26 November 2021

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS 200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Notice for a Collagenase Enzyme Preparation from *Streptomyces violaceoruber* pCol

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, NAGASE & CO., LTD., as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that a collagenase enzyme preparation obtained from a genetically modified production strain of *Streptomyces violaceoruber* (strain pCol), also referred to as DENAZYME XPP-011F, is Generally Recognized As Safe (GRAS) for use as a processing aid for direct use in meat processing, such as beef jerky, marinated and injected meat, and sausage casings. As such, these food uses of collagenase from *S violoceoruber* pCol are not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for NAGASE & CO., LTD.'s GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the Agency. Suitability data related to the use of collagenase from *S. violoceoruber* pCol in meat products that fall under purview of the United States Department of Agriculture are also included in the GRAS notice.

I certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus free using Symantec Endpoint Protection 12.1.5.

Should you have any questions or concerns regarding this GRAS notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

General Manager, Food Ingredients Department
NAGASE & CO., Ltd.

Email: koichiro.kojima@nagase.co.jp
Tel: +81-3-3665-3384

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Total Quality. Assured.

GRAS NOTICE FOR THE USE OF A COLLAGENASE ENZYME PREPARATION FROM *STREPTOMYCES VIOLACEORUBER* PCOL AS A PROCESSING AID IN FOOD PRODUCTION

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
NAGASE & CO., LTD.
5-1 Nihonbashi-Kobunacho Chuo-ku
Tokyo 103-8355
Japan

DATE:
26 November 2021

GRAS Notice for the Use of a Collagenase Enzyme Preparation From *Streptomyces violaceoruber* pCol as a Processing Aid in Food Production

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GRAS Notice for the Use of a Collagenase Enzyme Preparation From *Streptomyces violaceoruber* pCol as a Processing Aid in Food Production

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §§170.203 through 170.285, NAGASE & CO., LTD (hereinafter referred to as "Nagase") hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of a collagenase enzyme preparation obtained from a genetically modified production strain of *Streptomyces violaceoruber* (strain pCol), also referred to as DENAZYME XPP-011F, which is intended for use as a processing aid for direct use in meat processing, such as beef jerky, marinated and injected meat, and sausage casings, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Nagase's view that these notified uses are Generally Recognized as Safe (GRAS). Considering that the use of Nagase's enzyme preparation in meat products fall under the U.S. Department of Agriculture (USDA) jurisdiction, pertinent data regarding the suitability of collagenase enzyme preparation from *S. violaceoruber* (strain pCol) for use in USDA-regulated products have been included as part of this GRAS notice. Nagase authorizes U.S. FDA to share this information with the USDA Food Safety Inspection Service (FSIS). In addition, as a responsible official of Nagase, the undersigned hereby certifies that all data and information presented in this notice represents a complete and balanced submission that is representative of the generally available literature. Nagase considered all unfavorable as well as favorable information that is publicly available and/or known to Nagase and that is pertinent to the evaluation of the safety and GRAS status of the collagenase enzyme preparation as described herein.

Signed,

Koichiro Kojima
General Manager, Food Ingredients Department
NAGASE & CO., Ltd.

26 November 2021
Date

1.1 Name and Address of Notifier

NAGASE & CO., LTD.
5-1 Nihonbashi-Kobunacho Chuo-ku
Tokyo 103-8355
Japan

1.2 Common Name of Notified Substance

The subject of this Notice is a collagenase (EC 3.4.23.3) enzyme preparation from a genetically modified production strain of *Streptomyces violaceoruber* (strain pCol).

1.3 Conditions of Use

Collagenase from *S. violaceoruber* pCol is intended for direct use in meat processing such as beef jerky, marinated and injected meat, and sausage casings. The DENAZYME XPP-011F powder preparation will be marketed for use in meat processing. Although the use level of the enzyme is *quantum satis*, maximum use levels recommended by the enzyme manufacturer to achieve the desired technological effect for each individual food application are presented in Table 1.3-1.

The ingredient is not intended for use in infant formula or infant food products; however, the proposed food categories include food uses that are subject to the oversight by the U.S. Department of Agriculture (USDA) and the USDA Food Safety Inspection Service (FSIS). Suitability data for these proposed food uses are presented in Section 2.5.3.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Maximum Recommended Use Levels of Collagenase from *Streptomyces violaceoruber* pCol in the U.S.

Application	Maximum Proposed Collagenase Use Level (mg TOS/kg food)[a]	Maximum Level of Collagenase Potentially Present in Final Foods[b]
Beef Jerky	7.25	10.9 mg TOS/kg
Meat (marinated)	7.25	
Meat (injection)	10.9	
Sausage Casing	3.63	

TOS = total organic solids; U.S. = United States.

[a] The food enzyme preparation is used *quantum satis*. The use level indicated is the maximum use level recommended by the enzyme manufacturer to achieve the desired technological effect.

[b] Assumes that none of the collagenase from *Streptomyces violaceoruber* pCol used in meat processing is removed or denatured. In reality, the processing conditions of collagenase-treated meat products involve treatment at high temperatures which will inactivate the enzyme, thereby ensuring that the enzyme will not have any technological effect in final foods treated with collagenase.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the Code of Federal Regulations (CFR) (U.S. FDA, 2021), Nagase has concluded that the intended uses of the collagenase enzyme preparation from *S. violaceoruber* pCol as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

NAGASE & CO., LTD.
5-1 Nihonbashi-Kobunacho Chuo-ku
Tokyo 103-8355
Japan

Should the FDA have any questions or additional information requests regarding this Notification, Nagase will supply these data and information upon request.

1.6 Freedom of Information Act, 5 U.S.C. 552

It is Nagase's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the *Freedom of Information Act*, 5 U.S.C. 552.

Part 2. § 170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

2.1.1 Identity of the Enzyme

2.1.1.1 Names and Systematic Numbers

The collagenase enzyme is identified by the following names and systematic numbers:

Accepted Name:	Microbial collagenase
Synonyms:	collagenase; collagen peptidase; collagen protease; collagenase A; collagenase I; interstitial collagenase; matrix metalloproteinase; metallocollagenase
Trade Names:	DENAZYME XPP-011F (light brown powder)
International Union of Biochemistry and Molecular Biology (IUBMB) Number [Enzyme Commission (EC) Number]:	EC No. 3.4.24.3
Chemical Abstracts Service (CAS) Number:	9001-12-1

2.1.1.2 Amino Acid Sequence and Molecular Mass

The full amino acid sequence of the collagenase from *S. violaceoruber* pCol is presented below. The collagenase from *S. violaceoruber* pCol is a protein of 865 amino acids with a calculated molecular mass of 92.4 kDa.

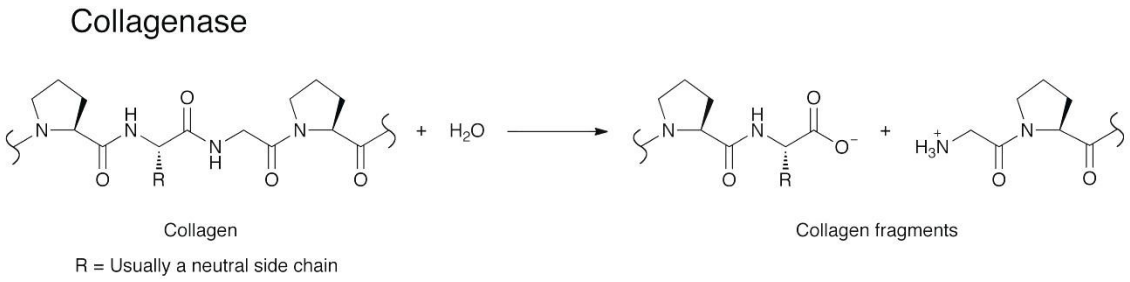
MRKSLVRRGLGAALPLALTVAMSVGLLSQPAGAAGNTGSVVHVAADDPEHAGPPPVAQSPTAETEHVAQGRTKA
SELPPVAASKDALKEVYGKTAKAPVRPSKSTDKAVAGKTGNSRARAAACNVSDFTSRSGGALVQQIKASTTDCVNT
LFNLTGNDAYYAFRESQMTSVAYALRDGSTSYPGNASTGMPQLVLYLRAGYYVHYYNAGTVGTYGSSLQTAIRAGL
DAFFASPHSRDVNDANGETLAEAVTLIDSAEENARYIHVVKRLLADYDSTWNSSWWMLNAVNNVYTVTFRGHQV
PAFVSAVQSDPGLIDALYNFASGHLALLGTDQSYLTSNAGRELGRFLQHSALRSKVSPLAGGLLNSSSIKGRTAPLWV
GVAEMTDYYDKANCSYYGTCDLQAQLARSVLTVTYPCSSSITIKAQQMTSGELSSSCSSLRNQDAYFHNVVRDNGP
VANDNNSTIEVVVFDSSTDYQTYAGAMYGIDTNNGGMYLEGNPSAAGNQPRFIAYEAEWLRPDFQIWNLNHEYT
HYLDGRFDMYGDFNANITTPTIWWVEGFAEYVSYSYRGVPYTEATTEAGRRTYALSTLFDTTYSHDTTRIYRWGYLA
VRYMLENHRADMDTVLSYYRAGNWNAARSYLTGTIGTRYDNDWYTWLAACAAGNCGGGGTNPPGNQAPTAA
FTTAVQGLNVTFTDQSTDADGTIASRSWSFGDGSTSTATNPVKTYGSAGSYTVKLTVTDDKGATATATRTVTVGSG

GGGGTECTGTDTRELGQNCQRGNQSATTGNYAYLYLYVPAGTTQLKITTSGGTGDADLYYSTSGWPGTTSYTQRA
TGAGNNHTLTITNPPAGANYISLHAVSSFSGVTVSSAY

2.1.1.3 Enzyme Properties

The collagenase enzyme catalyzes the hydrolysis of peptide bonds in collagen to produce collagen fragments according to the chemical reaction shown in Figure 2.1.1.3-1. Collagenases belong to the M9 peptidase family of enzymes (INTERPRO: IPR002169; PFAM: PF01752), which comprises bacterial metalloproteinases with collagenolytic activity (Harazono *et al*., 2020).

Figure 2.1.1.3-1 Hydrolytic Activity of Collagenase on Collagen



The optimal pH and temperature conditions for the collagenase food enzyme produced by *S. violaceoruber* pCol were determined to be pH 8.0 and a temperature of 50°C. It is important to note that the collagenase food enzyme activity is inactivated at temperatures above 50°C.

Collagenase activity was evaluated as a function of temperature and pH to determine the conditions for the stability of the enzyme and optimal collagenase activity. The results are summarized in Figures 2.1.1.3-1 through 2.1.1.3-4.

Figure 2.1.1.3-1 Enzymatic Activity of Collagenase from *Streptomyces violaceoruber* pCol as a Function of pH (30°C for 10 Minutes)

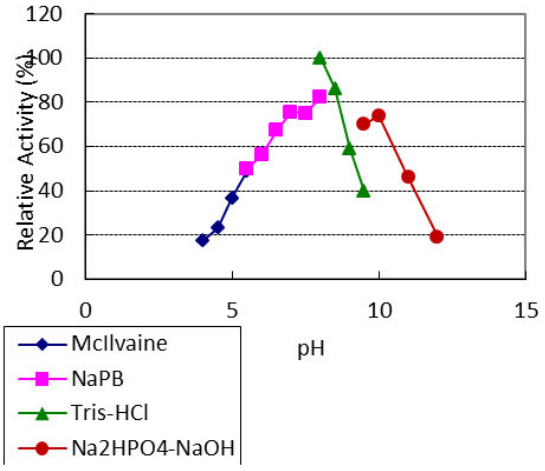


Figure 2.1.1.3-2 pH Stability of Collagenase from *Streptomyces violaceoruber* pCol (25°C for 24 Hours)

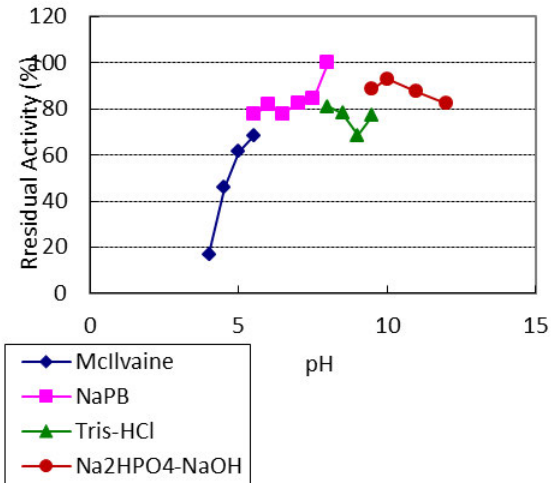


Figure 2.1.1.3-3 Enzymatic Activity of Collagenase from *Streptomyces violaceoruber* pCol as a Function of Temperature (pH 8.0 for 10 Minutes)

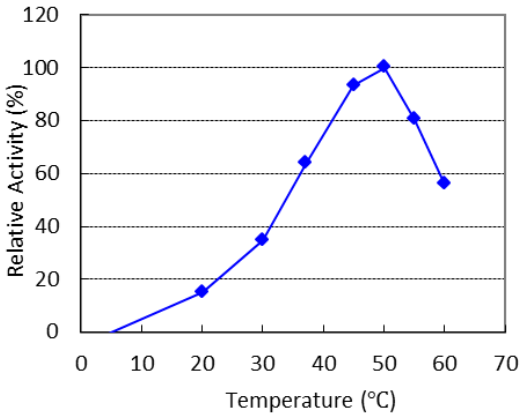
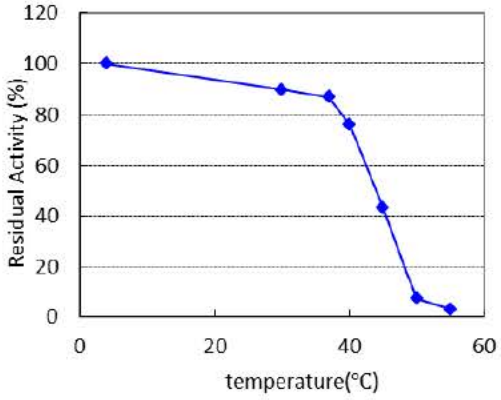


Figure 2.1.1.3-4 Thermal Stability of Collagenase from *Streptomyces violaceoruber* pCol (pH 8.0 for 60 Minutes)



2.1.2 Characterization of the Enzyme Source

The microorganism used to produce collagenase is *S. violaceoruber* pCol. It was constructed by transformation of a plasmid from *S. violaceoruber* ATCC 35287 and subsequent transfection into the host strain *S. violaceoruber* 1326. The host microorganism previously named *Streptomyces lividans* or *Streptomyces coelicolor*, is not pathogenic or toxigenic (Korn-Wendisch and Kutzner, 1992; Bergey's Manual, 1994) and was obtained from the John Innes Center (Norwich, United Kingdom).

The host strain complies with the Organisation for Economic Co-operation and Development's (OECD's) Good Industrial Large Scale Practice Microorganisms (GILSP) criteria (OECD, 1992) and the criteria on the safety of food enzymes established by Pariza and Foster (1983). The strain is not deposited in official culture collection for confidential and proprietary reasons to avoid any copy. The strain host is reported to be neither toxic nor pathogenic (see Section 5.4.2).

