Identity and Characterization

4.1 Collagenase

Collagenase catalyses the hydrolysis of collagen in the triple helical region at Gly bonds. It is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB) as follows:

Accepted name: Collagenase

Other name(s): microbial collagenase, collagenase; collagen peptidase; collagen protease; collagenase A; collagenase I; interstitial collagenase; matrix metalloproteinase; metallocollagenase

Reaction: Digestion of native collagen in the triple helical region at Gly bonds.

With synthetic peptides, a preference is shown for Gly at P3 and P1', Pro and Ala at P2 and P2', and hydroxyproline, Ala or Arg at P3'

Systematic name: Microbial collagenase

EC No.: 3.4.24.3

CAS No. 9001-12-1

Collagenase produced by *S. violaceoruber* is not known to have any subsidiary or secondary enzymatic activities. The primary sequence of collagenase has been determined to consist of 865 amino acids; its molecular weight by calculation from the determined amino acid sequence is 92.4 kDa.

Collagenase activity is determined spectrophotometrically by measuring the hydrolysis of a defined peptide substrate by collagenase at 570 nm; one unit of activity is defined as the quantity of enzyme required to liberate one µmol of glycine per minute under the conditions of the assay. The mean activities of collagenase from three batches each of the liquid and the two powder enzyme preparations were 477 U/g, 122 U/g and 2690 U/g, respectively.

4.2 Collagenase Enzyme Preparation

The collagenase 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 collagenase enzyme preparation is marketed as a powder formulation under the trade names DENAZYME CPO PEPRICH or DENAZYME PMC SOFTER and as a liquid under the trade name XPP-051. A representative composition of a commercial liquid formulation of the collagenase enzyme preparation is provided below:

Enzyme TOS:	2.8 %
Ash:	0.06 %
Water:	53.9 %
Excipients*:	43.2 %

*= glycerol

A representative composition of the powdered collagenase enzyme preparation is provided below:

Enzyme TOS:	20.9 %
Ash:	0.6 %
Water:	4.4 %
Dextrin:	74.1%

A representative composition of the second powdered collagenase enzyme preparation is provided below:

Enzyme TOS:	0.88 %
Ash:	0.05 %
Water:	3.56 %
Dextrin:	95 %

The specifications for the liquid collagenase preparation include activity (470-481 U/g), appearance, lead (< 5 mg/kg), arsenic < 3 ppm), mercury < 0.5 ppm, cadmium < 0.5 ppm, coliforms (NMT 30 CFU/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 (NMT 30 CFU/g), antimicrobial activity (absent by test), and loss on drying.

The specifications for the powdered collagenase preparations include activity (2670–2710 U/g) or activity (116 -126 U/g), appearance, lead (<5 mg/kg), arsenic <4 ppm), mercury <0.5 ppm, cadmium <0.5 ppm, total coliforms (NMT 30 CFU/g), total viable bacteria count (NMT 10,000 CFU/g), *Salmonella* (negative in 25 g), *E. coli* (negative in 25 g), *S. aureus* (negative in 1g), sulphur-reducing anaerobe (NMT 30 CFU/g), antimicrobial activity (absent by test), and loss on drying.

Collagenase 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 collagenase enzyme preparation is used as a processing aid for the degradation of collagen which is intended to reduce the toughness of connective tissue in meat products by selectively hydrolysing collagen resulting in enhancement of meat tenderness. For food supplement application, the collagenase catalyses the hydrolysis of peptide bonds at multiple sites in collagen. The collagenase enzyme preparation will be used at a maximum level of 36 mg TOS/kg raw material or 1566 mg/TOS/kg raw material (as a powder), and 1,188 mg TOS/kg raw material (as a liquid).

6. Fate in food

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

Collagenase enzyme preparation is intended to be used in the manufacture of meat, meat products, sausage casings and dietary supplements, which are intended to be consumed by the general population. While it is assumed that the collagenase 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, collagenase will be digested, as would any other protein occurring in food. Therefore, use of

collagenase in the processing of food categories described will not have a significant effect on the human body.

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