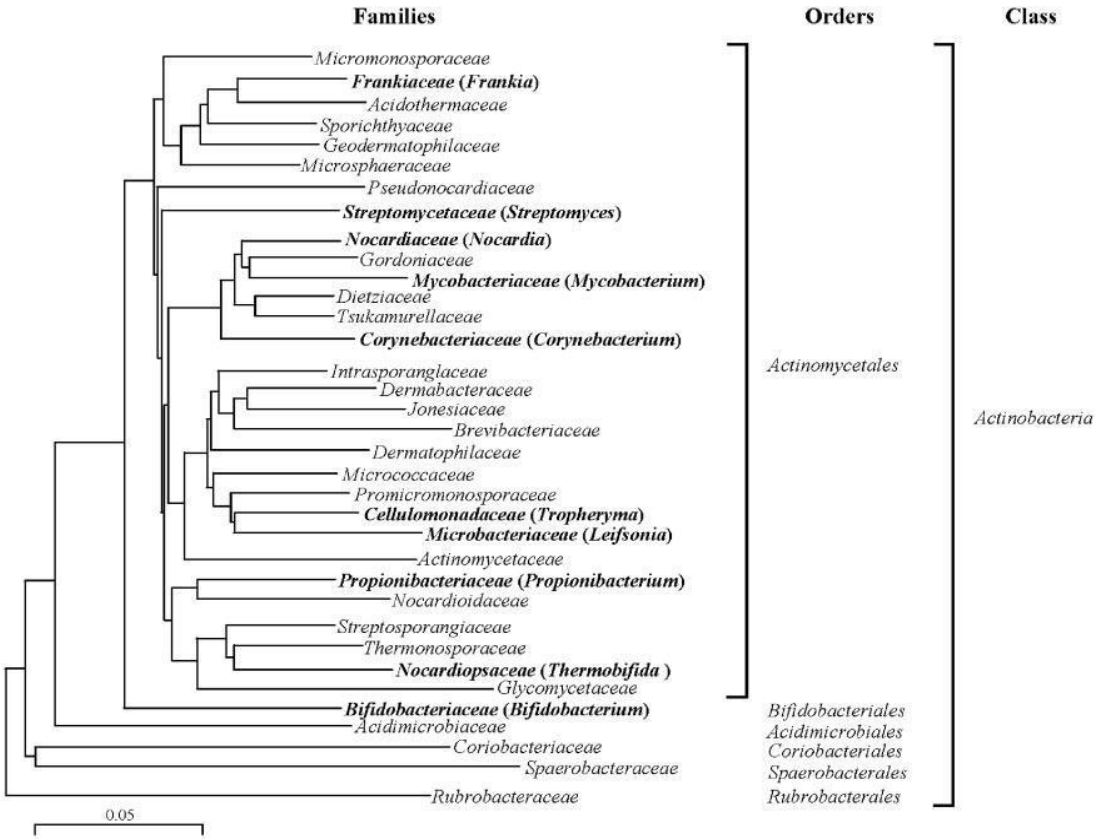
The taxonomic classification of *Streptomyces violaceoruber* 1326 is presented in Table 2.1.2-1, below (Taxonomy ID: 1935 on GenBank):

Table 2.1.2-1 Scientific Classification of *Streptomyces violaceoruber* 1326

Rank	Scientific Name
Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Streptomycineae
Family	Streptomycetaceae
Genus	*Streptomyces*
Species	*Streptomyces violaceoruber*

Streptomyces is the most abundant genera of Actinobacteria with more than 570 species of Streptomyces reported. The species of Streptomyces are Gram-positive bacteria with high proportion of guanine and cytosine in their DNA. Sequencing of 16S rRNA allowed identifying 39 families and 130 genera, such as Bifidobacteria or Micrococcaceae (see Figure 2.1.2-1) (Ventura *et al*., 2007).

Figure 2.1.2-1 Actinobacteria Phylogenetic Tree Based on 1,500 Nucleotides of the 16S rRNA



Scale bars equal 5 nucleotides.
Source: Ventura *et al.* (2007).

The production microorganism of collagenase is the self-cloned *S. violaceoruber* pCol. The plasmid pIJ702, which is extracted from *S. violaceoruber* ATCC 35287 and derived from the pIJ350 plasmid from *Streptomyces lividans*, is genetically modified to plasmid pCol, which is subsequently transfected to the host strain, *S. violaceoruber* 1326, to produce the production organism, *S. violaceoruber* pCol. The plasmid pCol contains:

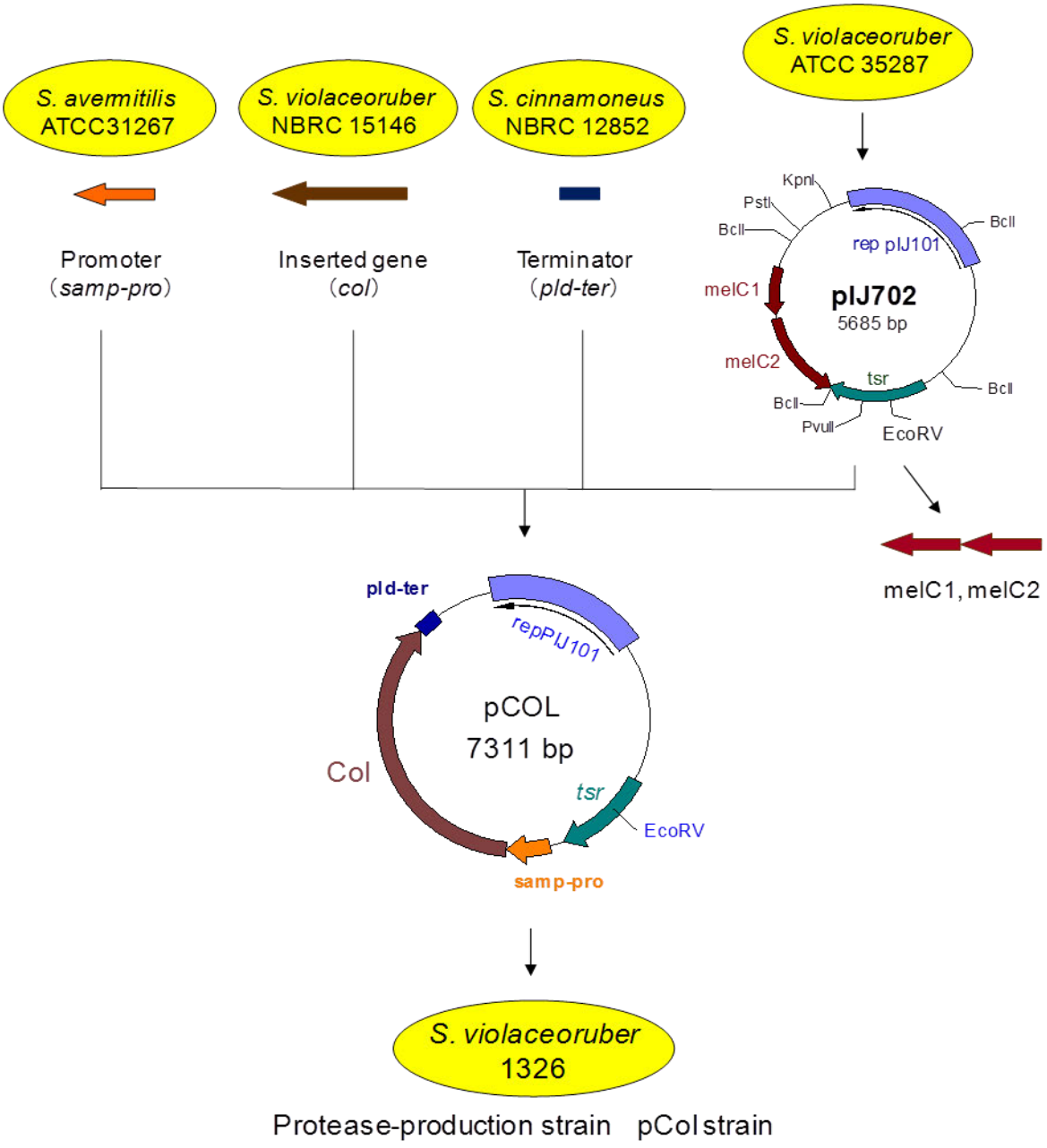
- A promoter sequence, *samp-pro*, obtained from *Streptomyces avermitilis* ATCC 31267;
- The structural gene of collagenase, *col*, obtained from *S. violaceoruber* NBRC 15146 (or *S. coelicolor* A3(2)) after identification and amplification of the structural gene by polymerase chain reaction (PCR); and
- A terminator sequence, *pld-ter*, obtained from *Streptomyces cinnamoneus* NBRC 12852.

The plasmid's sequence and the different restriction sites have been identified, and the plasmid is described by Kieser *et al.* (1982) to be non-conjugative, non-transferable, and it maintains its autonomous form in the cell. Consequently, the frequency of the inserted vector's mobilization and the ability for the transfer of genetic material are estimated to be very low.

The promotor sequence, *samp-pro,* is the promotor sequence of the gene of metallo-endopeptidase from *S. avermitilis* ATCC 31267. According to the American Type Culture Collection (ATCC), this strain belongs to the Biosafety Level 1: not known to consistently cause diseases in immunocompetent adult humans. The collagenase gene was obtained from the chromosome in *S. violaceoruber* NBRC 15146 (formerly *S. coelicolor* A3(2)) and amplification of the structural gene by PCR. In addition, to facilitate the insertion of the gene *col*, *Aor51H* I (dinucleotide) and *Sph* I (hexanucleotide) sites were added before the start codon and after the stop codon, respectively. Considering that these sites are outside the nucleotide sequence for the gene *col*, the production of the enzyme is not changed. The terminator sequence *pld-ter* is the terminator sequence of the gene of phospholipase D from *S. cinnamoneus* NBRC 12852, which has been used previously for the construction of a self-cloned strain of *S. violaceoruber* for the production of beta-glucanase (GRN 535). Modification of the host strain *S. violaceoruber* 1326 was performed by means of the protoplast method (Katsumata *et al*., 1984). This method had a long history of use in the introduction of plasmids into actinomycetes.

The tyrosinase genes (*melC1* and *melC2*) of pIJ702 were also removed. All DNA sequences used are derived from bacteria belonging to the genus *Streptomyces*. An overview of the construction process of the pCol plasmid and self-cloned strain is provided in Figure 2.1.2-2.

Figure 2.1.2-2 Construction of the *Streptomyces violaceoruber* pCol



A study was conducted to compare the sequences of collagenase genes from the donor organism (*S. violaceoruber* NBRC 15146) and the self-cloned organism (*S. violaceoruber* pCol) by PCR amplification. The results showed complete homology between the collagenase gene sequences. Additionally, the plasmid is not considered to be transferable (Chater *et al*., 1982; Katz *et al*., 1983) and the vector can only be replicated by microorganisms from the genera *Streptomyces*. The plasmid is also known to be stable only under specific cultural conditions.

The stability of the introduced collagenase gene sequence was assessed by culturing the production strain for several generations, including cultures in the absence of selective pressure. Productivity of the enzyme was measured for each generation. The results indicate that the efficiency remains constant over several generations. A review of the publicly available scientific literature was conducted to assess the potential pleiotropic effects of the bacterial collagenase gene. No evidence of pleiotropy effects was identified with sequences from the *pIJ702* vector or inserted gene of collagenase. As such, it can be concluded that pleiotropic effects for this self-cloned strain are unlikely.

The plasmid pIJ702 contains a gene, *tsr*, encoding for a thiostrepton-resistance protein. Thiostrepton is a cyclic oligopeptide antibiotic of the thiopeptide class and its resistance gene, *tsr*, is naturally present in *Streptomyces azereus* (Bibb *et al*., 1985). The presence of this antibiotic resistance gene is only considered to be a concern if the gene is transferable (Pariza and Johnson, 2001), and it was previously indicated in this section that the plasmid is not transferable (Chater *et al*., 1982; Katz *et al*., 1983). Furthermore, it has been noted by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Consultation on Biotechnology and Food Safety (FAO/WHO, 1996), that the presence of antibiotic resistant genes is not of concern if it is not present in foods or released intentionally into the environment. Considering that the collagenase enzyme preparation is subjected to multiple steps during production that are designed to remove the production organism, it can be concluded that the presence of the *tsr* antibiotic resistance gene is not a safety concern. Additionally, U.S. FDA (1998) has reported that the transfer of antibiotic resistant genes from transgenic plants to microorganisms in the digestive tracts of animals and humans or the environment is unlikely due to the degradation of DNA.

Considering that the *tsr* gene sequence is available, its digestibility was studied using the software Gene/Protein Analysis software (GENETYX Corporation). Several peptide bonds in the amino acid sequence can be recognized and hydrolyzed by chymotrypsin and trypsin. Moreover, pepsin, although less specific, may act on other residues and complete the catabolism. It is concluded that the thiostrepton resistance protein can be easily digested by enzymes in the human digestive tract and will not raise a safety concern.

2.1.3 Composition of the Enzyme Preparation

The collagenase enzyme preparation from *S. violaceoruber* pCol is directly purified from the fermentation medium without any added process except dilution. Collagenase will be marketed in a dry (powdered) form under the trade name DENAZYME XPP-011F. The collagenase enzyme concentrate (DENAZYME CO-BOC) produced by *S. violaceoruber* pCol and used for the production of DENAZYME XPP-011F is obtained by freeze-drying and subsequently standardized to the powdered preparation.

Prior to the formulation of the commercial product, the collagenase enzyme is prepared as a concentrate and standardized by the addition of dextrin. To produce the commercial DENAZYME XPP-011F powder preparation, the collagenase enzyme concentrate is further standardized with dextrin as a diluent at approximately 96%. The total organic solids (TOS) content of the DENAZYME XPP-011F powder collagenase preparation is 1.45%, which corresponds to collagenase activity at approximately 13.8 U/mg TOS.

2.2 Manufacturing

2.2.1 Raw Materials and Processing Aids

The collagenase production process comprises a cultivation step, followed by several filtration and purification steps. The collagenase preparations are produced under Quality Assurance Certification and current Good Manufacturing Practice (cGMP) and comply with the guidelines for the safe handling of microbial enzyme preparations by the Association of Manufacturers of Fermentation Enzyme Products (AMFEP). All raw materials used during fermentation of *S. violaceoruber* pCol and for production of collagenase are food-grade, permitted for their respective uses by an appropriate federal regulation, or have been previously determined to be GRAS for their intended uses. None of the raw materials or processing aids used in the fermentation, purification, or formulation of collagenase contain major allergens or are derived from allergenic sources.

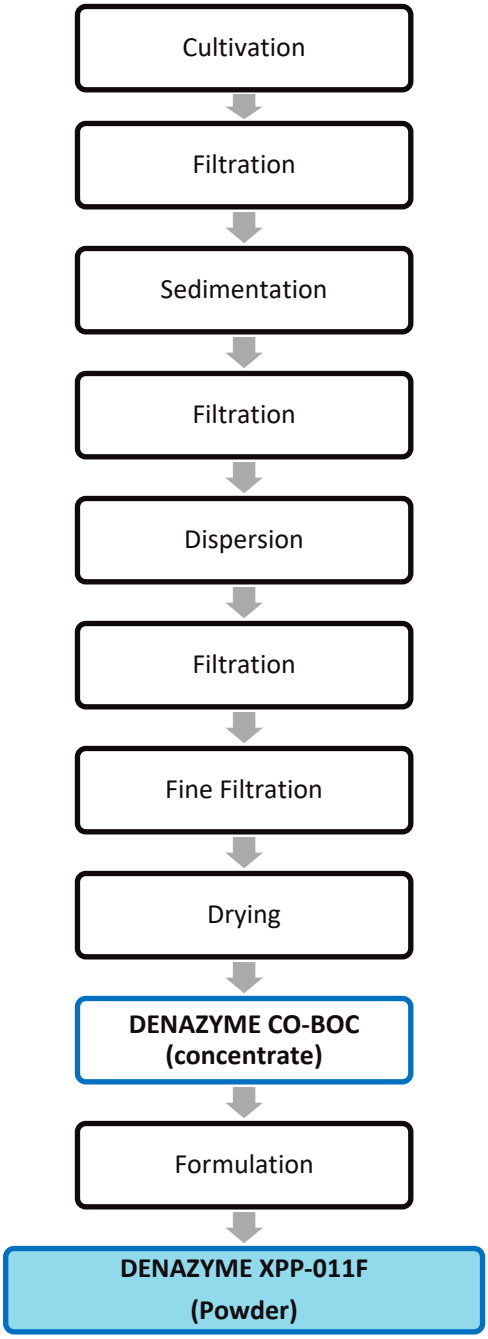
2.2.2 Manufacturing Process

Collagenase will be marketed in a dry (powdered) form under the trade name DENAZYME XPP-011F. The collagenase enzyme concentrate (DENAZYME CO-BOC) produced by *S. violaceoruber* pCol and used for the production of DENAZYME XPP-011F is obtained by freeze-drying and subsequently standardized to the powdered preparation.

Prior to the formulation of the commercial product, the collagenase enzyme is prepared as a concentrate and standardized by the addition of dextrin. To produce the commercial DENAZYME XPP-011F powder preparation, the collagenase enzyme concentrate is further standardized with dextrin as a diluent at approximately 96%. The total organic solids (TOS) content of the DENAZYME XPP-011F powder collagenase preparation is 1.45%, which corresponds to collagenase activity at approximately 13.8 U/mg TOS.

Collagenase is produced by *S. violaceoruber* pCol, which is cultivated in 3 different phases: seed culture, pre-culture, and main culture. At each phase, *S. violaceoruber* pCol is grown in culture medium containing nutrients and antifoam material (refer to Table 3.1-1 for raw materials) and controlled for temperature, pH, and microscopic observations. No antibiotics are used in the fermentation media as a selection marker for plasmid uptake in any of the 3 phases. A schematic overview of the manufacturing process of collagenase from *S. violaceoruber* pCol is provided in Figure 2.2.2-1.

Figure 2.2.2-1 Schematic Overview of the Production Process for DENAZYME XPP-011F



Firstly, *S. violaceoruber* pCol is isolated from a pure culture and cultivated in culture medium at temperatures ranging from 27 to 29°C for 72 hours to be used as the seed inoculum. The resulting biomass is then transferred to a 3-L flask to be further cultivated at temperatures ranging from 27 to 29°C for 105 hours before being transferred to a 2,000-L container. The main culture undergoes quality control to ensure collagenase activity exceeds 40 U/mL.

Subsequently, the culture undergoes 3 separate phases of filtration. Initially, the main culture is combined with pH stabilizers and processing aids to maintain pressure (not more than 4 kg/cm^2), ambient temperature, and pH (between 6.0 and 6.3) and isolate the permeates from the solution using a 20 m^2 (total area) filter at a flow rate of 2 m^3/hour. The pH and turbidity [≤0.2 (absorbance at 660 nm)] of the solution is used as a control point during the first filtration. The permeate (approximately 2,100 L) is then concentrated using a 20 m² (total area) ultrafiltration membrane at an average flow rate of 0.6 m^3/hour under controlled temperature (≤15°C) and pH (5.5 to 8.5) to a 100-L concentrate. The molecular weight threshold for the ultrafiltration is 30 kDa.

Ammonium sulfate (40% concentration) is then added to the resulting permeate as a processing aid to precipitate the protein from solution. The solution containing the protein sediments undergoes a second filtration step using diatomaceous earth at ambient temperature and controlled pressure (not more than 4 kg/cm^2). The precipitate from the second filtration is expected to be 18 kg and the quality is controlled to contain collagenase activity at <5%. The precipitate undergoes dispersion and a third filtration step using diatomaceous earth at ambient temperatures and controlled pressure (not more than 4 kg/cm²) and pH (between 5.5 and 8.5).

To prepare the final powdered preparation of the collagenase enzyme concentrate, the product undergoes further filtration using a 0.45 µm, eq. fine filter and assessed for microbiological contamination (*i.e*., microbial count, coliform, and Salmonella). The product is then freeze-dried at an initial temperature of -30°C and then raised to a temperature of 20°C. The product is subsequently standardized with dextrin (75%) to produce the collagenase powder at a concentration of 25%. The final collagenase enzyme concentrate is then analyzed to ensure that the product meets its specifications (see Section 2.3.1).

2.2.3 Quality Control

Following the production process as outlined above, 2 studies were performed to demonstrate the efficiency of the strain removing process. First, a search for the plasmid pCol was performed by DNA purification from DENAZYME CO-BOC (concentrate) and compared with the plasmid DNA. This study demonstrated that the amount of DNA in the enzyme concentrate is very low and differs greatly from the plasmid DNA, as its size is smaller.

As *S. violaceoruber* pCol contains the resistance gene, *tsr*, the amount of *tsr* in DENAZYME CO-BOC (concentrate) was also determined. This study was performed by PCR amplification. It was concluded that 1.028×10^{-8} U collagenase contains less than 1 molecule *tsr*.

Finally, a sample of collagen peptide treated with a powdered collagenase enzyme preparation characterized by a TOS content of 21.1% was incubated in a thiostrepton-rich medium at 30°C for 5 days. No colonies appeared on the plates indicating the absence of the production strain in the final food ingredient.

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

Product specifications for the DENAZYME XPP-011F collagenase food enzyme preparation comply with the purity and microbial limits established for enzyme preparations by JECFA (2006) and FCC (2018). A summary of the specifications for this enzyme preparation is provided in Table 2.3.1-1. Analytical methods to test for the specification parameters are consistent with the corresponding methods outlined in the *Combined Compendium of Food Additive Specifications* (JECFA, 2006).

Table 2.3.1-1 General Specifications for the DENAZYME XPP-011F Collagenase Enzyme Preparation from *Streptomyces violaceoruber* pCol

Parameter	Method	Specification
Appearance	Visual examination	Light brown powder
Activity		
Collagenase activity	Colorimetric determination (Validated in-house method)	>15 U/mg TOS or >200 U/g
Antimicrobial activity	JECFA Method	Absent
Heavy Metals		
Lead	Atomic absorption spectroscopy	NMT 5 mg/kg
Arsenic (as As_2O_3)	Japanese Pharmacopoeia Chemical Method 1.11 (Method 4)	NMT 4 mg/kg
Microbiological Criteria		
Total viable bacteria count	Japanese Pharmacopoeia Microbial Test 4.05 (Method I)	NMT 10,000 CFU/g
Salmonella species	Japanese Pharmacopoeia Microbial Test 4.05 (Method II)	Absent in 25 g
Escherichia coli	Japanese Standard Methods of Analysis in Food Safety Regulation	Absent in 25 g
Total coliforms	Japanese Standard Methods of Analysis in Food Safety Regulation	NMT 30 per g

CFU = colony forming units; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NMT = not more than; TOS = total organic solids; U = enzymatic unit of activity.

2.3.2 Batch Analysis

Full analytical data on 6 batches of the collagenase enzyme preparation from *S. violaceoruber* pCol (Lot Nos 8308801, 8308802, 8308803, 2142243, 01138125, and 0114514202) are presented below in Table 2.3.2-1 and demonstrate that the manufacturing process produces a consistent product that meets the product specifications defined in Section 2.3.1.

Table 2.3.2-1 Batch Analyses for Batches of DENAZYME XPP-011F Collagenase Enzyme Preparation from *Streptomyces violaceoruber* pCol

Parameters	Lot Nos					
	8308801	8308802	8308803	2142243[c]	01138125[c]	0114514202[c]
Appearance	Light brown powder	Light brown powder	Light brown powder	White to brown powder	White to brown powder	White to brown powder
Ash (%)	0.03	0.05	0.05	N/A	N/A	N/A
Moisture (%)	5.50	5.60	5.60	N/A	N/A	N/A
Dextrin (%)[a]	92.94	92.94	92.94	N/A	N/A	N/A
TOS (%)[b]	1.53	1.41	1.41	N/A	N/A	N/A

Table 2.3.2-1 Batch Analyses for Batches of DENAZYME XPP-011F Collagenase Enzyme Preparation from *Streptomyces violaceoruber* pCol

Parameters	Lot Nos					
	8308801	8308802	8308803	2142243[c]	01138125[c]	0114514202[c]
Collagenase activity	16.46 U/mg TOS	17.70 U/mg TOS	17.84 U/mg TOS	270 U/g	220 U/g	230 U/g
Antimicrobial activity	Negative	Negative	Negative	N/A	N/A	N/A
Lead (mg/kg)	<0.1	<0.1	<0.1	<5	<5	<5
Arsenic (as As_2O_3) (mg/kg)	<1	<1	<1	<3	<3	<3
Cadmium (mg/kg)	<0.01	<0.01	<0.01	N/A	N/A	N/A
Mercury (mg/kg)	<0.01	<0.01	<0.01	N/A	N/A	N/A
Total viable bacteria count (CFU/g)	<100	<100	<100	<10,000	<10,000	<10,000
Staphylococcus aureus (negative in 1 g)	Negative	Negative	Negative	N/A	N/A	N/A
Salmonella species (negative in 25 g)	Negative	Negative	Negative	Negative	Negative	Negative
Escherichia coli (negative in 25 g)	Negative	Negative	Negative	Negative	Negative	Negative
Total coliforms (/g)	<30	<30	<30	Negative	Negative	Negative
Sulfur-reducing anaerobes (CFU/g)	<30	<30	<30	N/A	N/A	N/A
Visible foreign object	N/A	N/A	N/A	Absent	Absent	Absent

CFU = colony forming units; N/A = not available; Nos = numbers; TOS = total organic solids; U = enzymatic unit of activity.
[a] Concentrate of collagenase is standardized by dilution with dextrin and enzyme preparations further diluted by addition of dextrin.
[b] TOS content was calculated according to the following equation: TOS = 100 - (A + W + D).
Where A = ash content, W = moisture content, and D = diluent content.
[c] Representative of scale up batches.

In addition to the testing for the chemical and microbiological purity specifications presented in Section 2.3.1, the enzyme preparation also was analyzed for potential presence of mycotoxins. Results of the analysis are presented in Table 2.3.2-2 and confirm absence of a number of recognized mycotoxins, including aflatoxins B1, B2, G1, and G2, in the DENAZYME XPP-011F collagenase enzyme preparation obtained from *Streptomyces violaceoruber* pCol.

Table 2.3.2-2 Mycotoxin Analysis on 3 Representative Batches of DENAZYME XPP-011F Collagenase Enzyme Preparation from *Streptomyces violaceoruber* pCol

Parameters	Lot Nos		
	8308801	8308802	8308803
Aflatoxin B_1 (µg/kg)	<1	<1	<1
Aflatoxin B_2 (µg/kg)	<1	<1	<1
Aflatoxin G_1 (µg/kg)	<1	<1	<1
Aflatoxin G_2 (µg/kg)	<1	<1	<1

Nos = numbers.

In addition to mycotoxin analysis, 3 batches of the DENAZYME XPP-011F enzyme preparation were tested for potential subsidiary enzyme activity, and the results are provided in Table 2.3.2-3. Activity per mg TOS

was calculated based on the TOS content of the individual batches from Table 2.3.2-1. Apart from the main intended enzymatic activity, small levels of lipase subsidiary activity were also detected. The subsidiary lipase enzymatic activity is not considered to raise a safety concern, considering that (i) the activity levels are relatively low in relation to the collagenase primary activity; (ii) the lipase enzyme has a long history of safe use in food processing; and (iii) the lipase enzyme occurs naturally in most edible plants or animals already consumed by humans.

Table 2.3.2-3 Subsidiary Enzyme Activity in 3 Representative Batches of DENAZYME XPP-011F Collagenase Enzyme Preparation from *Streptomyces violaceoruber* pCol

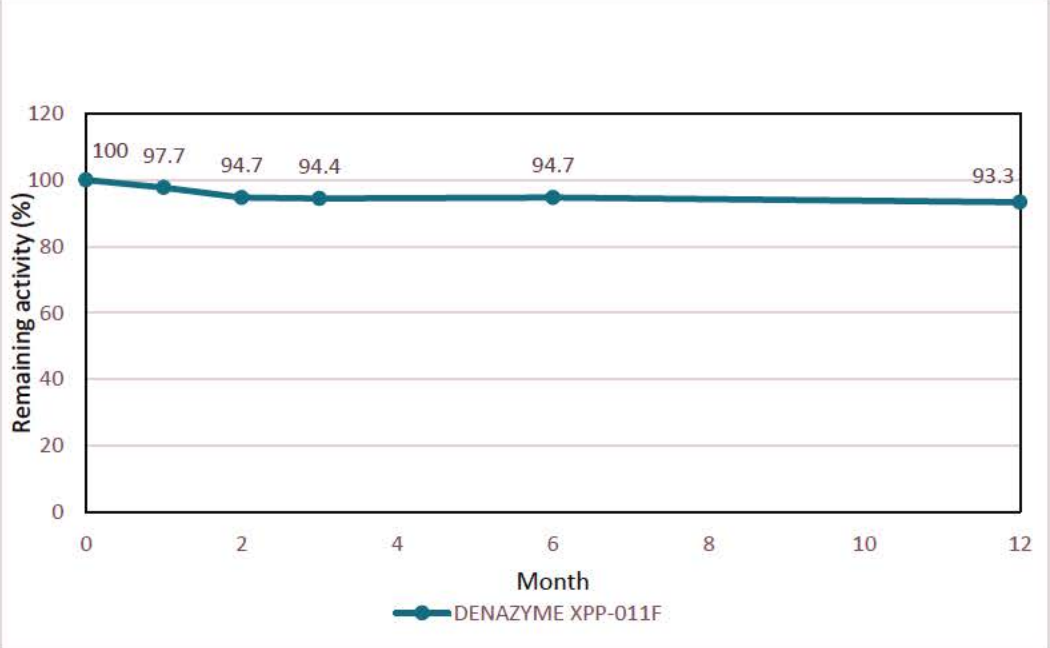
Parameters	Lot Nos		
	8308801	8308802	8308803
Collagenase[a] (U/mg TOS)	16.41	17.66	17.80
Amylase[b] (DUN/mg TOS)	<0.065	<0.071	<0.071
Lipase[c] (LUN/mg TOS)	0.85	1.35	1.28

DUN = dextrinogenic unit; LUN = lipolytic unit; Nos = numbers; TOS = total organic solids; U = enzymatic unit.
[a] Limit of quantification = 0.04 U/g.
[b] Limit of quantification = 1 DUN/g.
[c] Limit of quantification = 10 LUN/g.

2.4 Stability

The stability of the DENAZYME XPP-011F collagenase enzyme preparation was studied for a period of 12 months. The powdered DENAZYME XPP-011F collagenase enzyme preparations were sealed in aluminum bags and stored at 5°C. No significant loss of enzyme activity was reported. Based on the results, it can be concluded that the DENAZYME XPP-011F collagenase enzyme preparations are stable for a period of at least 12 months (see Figure 2.4-1).

Figure 2.4-1 Stability of the DENAZYME XPP-011F Collagenase Enzyme Preparation During Long-Term Storage at 5°C



2.5 Intended Technical Effects

2.5.1 Technological Function

The collagenase enzyme catalyzes the hydrolysis of peptide bonds in collagen. As such, the collagenase enzyme preparation is used as a processing aid for the degradation of collagen, which is intended to reduce the toughness of connective tissues in meat products by selectively hydrolyzing collagen resulting in enhancement of meat tenderness. Based on its enzymatic activity, the uses of collagenase are intended to improve the consistency, organoleptic, or the physical properties of meat products. It is noted that all food uses of collagenase involve a heating step to inactivate the enzyme.

It has been indicated in the publicly available literature that hydrolyzed collagen is also utilized in the production of several foodstuffs, such as confectionery, bakery, or dairy products (Dybka and Walczak, 2009). These uses are not covered by Nagase's application.

Collagenase is inactivated when held at temperatures above 50°C for 60 minutes (see Section 2.4). Consequently, no collagenase activity is expected in the final foods, as the manufacturing processes of meat products include a heating step and the manufacturing processes of collagen hydrolysates involves a sterilization step, both of which involve heating to temperatures much higher than 50°C (refer to Figures 2.5.1-1 to 2.5.1-3).

Figure 2.5.1-1 Schematic Manufacturing Process of Beef Jerky

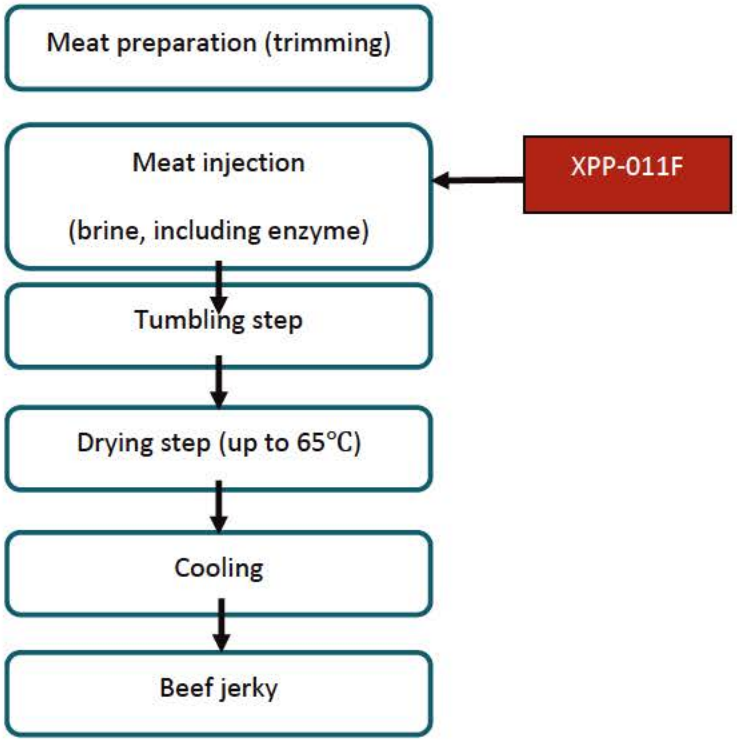


Figure 2.5.1-2 Schematic Manufacturing Process of Meat Products

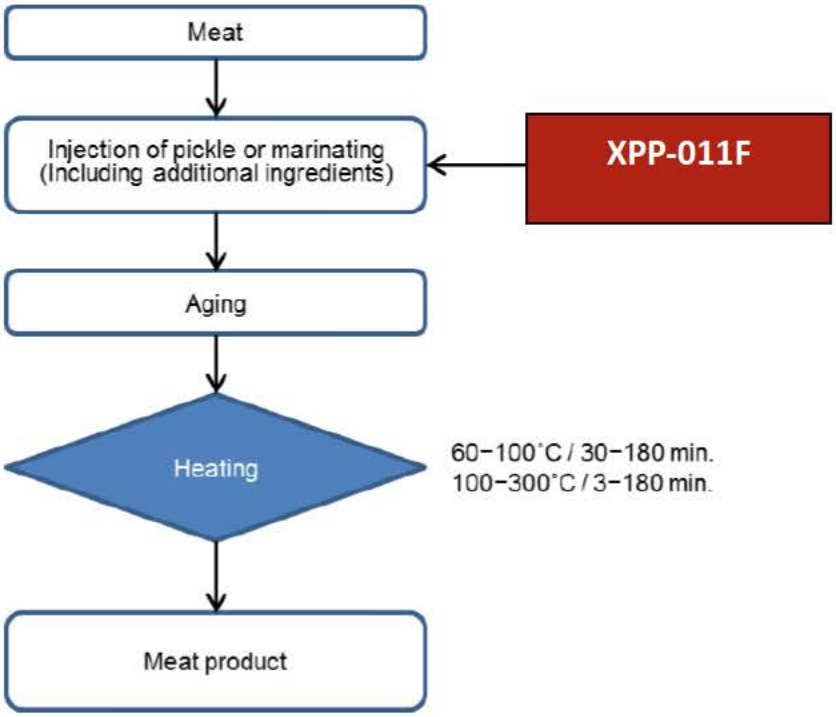
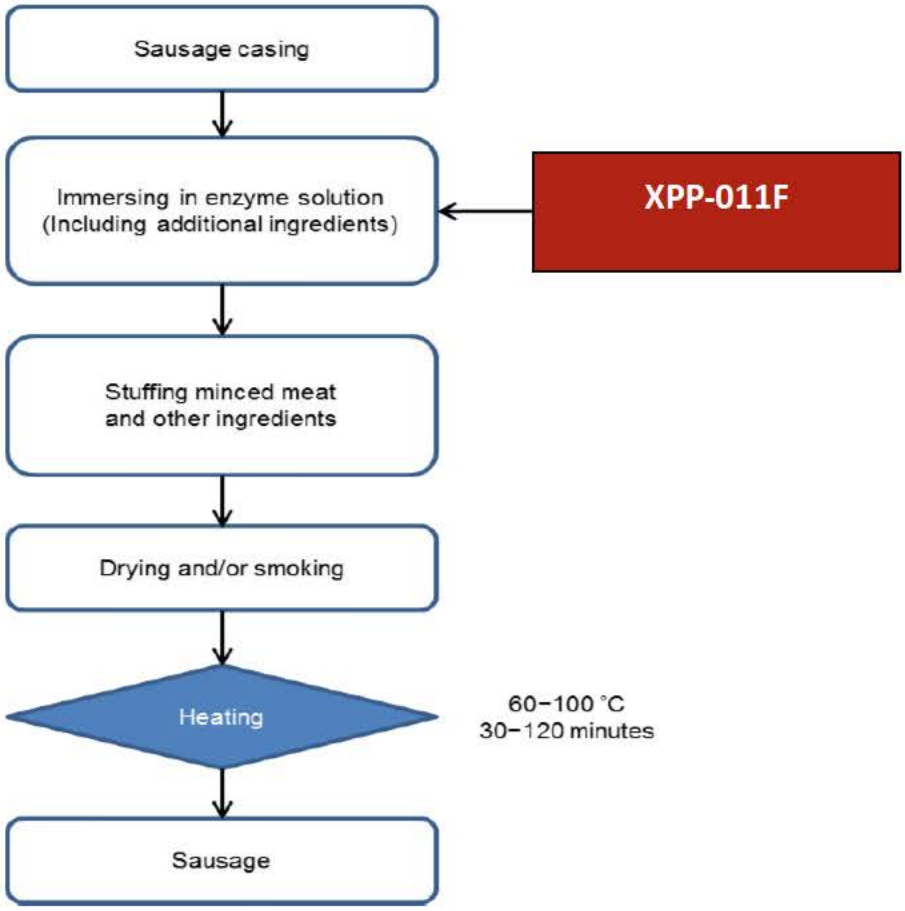


Figure 2.5.1-3 Schematic Manufacturing Process of Sausage Casing



To confirm the inactivation of the enzyme preparation during food processing, a study was performed on chicken meat treated with and without a powdered collagenase enzyme preparation characterized by a TOS content of 21.1% at a concentration of 0.025%. After treatment, the meat was left overnight in a refrigerator. After having it covered with commercial fried chicken powder, the chicken is fried with vegetable oil at 160 to 170°C for 3 minutes and subsidiary enzymatic activities were measured. The meat was neither filtered nor sterilized before measurements. As presented in Table 2.5.1-1, no secondary enzymatic activities were detected with or without powdered collagenase treatment following processing of the chicken meat.

Table 2.5.1-1 Residual Enzyme Activities in Chicken Meat Treated with and without Powdered Collagenase

Treatment	Replicate	Residual Enzyme Activity	
		Lipase (LUN/g)	Amylase (DUN/g)
- Enzyme	1	N.D.	N.D.
	2	N.D.	N.D.
	3	N.D.	N.D.
+ Enzyme	1	N.D.	N.D.
	2	N.D.	N.D.
	3	N.D.	N.D.

DUN = dextrinogenic unit; LOQ = limit of quantitation; LUN = lipolytic unit; N.D. = not detected.
Note: LOQ for lipase: 10 LUN/g; LOQ for α-amylase: 1 DUN/g.

A similar experiment was conducted on the production of gelatin with and without collagenase. The collagen hydrolysate was treated with a powdered collagenase enzyme preparation characterized by a TOS content of 21.1% at a concentration of 0.1%. The liquid is then concentrated and refined in order to obtain the peptide. As presented in Table 2.5.1-2, no secondary enzymatic activities were detected with or without powdered collagenase treatment following processing of gelatin.

Table 2.5.1-2 Residual Enzyme Activities in Gelatin Treated with or without Powdered Collagenase

Treatment	Replicate	Residual Enzyme Activity	
		Lipase (LUN/g)	Amylase (DUN/g)
- Enzyme	1	N.D.	N.D.
	2	N.D.	N.D.
	3	N.D.	N.D.
+ Enzyme	1	N.D.	N.D.
	2	N.D.	N.D.
	3	N.D.	N.D.

DUN = dextrinogenic unit; LOQ = limit of quantitation; LUN = lipolytic unit; N.D. = not detected.
Note: LOQ for lipase: 10 LUN/g, LOQ for α-amylase: 1 DUN/g.

Considering that the available method for measuring collagenase activity consists of measuring Gly-Pro linkages, and because such peptides are present at high levels in the treated meat or gelatin, collagenase residual activities could not be measured. In summary, it was reported that no subsidiary activities were detectable in foodstuffs or collagen hydrolysates processed with the enzyme preparation. Collagen is the only substrate for collagenase and the main intended reaction product is hydrolyzed collagen. No other unintended reaction products have been identified. Collagenase is not known to exhibit any effect on other food components, such as lipids and hydrocarbons or other micro-organisms in food. As such, it can be concluded that the food enzyme does not exert any unintentional enzymatic activity in the final food.

2.5.2 Food Applications and Use Levels

Collagenase from *S. violaceoruber* pCol is intended for direct use in meat processing such as beef jerky, marinated and injected meat, and sausage casings, as presented in Table 2.5.2-1. The DENAZYME XPP-011F powder preparation will be marketed for use in meat processing and sold exclusively for meats undergoing further processing (*i.e.,* it will be sold to businesses and not to final consumers). Furthermore, it was shown in Section 2.4 that the DENAZYME CPP-011F preparation is stable for a period of at least 12 months under long-term storage at 5˚C, demonstrating that the enzyme is suitable for use on refrigerated meat products. Although the use level of the enzyme is *quantum satis*, maximum use levels recommended by the enzyme manufacturer to achieve the desired technological effect for each individual food application are presented in Table 2.5.2-1.

Table 2.5.2-1 Summary of the Individual Proposed Food Uses and Maximum Recommended Use Levels of Collagenase from *Streptomyces violaceoruber* pCol in the U.S.

Application	Maximum Proposed Collagenase Use Level (mg TOS/kg food)[a]	Maximum Level of Collagenase Potentially Present in Final Foods[b]
Beef Jerky	7.25	10.9 mg TOS/kg
Meat (marinated)	7.25	
Meat (injection)	10.9	
Sausage Casing	3.63	

TOS = total organic solids; U.S. = United States.

[a] The food enzyme preparation is used *quantum satis*. The use level indicated is the maximum use level recommended by the enzyme manufacturer to achieve the desired technological effect.

[b] Assumes that none of the collagenase from *Streptomyces violaceoruber* pCol used in meat processing is removed or denatured. In reality, the processing conditions of collagenase-treated meat products involve treatment at high temperatures which will inactivate the enzyme, thereby ensuring that the enzyme will not have any technological effect in final foods treated with collagenase.

The dietary exposure assessment considered maximum recommended use levels of collagenase from *Streptomyces violaceoruber* pCol for meat processing applications. Assuming collagenase is not removed and/or denatured during the final processing stages, and that 100% of the enzyme preparation is carried over to finals foods, the maximum level of collagenase potentially present in final meat products is 10.9 mg TOS/kg.

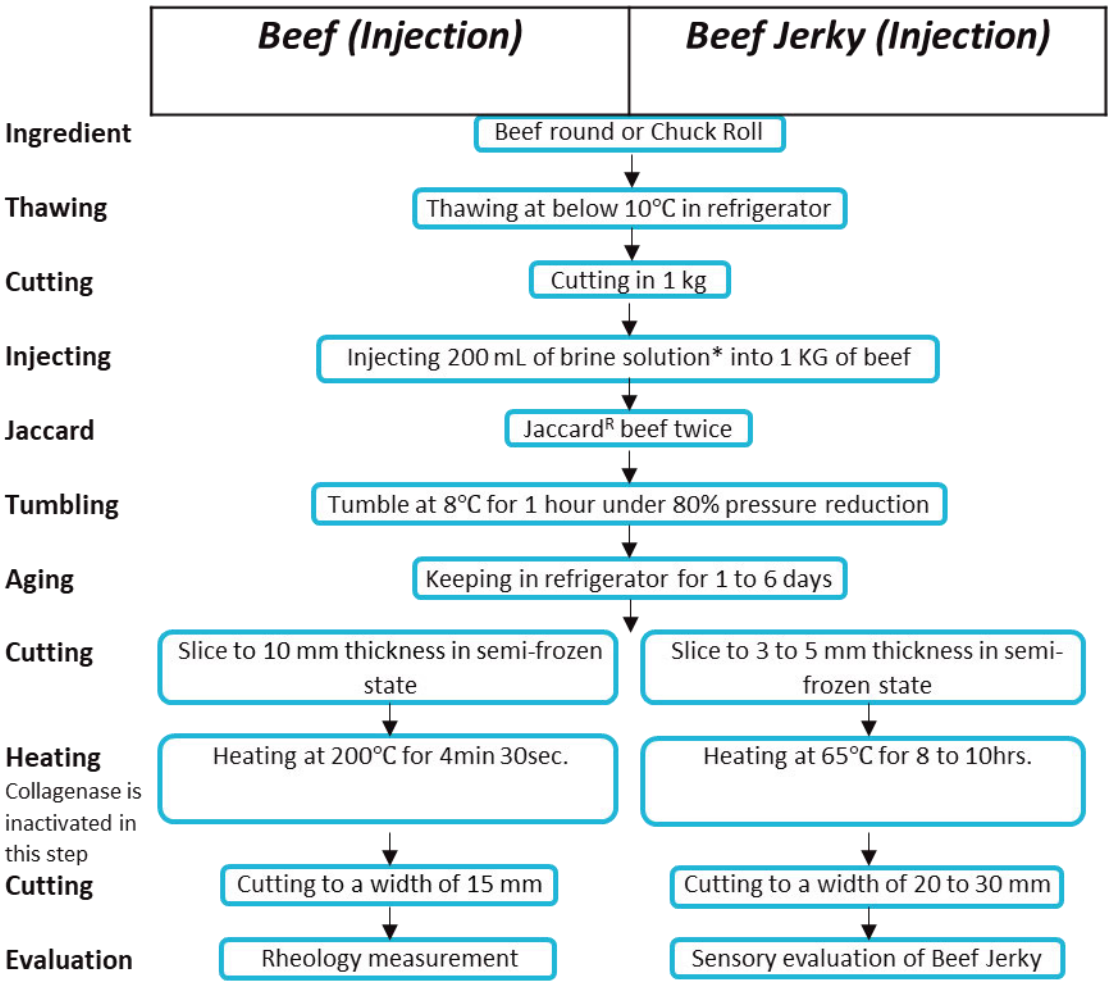
In addition to the assumption that collagenase is added at the maximum recommended use level for meat processing, it was assumed in the dietary exposure assessment that all target foods are processed with collagenase. In reality, the recommended use level of collagenase is *quantum satis,* and it is unlikely that collagenase will have 100% market penetration in all identified food categories. Furthermore, it was assumed in the dietary exposure assessment that collagenase is not removed and/or denatured during the final processing stages of meat products and that 100% of the enzyme preparation is carried over to final foods. In reality, as indicated in Section 2.5.1, the enzyme is inactivated during the final processing stages by treatment at high temperatures, and thereby, ensuring that the enzyme will not have any technological effect in final foods.

2.5.3 Suitability Data

Suitability data for the DENAZYME XPP-011F powdered preparation for its intended uses has been prepared, the results of which demonstrate that the collagenase enzyme preparation accomplishes its intended function across the range of its intended uses, as described in Section 1.3. For the intended uses

of injecting beef and beef jerky, a brine solution containing 2.2% collagenase was injected and the meats were subjected to typical processing (see Figure 2.5.3-1). The enzyme is expected to be heat-inactivated during the Heating step shown in Figure 2.5.3-1. The beef was evaluated using rheology measurements and the beef jerky was evaluated using sensory evaluations, and injection of the brine solution resulted in good, tenderized texture (see Figure 2.5.3-2 and 3).

Figure 2.5.3-1 Processing Procedure for Beef and Beef Jerky Injected with DENAZYME XPP-011F



* Brine solution either contains water ("Brine (Control)") or XPP-011F ("Brine + XPP-011F") at levels of 2.2% which corresponds to 10.9 mg TOS/kg food based on a TOS content of 1.5% when injected at 200 mL/kg food.

Figure 2.5.3-2 Rheology Evaluation of Beef Injected with DENAZYME XPP-011F

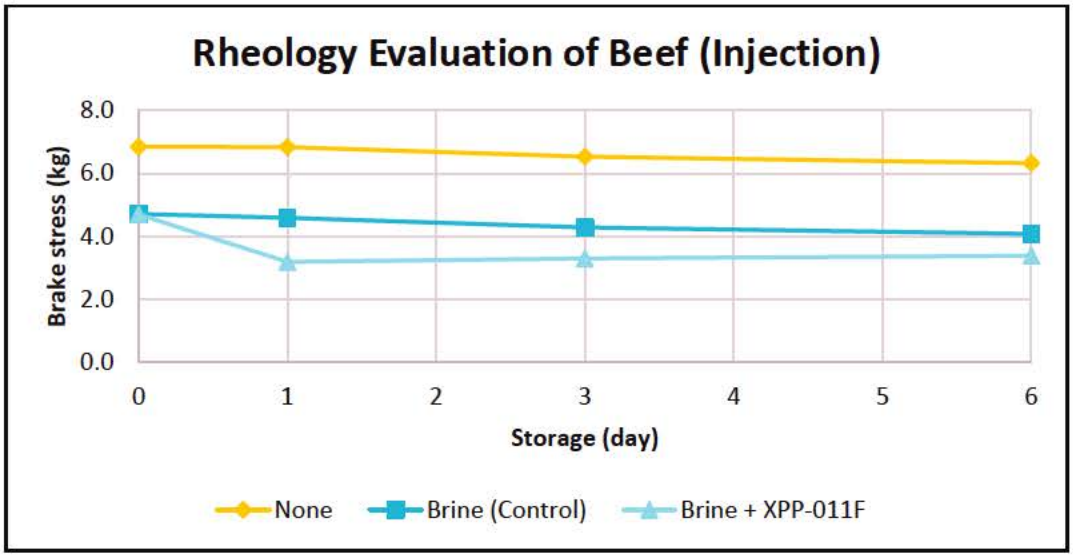
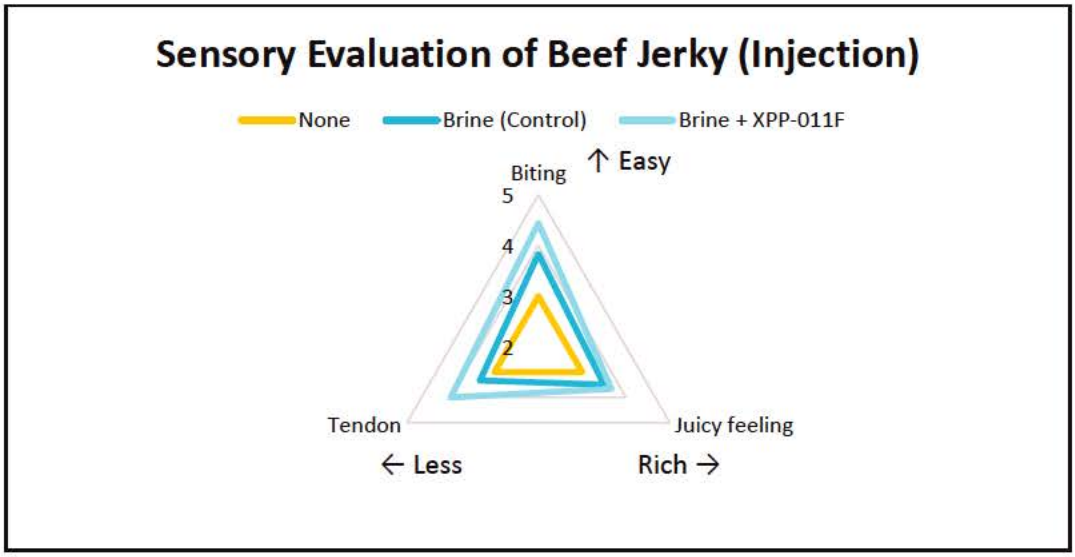
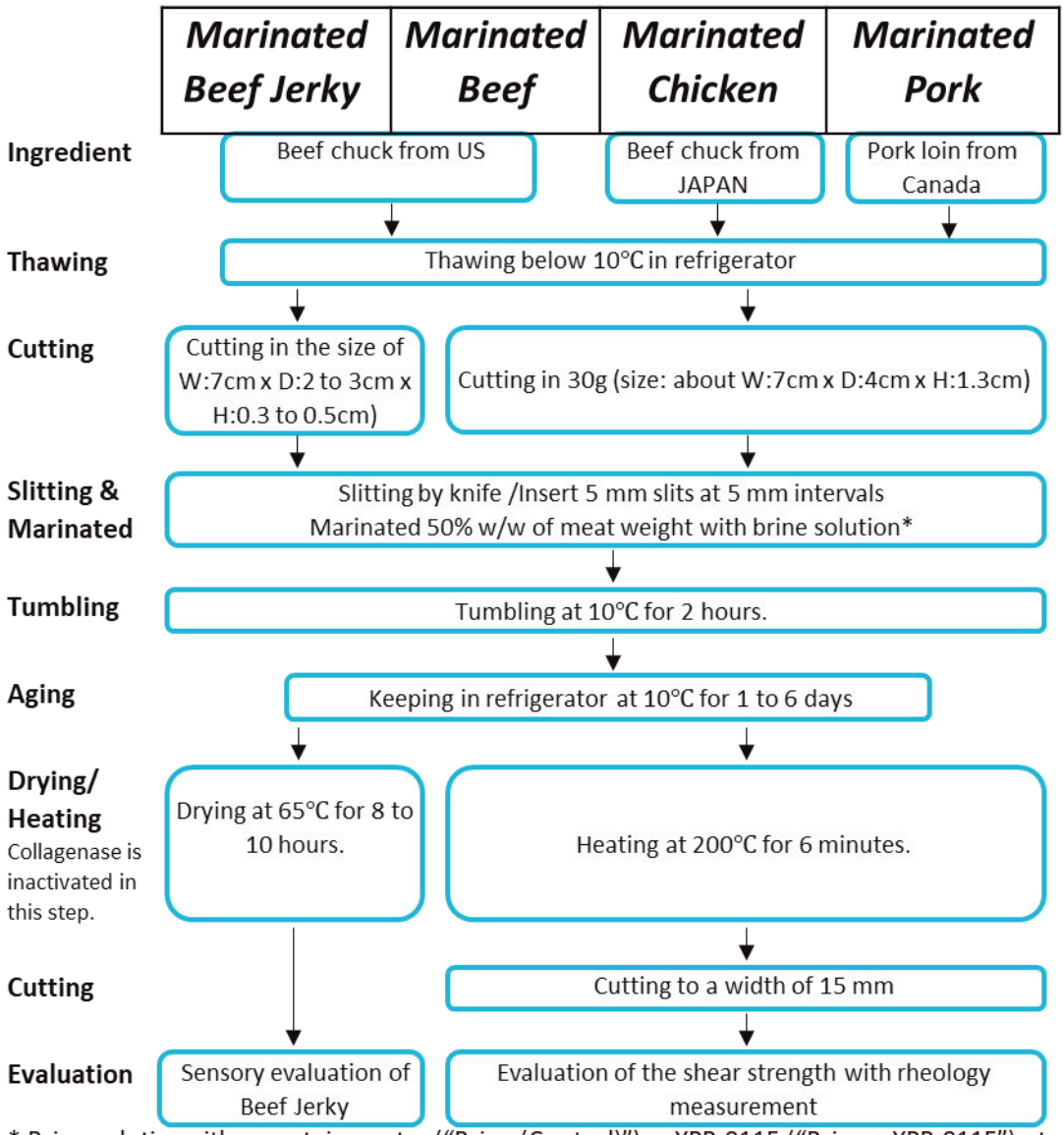


Figure 2.5.3-3 Sensory Evaluation of Beef Jerky Injected with DENAZYME XPP-011F



For the intended uses of marinating beef jerky, beef, chicken, and pork, these meats were marinated with a brine solution containing 5.3% collagenase and subjected to typical processing (see Figure 2.5.3-4). The enzyme is expected to be heat-inactivated during the Drying/Heating step shown in Figure 2.5.3-4. The beef jerky was evaluated using sensory evaluations, and the beef, chicken, and pork were evaluated using rheology measurements. Marinating of these meats with the brine solution resulted in good, tenderized texture (see Figures 2.5.3-5 to 8).

Figure 2.5.3-4 Processing Procedure for Beef Jerky, Beef, Chicken, and Pork Marinated with DENAZYME XPP-011F



* Brine solution either contains water ("Brine (Control)") or XPP-011F ("Brine + XPP-011F") at levels of 5.3% which corresponds to 7.25 mg TOS/kg food based on a TOS content of 1.5% when marinated with 100 mL/kg food.

Figure 2.5.3-5 Sensory Evaluation of Beef Jerky Marinated with DENAZYME XPP-011F

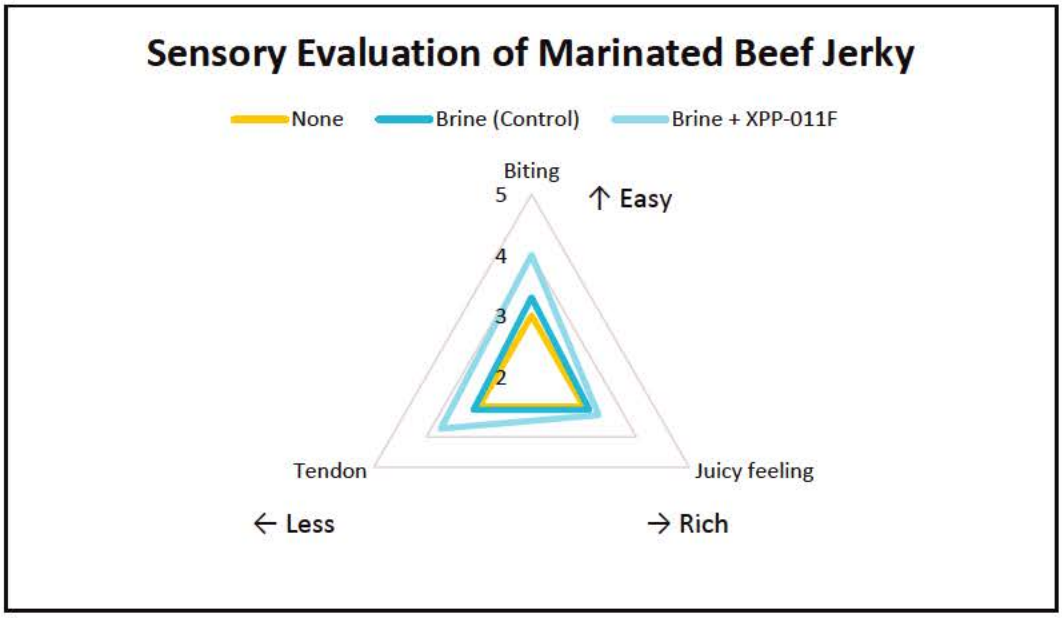


Figure 2.5.3-6 Rheology Evaluation of Beef Marinated with DENAZYME XPP-011F

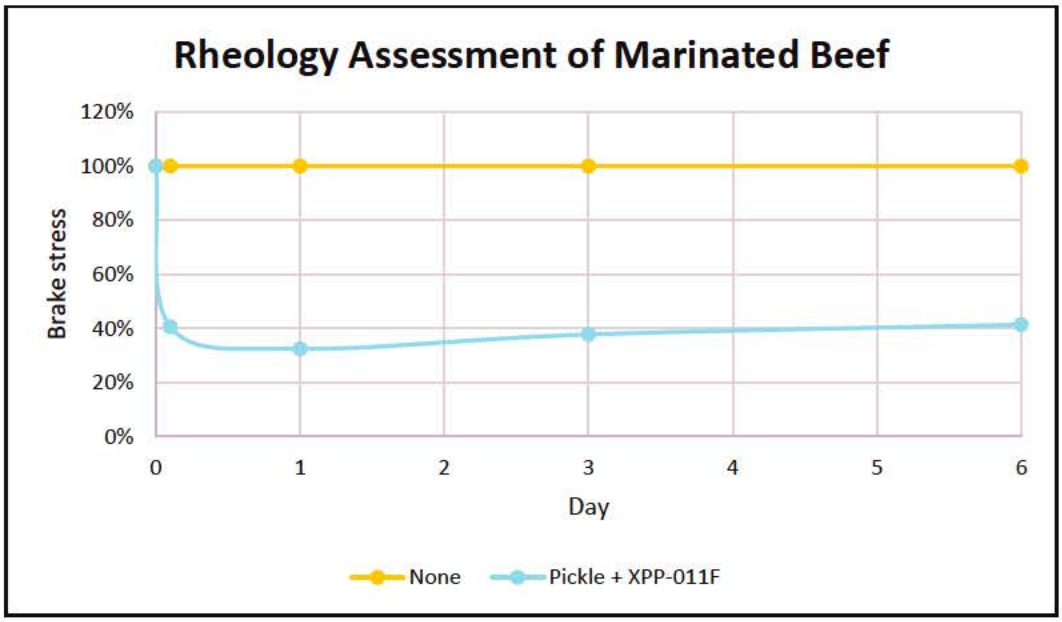


Figure 2.5.3-7 Rheology Evaluation of Chicken Marinated with DENAZYME XPP-011F

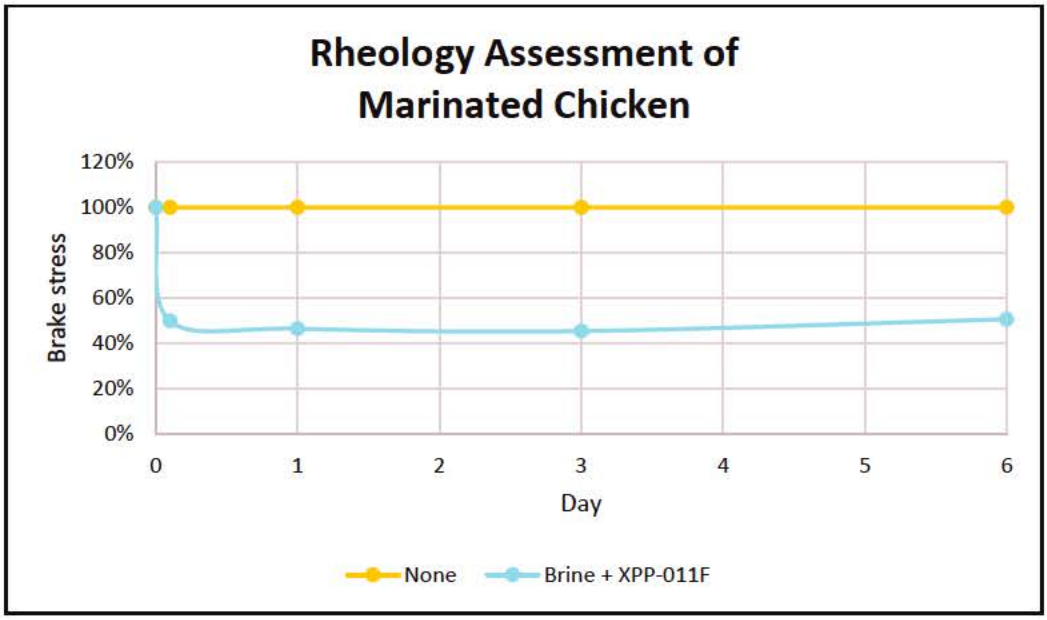
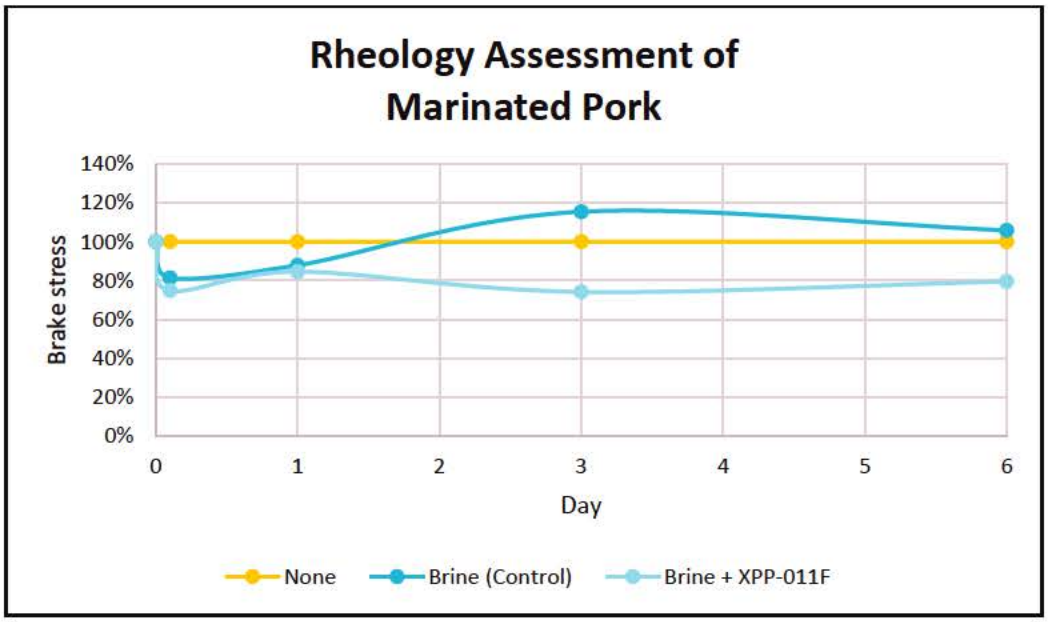
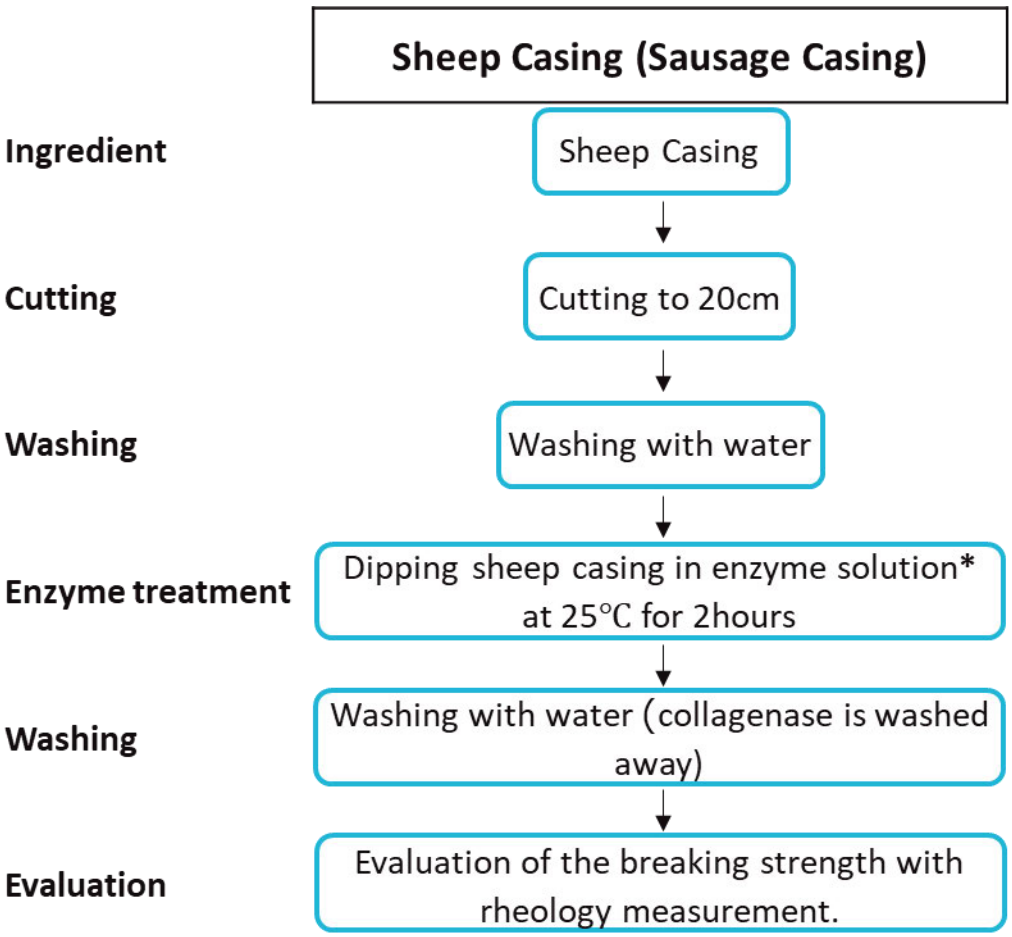


Figure 2.5.3-8 Rheology Evaluation of Pork Marinated with DENAZYME XPP-011F



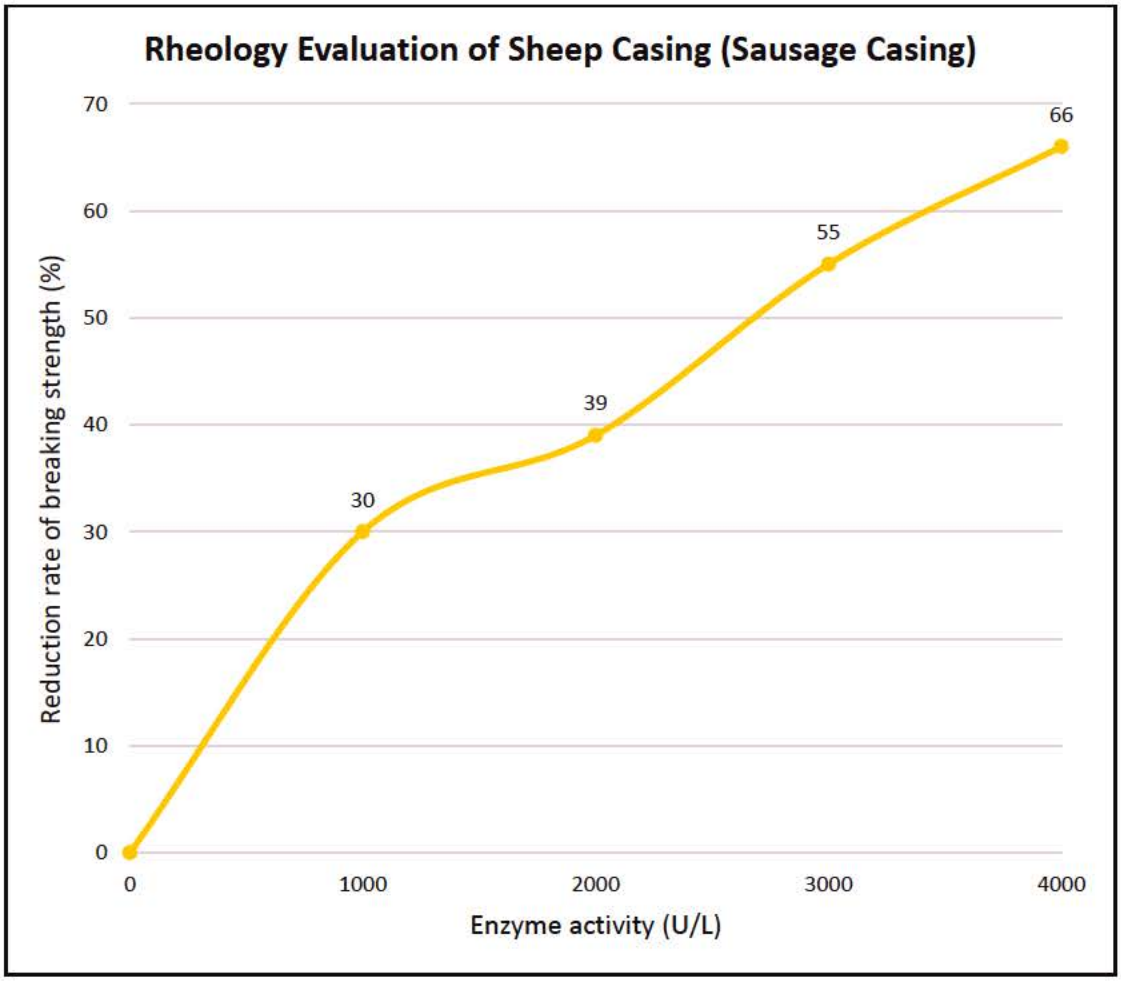
For the intended use in sausage casing, the sheep casing was subjected to typical processing and dipped in a solution containing 0, 0.5, 1, 1.5, or 2% collagenase (corresponding to 0, 1,000 2,000, 3,000, or 4,000 enzymatic units/L, respectively) at 25˚C for 2 hours (see Figure 2.5.3-9). The enzyme is expected to be washed away during the second Washing step in Figure 2.5.3-9. The sausage casing was evaluated using rheology measurements. Nagase reported that the optimal crispy texture occurs between 20 and 40% reduction in break strength. Given the gradual increase in reduction of break strength accomplished using increasing concentrations of DENAZYME-XPP-011F, including a reduction of 30 and 39% using 0.5% and 1.0% XPP-011F, respectively, both of which fall into the optimal texture range, the enzyme preparation is suitable for obtaining optimal crispy texture of sausage casings (see Figure 2.5.3-10).

Figure 2.5.3-9 Processing Procedure for Sausage Casing Treated with DENAZYME XPP-011F



* The enzyme solution contained XPP-011F at concentrations of 0%, 0.5%, 1.0%, 1.5%, or 2.0% (corresponding to 1,000, 2,000, 3,000, or 4,000 U/L, respectively).

Figure 2.5.3-10 Rheology Evaluation of Sausage Casings Treated with DENAZYME XPP-011F



Part 3. §170.235 Dietary Exposure

Potential human exposure to the collagenase enzyme preparation under the proposed conditions of use was estimated. The assessment was performed using the "Budget" method, as described below.

3.1 Overview of the Budget Method

The potential human exposure to collagenase has been estimated using the Budget Method, which is a widely-accepted preliminary screening tool used to assess the intake of chemicals such as food additives (FAO/WHO, 2020). The Budget Method allows for the calculation of a theoretical maximum daily intake (TMDI) based on assumptions regarding the maximum human physiological levels of daily food and beverage consumption, rather than on food consumption data collected from dietary surveys. Specifically, the Budget Method relies on conservative assumptions made regarding (i) the level of consumption of solid foods; (ii) the level of presence of the substance in solid foods; and (iii) the proportion of solid foods that may contain the substance (FAO/WHO, 2020). The levels of anticipated exposure to food enzymes that are derived using the Budget Method are, thus, considered to be conservative estimates (FAO/WHO, 2020).

The results of this assessment are described in Section 3.1.1 below.

3.1.1 Assumptions of the Budget Method

Level of Consumption of Solid Foods

The FAO/WHO report on the *Principles and Methods for the Risk Assessment of Chemicals in Food* (FAO/WHO, 2020) specifies the standard value for food intake to be 0.05 kg/kg body weight/day for solid foods.

Level of Presence of Food Enzyme in Solid Foods

To estimate the exposure to collagenase from its intended uses in foods, it is assumed that the entire enzyme preparation added during processing will be present in the final foods as consumed (*i.e.*, assuming no removal and/or inactivation). Thus, the amount of enzyme assumed to be present in solid foods is based on the maximum recommended use level of the enzyme in meat processing. It is anticipated that all of the collagenase added remains in meat products. Therefore, the maximum amount of collagenase that could potentially be present in solid foods processed with the enzyme is 10.9 mg TOS/kg food.

Proportion of Solid Foods That May Contain the Food Enzyme

According to the FAO/WHO report, the default proportion that is typically assumed is that 12.5% of all solid foods consumed will contain the food enzyme (FAO/WHO, 2020). However, the proportion of solid foods containing the food enzyme may be increased to 25% in cases where the substance is used in a wide range of food categories (FAO/WHO, 2020). The food uses listed in Table 2.5.2-1 are representative of some of the final foods processed with collagenase. However, since the substance is not used in a wide range of food categories, the proportion of solid foods that are assumed to contain the enzyme was not increased to 25% for the TMDI assessment.

3.2 Theoretical Maximum Daily Intake of the Collagenase Enzyme Preparation from *Streptomyces violaceoruber* pCol

Based on conservative assumptions for the Budget Method described in Section 3.1.1 above, the TMDI of collagenase, from its intended use in the processing of meat products, was calculated to be 0.068 mg TOS/kg body weight/day, as shown in Table 3.2-1.

Table 3.2-1 Estimated TMDI of Collagenase from Foods Intended for the General Population that Contain the Ingredients Made with the Enzyme Preparation

Products	A Level of Consumption of Foods (kg/kg bw/day)	B Proportion of Foods Containing Collagenase (%)	C Level of Consumption of Foods Containing Collagenase (kg/kg bw/day)[a]	D Maximum Level of Collagenase in Foods (mg TOS/kg)	Exposure to Collagenase (mg TOS/kg bw/day)[b]
Solid Foods	0.05	12.5	0.00625	10.9	0.068

bw = body weight; TMDI = theoretical maximum daily intake; TOS = total organic solids.
[a] Calculation: (A)*(B/100).
[b] Calculation: (C)*(D).

3.3 Summary of the Estimated Daily Intake

The potential human exposure to collagenase was calculated assuming that all of the enzyme used in the processing of meat products is carried over to final foods. However, in most cases collagenase will be heat-denatured and inactivated during the final stages of processing for the final food products, which involves treatment at high temperatures, such as those that occur during pasteurization or sterilization. As such, even though the enzyme may be present, it will not have any technological effect in final foods as consumed. Furthermore, it was assumed that collagenase is added at maximum recommended use levels of the enzyme preparation to target foods intended for the general population at an inclusion rate of 100% (*i.e.*, all target foods are processed with collagenase).

Using the budget method, the TMDI of collagenase from the consumption of meat products processed with collagenase was estimated at 0.068 mg TOS/kg body weight/day in the general population.

Part 4. §170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with the collagenase enzyme preparation from *S. violaceoruber* pCol (DENAZYME XPP-011F).

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. §170.250 Narrative and Safety Information

6.1 Introduction

Nagase's collagenase enzyme preparation is isolated from the fermentation of *S. violaceoruber* pCol. The collagenase enzyme preparation is intended for processing of beef jerky and production of collagen hydrolysates, which are used as ingredients in food supplements to catalyze the hydrolysis of peptide bonds in collagen. Under the conditions of intended use in foods, the total TMDI was calculated to be 0.068 mg TOS/kg body weight/day. However, it should be noted that considering the intended uses of the collagenase enzyme preparation, the enzyme was reported to be inactivated during the processing of the final food product.

In the U.S., the safety evaluation of enzyme preparations is typically conducted in accordance with the safety assessment paradigm established by Pariza and Foster (1983). This paradigm was updated in 2001 to incorporate safety considerations unique to enzyme preparations produced by modern practices of biotechnology (Pariza and Johnson, 2001). An evaluation of the safety of the collagenase enzyme preparation using the Pariza decision tree approach involves 2 pivotal elements: (i) an assessment of the safety of the production strain lineage; and (ii) data and information supporting the safety of the enzyme preparation under its conditions of intended use in foods. A discussion of available safety studies conducted using the collagenase enzyme preparation is presented in Section 5.2, bioinformatic evaluation of the enzyme is presented in Section 5.3, and information relevant to the safety of the *S. violaceoruber* pCol production strain is discussed in Section 5.4.

6.2 Toxicological Studies on Nagase's Collagenase Enzyme Preparation

Toxicological studies of collagenase from *S. violaceoruber* pCol have been conducted using a powdered collagenase concentrate (93.96% TOS and enzyme activity of 10,287 U/g).

6.2.1 Acute Toxicity

An acute oral toxicity study of collagenase concentrate was conducted in accordance with the OECD Test Guideline 423 (*Acute oral toxicity - acute toxic class method*) (OECD, 2001) and Principles of Good Laboratory Practice (GLP) (OECD, 1998a; Harazono *et al*., 2020). The study was conducted in 8-week-old Sprague-Dawley SPF rats that were given a single gavage administration of collagenase concentrate from *S. violaceoruber* pCol (Lot No. 3T-1) characterized by an enzyme activity of 10,287 U/g or 10,948 U/mg TOS (based on the TOS content of 93.96%). The powder test material was dissolved in water to a concentration of 200 mg/mL and 2 groups of 3 females each received the test material solutions at a dose volume of 10 mL/kg body weight *via* gavage providing doses of 2,000 mg/kg body weight or 1,879.2 mg TOS/kg body weight. This dose is the upper limit level described in the test guideline and was selected because enzymes are known to have low acute toxicity. Clinical observations were made 4 hours after administration and daily thereafter for 14 days and body weights were recorded at the time of administration and on Days 1, 3, 7, and 14 after administration. At the end of the 14-day observation period, the animals were euthanized by exsanguination and subjected to pathological examination.

No deaths or abnormalities were reported throughout the study. No significant changes due to administration of the collagenase enzyme were reported in the general condition, body weight, body weight gain, or gross pathology compared to controls. The median lethal dose of collagenase from *S. violaceoruber* pCol *via* gavage was estimated to be >1,879.2 mg TOS/kg body weight (Harazono *et al*., 2020).

6.2.2 Subchronic Toxicity

A 13-week repeated-dose oral toxicity study of collagenase concentrate was conducted in accordance with the OECD Test Guideline 408 (*Repeated dose 90-day oral toxicity study in rodents*) (OECD, 1998b) and Principles of GLP (OECD, 1998a; Harazono *et al*., 2020). The study was conducted in 6-week-old Sprague-Dawley SPF rats *via* gavage with collagenase concentrate from *S. violaceoruber* pCol (Lot No. 3T-1) characterized by an enzyme activity of 10,287 U/g or 10,948 U/mg TOS (based on the TOS content of 93.96%) for 13 weeks. The powder test material was dissolved in water and 4 groups, each comprising 10 males and 10 females, received the test article at concentrations of 0 (distilled water), 58.7, 234.9, and 939.6 mg TOS/kg body weight/day. The dose levels were selected based on the results of a preliminary 2-week repeated-dose oral toxicity study in which animals were administered 0 (distilled water), 58.7, 234.9, or 939.6 mg TOS/kg body weight/day *via* gavage (Harazono *et al*., 2020). No compound-related adverse effects were reported in any of the dose groups in the 2-week preliminary study, and therefore, the high-dose level for the 13-week study was set at 939.6 mg TOS/kg body weight/day, the highest tested dose from the 2-week preliminary study.

Clinical observations and mortality were monitored at least twice daily except on the day of necropsy (Day 91) when clinical observations were only monitored once. Body weights and food consumption were recorded weekly as well as on the day of necropsy and the mean daily food consumption was calculated. Ophthalmological examinations consisting of appearance, light reflex, and examination of the anterior segment of the eyeball, optic media, and fundus oculi were conducted on surviving animals from the control and high-dose (939.6 mg TOS/kg body weight/day) groups on Day 82 and 83 in males and females, respectively. Blood samples were collected from the abdominal aorta from fasted animals under anesthesia

prior to necropsy and were analyzed for routine hematology, coagulation, and serum chemistry parameters. Hematology parameters included hematocrit, hemoglobin concentration, red blood cell count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte ratio, platelet count, white blood cell count, differential leukocyte ratios, neutrophil count, lymphocyte count, monocyte count, eosinophil count, basophil count, and large unstained cell count. Coagulation parameters included prothrombin time, activated partial thromboplastin time, and fibrinogen concentration. Serum chemistry parameters included total protein, glucose, triglyceride, total cholesterol, blood urea nitrogen, creatinine, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase activity, calcium, inorganic phosphorus, sodium, potassium, chloride, albumin ratio, α1-, α2-, β-, and γ-globulin ratios, and the protein fraction and albumin:globulin (A/G) ratio were calculated. Urine samples were also collected prior to necropsy following an overnight fasting and deprivation of water and were subjected to urinalysis. Urinalysis parameters included pH, occult blood, ketone bodies, glucose, protein, bilirubin, and urobilinogen, urinary volume and color, osmotic pressure, and urine sediments were microscopically examined. Animals were euthanized by exsanguination following the final test-article administration and blood sample collection and were subjected to pathological examinations. Organ weights were recorded, and organ-to-body (relative) weights were calculated based on the body weights on the day of necropsy. Histopathological examinations were conducted on all organs outlined in the OECD test guidelines for animals from all groups.

All animals survived until study termination and no treatment-related clinical signs of toxicity were reported in any group. No significant and treatment-related differences in body weight, body weight gain, or food consumption were reported between control and treatment groups. A statistically significant increase in food consumption was recorded on Day 21 in females of the low dose group (58.7 mg TOS/kg body weight/day) compared to controls, and a statistically significant reduction in food consumption was recorded on Day 42 in males of the low-dose group compared to the control group. The study authors considered these effects not toxicologically relevant, as there was no clear dose response relationship and there were no observed effects on body weight gain in these groups. No significant and treatment-related differences were reported in any of the measured ophthalmological, hematology, urinalysis, or coagulation parameters. A significantly decreased value for blood chloride was reported in males of the high-dose group. Abnormally high values in aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic acid dehydrogenase (LDH), total cholesterol, triglyceride, and phospholipids were reported in 1 female of the high-dose group, but no significant changes were reported in this group compared to the controls. A statistically significant increase in the relative kidney weight in males of the high-dose group compared to the controls was reported. No other statistically significant differences in relative or absolute organ weights were reported and no treatment-related histopathological findings were reported.

Based on the results of this study, administration of 939.6 mg TOS/kg body weight/day, the highest dose tested, resulted in a statistically significant reduction in blood chloride levels and a statistically significant increase in relative kidney weights in males and abnormally high values AST, ALT, LDH, total cholesterol, triglycerides, phospholipids, and total bilirubin in 1 female. The study authors reported that the no-observed-adverse-effect level (NOAEL) was 234.9 mg TOS/kg body weight/day, the mid-dose group, providing a margin of safety of 3,454 compared to the TMDI of 0.068 mg TOS/kg body weight/day. However, it should be noted that the reported effects were isolated, only seen in 1 female, were not dose-related, and were not accompanied by any histopathological findings. As such, these effects are unlikely to be toxicologically relevant.

6.2.3 Genotoxicity/Mutagenicity

Bacterial Reverse Mutation Assays

The potential mutagenicity of the powder concentrate of collagenase from *S. violaceoruber* pCol was evaluated in a bacterial reverse mutation assay conducted in 5 tester strains: *Salmonella typhimurium* TA100, TA98, TA1535, and TA1537, and *Escherichia coli* WP2uvrA using the preincubation method (Harazono *et al*., 2020). The study was conducted in accordance with the OECD Test Guideline 471 (*Bacterial reverse mutation test*) (OECD, 1997a) and the Principles of GLP (OECD, 1998a). Preliminary and concentration range-finding tests were conducted using the preincubation method to investigate bacterial growth inhibition and mutagenicity and to determine the concentrations to be used in the main tests. In the preliminary and concentration range-finding tests, concentrations up to 4,698 µg TOS/plate were tested in all 5 tester strains with and without metabolic activation. Growth inhibition was reported at the highest tested concentration of 4,698 µg TOS/plate in all tester strains of *S. typhimurium* with and without metabolic activation; however, no significant increases in the number of revertant colonies were reported. Concentrations for the main test were established based on the results of the preliminary and concentration range-finding tests such that plates with strains of *S. typhimurium* were treated with concentrations of 0 (water), 147, 294, 587, 1,175, 2,349, or 4,698 µg TOS/plate and plates of *E. coli* WP2uvrA were treated with concentrations of 0 (water), 294, 587, 1,175, 2,349, or 4,698 µg TOS/plate with and without metabolic activation. The compounds used as positive controls in the assays conducted in the absence of metabolic activation included: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2), sodium azide (NaN_3), or 2-methoxy-6-chloro-9-[3-82-chloroethyl9-aminopropylamino] acridine 2HCl (ICR-191). The compounds used as positive controls in the assays conducted in the presence of metabolic activation were either 2-aminoanthracene (2-AA) or benzo[α]pyrene (B[α]P).

In the first test, the number of revertant colonies exceeded twice that of the negative control group at concentrations of 2,349 and 4,698 µg TOS/plate in *S. typhimurium* strain TA1535 in the presence of metabolic activation; however, growth inhibition was also reported at 4,698 µg TOS/plate in *S. typhimurium* strain TA1535 in the presence of metabolic activation. A dose-dependent increase in the number of revertant colonies was also reported in *S. typhimurium* strain TA100 in the absence of metabolic activation and in strains TA100 and TA1535 in the presence of metabolic activation. The number of revertant colonies in all other tester strains was less than twice that of the negative control at all tested concentrations in the presence and absence of metabolic activation, and precipitation of the test article was not observed. The number of revertant colonies in the positive controls were greater than 2 times those reported in the negative controls. Based on the increase in the number of revertant colonies in *S. typhimurium* strain TA1535 at 2,349 and 4,698 µg TOS/plate in the presence of metabolic activation and the dose-dependent increase in *S. typhimurium* strain TA100 in the absence of metabolic activation and in strains TA100 and TA1535 in the presence of metabolic activation, the study authors reported that "*COL* [collagenase] *produced a positive response in the in vitro bacterial reverse mutation test using the pre-incubation methods*" (Harazono *et al*., 2020).

A second bacterial reverse mutation assay was then conducted using the preincubation and treat-and-wash methods in *S. typhimurium* strains TA100 and TA1535 to determine if the findings from the first study were due to free amino acids. Concentrations of 0 (water), 939.6, 1,879, 2,819, 3,758, or 4,598 µg TOS/plate with and without metabolic activation were used for this second main test. The positive controls used for these strains, with and without metabolic activation, from the first main test were also included in this second main test. The number of revertant colonies was similar to the negative controls in all of the tester strains up to the highest tested concentration (4,698 µg TOS/plate) using the preincubation or treat-and-wash methods in the presence and absence of metabolic activation. The number of revertant colonies in the

positive controls were greater than 2 times those reported in the negative controls. The study authors concluded that "*based on the results of the treat-and-wash method, it was concluded that COL* [collagenase] *was non-mutagenic in the in vitro bacterial reverse mutation test*" and the positive results observed in the first study were likely due to the presence of free amino acids (Harazono *et al*., 2020).

In Vitro Mammalian Cell Gene Mutation Assays

The potential mutagenicity of the powder concentrate of collagenase was further evaluated in an *in vitro* mammalian cell gene mutation assay (mouse lymphoma TK assay) using mouse lymphoma cultured cells (L5178Y) under short-term and continuous exposure conditions (Harazono *et al*., 2020). The study was based on OECD Test Guideline N 476 (*In vitro mammalian cell gene mutation test*) (OECD, 1997b) and 490 (*In vitro thymidine kinase mutation test*) (OECD, 2016a) and conducted in accordance with the Principles of GLP (OECD, 1998a). A preliminary concentration range-finding test was conducted in which cells were incubated with concentrations of 0 (water), 73.4, 147, 293, 587, 1,175, 2,349, or 4,698 µg TOS/mL without metabolic activation. The relative cell survival rate remained above 20% for all the concentration groups. Concentrations for the main test were selected based on the results of the preliminary concentration range-finding test such that cells were incubated with concentrations of 0 (water), 147, 294, 587, 1,175, 2,349, or 4,698 µg TOS/mL for 24-hour continuous treatment without metabolic activation. Positive control groups were treated with methyl methanesulfonate in the absence of metabolic activation or cyclophosphamide in the presence of metabolic activation (data not shown in study report). All samples were plated in duplicate to determine mutation frequency.

No precipitation was reported and the total mutant frequency (TMF) was less than twice that of the negative control group and less than 200×10^{-6} up to the highest tested concentration of 4,698 µg TOS/mL with and without metabolic activation under short-term exposure conditions (data not shown in study report). Under continuous treatment conditions, the TMF exceeded 200×10^{-6} (299.75×10^{-6}) at the highest tested concentration of 4,698 µg TOS/mL without metabolic activation. Due to a lack of a clear dose-response relationship, a confirmatory test was conducted to further investigate the results obtained at the highest tested dose in this test.

In the confirmatory test, no precipitation was reported and the TMF was similar to the negative controls and did not exceed 200×10^{-6} up to the highest tested concentration of 4,698 µg TOS/mL (presence or absence of metabolic activation was not reported). In addition, the percentage of small colonies (indicators of chromosomal damage) was 83% at concentrations of 4,698 µg TOS/mL which is higher than the negative controls (36%) (statistical significance not reported). The study authors reported that this finding "…*might suggest that the results were reactions caused by chromosome aberrations*" (Harazono *et al*., 2020). In order to elucidate the possible role of chromosomal aberrations, an *in vivo* micronucleus test was conducted.

In Vitro Micronucleus Induction Assay

The genotoxic potential of the powder collagenase concentrate was evaluated in an *in vitro* micronucleus induction assay in cultured human peripheral lymphocytes (Laboratory of Pharmacology and Toxicology, 2020 [unpublished]; Appendix A). The study was conducted in accordance with OECD Test Guideline No. 487 (*In vitro mammalian cell micronucleus test*) (OECD, 2016b) and the Principles of GLP (OECD, 1998a). A preliminary cytotoxicity test was conducted to determine the concentrations to be used in the main study in which cell cultures were exposed to concentrations of 0 (purified water), 125, 250, 500, 1,000, or 2,000 µg/mL (TOS content not reported) with and without metabolic activation for 4 or 24 hours. Test article precipitation was reported at concentrations of 1,000 and 2,000 µg/mL in the presence and absence of

metabolic activation in both 4- and 24-hour exposure conditions; however, no signs of cytotoxicity or relevant changes in pH or osmolality were reported when compared to the negative control group at any tested concentration. The concentrations used in the main study were, therefore, set to match the concentrations used in the preliminary cytotoxicity test such that cells were incubated with concentrations of 0 (purified water), 125, 250, 500, 1,000, or 2,000 µg/mL with and without metabolic activation for 4 or 24 hours. The compounds used as positive controls in the assays conducted in the absence of metabolic activation included mitomycin C and colchicine to demonstrate clastogenic and aneugenic effects, respectively. In the assays conducted in the presence of metabolic activation, cyclophosphamide was used as the positive control to demonstrate clastogenic effects.

No cytotoxicity was reported in the 4-hour exposure conditions in the presence and absence of metabolic activation or the 24-hour exposure conditions in the absence of metabolic activation up to the highest tested concentration of 2,000 µg/mL. Test item precipitation was reported at concentrations of 1,000 and 2,000 µg/mL under all treatment conditions. No significant differences were reported in the mean micronucleus frequencies in any treated group up to the highest tested concentration of 2,000 µg/mL under all treatment conditions compared to the negative controls. The mean micronucleus frequency in the negative control group was within the range of historical control data and a significant increase was reported in the positive control group compared to the negative control group. The test was, therefore, considered to have been conducted appropriately and the study authors concluded that:

> "*…under the present test conditions, pCol Protease [collagenase] tested up to the top concentration of 2,000 µg pCol Protease/mL medium in the absence and in the presence of metabolic activation employing two exposure times without S9 mix and one exposure time with S9 mix revealed no indications of chromosomal damage in the in vitro micronucleus test*" (Laboratory of Pharmacology and Toxicology, 2020 [unpublished]; Appendix A).

In Vivo Micronucleus Induction Assay

The genotoxic potential of the powder collagenase concentrate was further evaluated in an *in vivo* micronucleus induction assay in 8-week-old Sprague-Dawley SPF rats (Harazono *et al*., 2020). The study was conducted in accordance with OECD Test Guideline No. 474 (*Mammalian erythrocyte micronucleus test*) (OECD, 1997c) and the Principles of GLP (OECD, 1998a). The rats were received at 7 weeks of age and acclimatized for 6 days. A preliminary dose range-finding study was conducted in which 4 groups of 3 males and 3 females were administered doses of 0 (water), 234.9, 469.8, 939.6, or 1,879 mg TOS/kg body weight/day by gavage for 2 consecutive days. No noteworthy effects related to general condition or mortality were reported and no significant and treatment-related differences in body weights were reported. The doses in the main study were based on these findings. Due to a lack of sex-specific difference in the preliminary study, the main study was conducted only with males. In the main test, 4 groups of 5 males were administered doses of 0 (water), 234.9, 469.8, 939.6, or 1,879 mg TOS/kg body weight/day by gavage for 2 consecutive days. Mitomycin C was used as the positive control substance. General condition and mortality were monitored immediately before and after dosing, approximately 2 hours after dosing, and prior to collecting the bone marrow samples. Body weights were measured upon receiving the animals, after the acclimatization period, and prior to collecting the bone marrow samples. Animals were terminated by exsanguination and bone marrow samples were collected 24 hours after the second administration. Bone marrow smears were prepared and scored for normochromatic erythrocytes and polychromatic erythrocytes (PCE) per 200 erythrocytes and the number of micronucleated polychromatic erythrocytes (MNPCE) per 2,000 PCE were also counted, and the proportion was calculated.

No clinical signs of toxicity or significant differences in body weight gain were reported in any animals including the highest dose tested (1,879 mg TOS/kg body weight/day). No significant differences in the proportion of MNPCE and PCE were reported between treatment and negative control groups and the positive control resulted in a significant increase in the proportion of MNPCE compared to the negative control. The study was therefore conducted appropriately and study authors concluded that collagenase from *S. violaceoruber* pCol is non-genotoxic under the conditions of this assay (Harazono *et al.*, 2020).

The results and study details for genotoxicity/mutagenicity assays conducted with the powder collagenase concentrate are summarized in Table 6.2.3-1.

Table 6.2.3-1 Genotoxicity of Collagenase from *Streptomyces violaceoruber* pCol

Assay	Test system	Concentration	Result	Reference
In vitro				
Reverse mutation (preincubation)	*Salmonella typhimurium* TA100, TA98, TA1535 and TA1537 and Escherichia coli WP2uvrA	0, 147, 294, 587, 1,175, 2,349, or 4,698 µg TOS/plate ± S9	Positive in *S. typhimurium* TA1535 at 4,698 µg TOS/plate + S9	Harazono *et al.* (2020)
Reverse mutation (preincubation/treat-and-wash)	*S. typhimurium* TA100 and TA1535	0, 939.6, 1,879, 2,819, 3,758, or 4,698 µg TOS/plate ± S9	Negative	Harazono *et al.* (2020)
Mammalian cell gene mutation test	L5178Y mouse lymphoma cells	0, 147, 294, 587, 1,175, 2,349, or 4,698 µg TOS/mL ± S9	Equivocal	Harazono *et al.* (2020)
Micronucleus test	Human peripheral lymphocytes	0, 125, 250, 500, 1,000, 2,000 µg/mL ± S9	Negative	Laboratory of Pharmacology and Toxicology (2020) [unpublished]; Appendix A
In vivo				
Micronucleus test	Sprague-Dawley SPF rats	0, 469.8, 939.6, or 1,879 mg TOS/kg bw/day	Negative	Harazono *et al.* (2020)

+ = presence; - = absence; ± = presence or absence; bw = body weight; NR = not reported; S9 = 9,000 x g microsomal fraction from rat livers serving as metabolic activation; SPF = specific-pathogen free; TOS = total organic solids.

6.3 Safety of the *Streptomyces violaceoruber* pCol Production Strain

6.3.1 History of Safe Use of the Production Strain

Streptomyces violaceoruber is a Gram-positive bacterium that occurs naturally in soil (Duangmal *et al.*, 2005). Species of bacteria from the genus, *Streptomyces,* have been used in the production of antibiotics as early as 1942 and up to 80% of antibiotics are now produced by species of this genus (de Lima Procopio *et al.*, 2012). Species of bacteria from this genus have also been widely used in the production of industrially important enzymes, including those intended for use in food processing (Mukhtar *et al.*, 2017). The GRAS status of several food enzymes derived from *S. violaceoruber*, including Genencor's phospholipase A2 enzyme preparation from *Streptomyces violaceoruber*, Nagase's phospholipase A2 enzyme preparation from *Streptomyces violaceoruber* expressing a gene encoding phospholipase A2, and beta-glucanase from *Streptomyces violaceoruber* has been notified to the U.S. FDA in 2004, 2007, and 2015, respectively, and all

received letters of "no questions" from the Agency for use in food processing (Genencor International, Inc., 2004; U.S. FDA, 2004, 2007, 2015; Nagase ChemteX Corporation, 2006; Nagase America Corporation, 2014).

Both the production and host strains of *S. violaceoruber* are classified in Group 1 of National Institutes of Health (NIH) Guidelines (November 2013 revision) concerning risk groups for host and production microorganisms: "Agents that are not associated with disease in healthy adult humans."

JECFA expressed several positive opinions on the use of products derived from the *Streptomyces* genus, but did not specify any acceptable daily intakes (ADIs) for glucose isomerase from *Streptomyces violaceoniger*, *Streptomyces olivochromogenes*, or *Streptomyces rubiginosus,* and for protease from *Streptomyces fradiae*.

In 2017, EFSA evaluated *Streptomyces violaceoruber* for the qualified presumption of safety (QPS) status, and concluded that "*S. violaceoruber cannot be recommended for the QPS list because safety concerns cannot be excluded*" (EFSA, 2017). These safety concerns are related to the presence of granaticin, an aminoacyl tRNA synthetase inhibitor, and phospholipase A as well as the possibility that *S. violaceoruber* may produce secondary metabolites, similar to the related strain, *S. coelicolor* (EFSA, 2017). *S. violaceoruber* species are known for their ability to produce secondary metabolites granaticin and phospholipase A2 (Ichinose *et al*., 1998; Sugiyama *et al*., 2002).

6.3.2 Pathogenicity of the Production Strain

A review of the publicly available scientific literature was conducted to identify the relevant literature on the pathogenicity of strains of *S. violaceoruber.* The pathogenicity of species of the genus *Streptomyces* in humans has been reviewed (Kieser *et al*., 2000), the results of which indicated that infections caused by actinomycetes can mostly be attributed to species *Mycobacterium*, *Actinodura*, *Nocardia*, and *actinomyces,* rather than *Streptomyces*. Although *Streptomyces somaliensis*, *Streptomyces madurae*, and *Streptomyces sudanenisis* have been identified as pathogens (Boiron *et al*., 1998; Khatri *et al*., 2002; Dieng *et al*., 2003, 2005; Quintana *et al*., 2008), no publications were identified indicating that strains of *S. violaceoruber* are pathogenic.

6.3.3 Pariza Decision Tree

The safety of the production strain was determined using the guidelines developed by Pariza and Foster (1983), Pariza and Johnson (2001), and IFBC (1990), presented in the form of a decision tree. These guidelines are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial enzyme preparations used in foods. Using this Pariza decision tree approach (see Figure 6.3.3-1), *S. violaceoruber* pCol is genetically modified using recombinant DNA technology; however, it is derived from a safe lineage, as previously demonstrated by repeated assessment *via* this evaluation procedure. As mentioned in Section 6.3.2, the GRAS status of several food enzymes derived from *S. violaceoruber* (U.S. FDA, 2004, 2007, 2015) demonstrate that strains of this species have been repeatedly evaluated to be derived from a safe lineage. Furthermore, the collagenase food enzyme does not have a history of safe use in food, and as such, the following questions in the Pariza decision tree must be addressed in order for the enzyme to be considered safe:

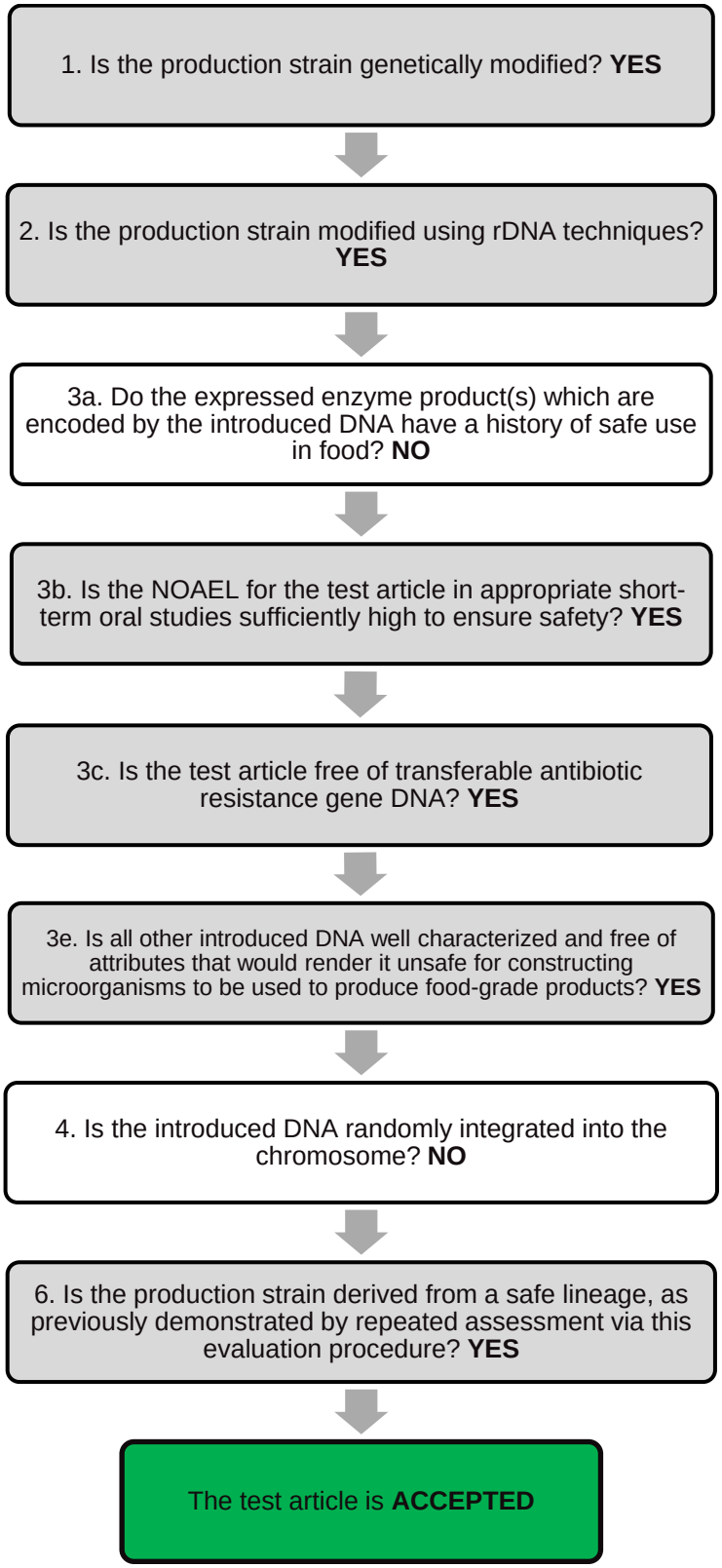
3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?

3c. Is the test article free of transferable antibiotic resistance gene DNA?

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?

4. Is the introduced DNA randomly integrated into the chromosome?

Figure 6.3.3-1 Determining the Safety of *Streptomyces violaceoruber* pCol for Use in Food Based on the Pariza Decision Tree



With respect to Point 3b above, a NOAEL of 234.9 mg TOS/kg body weight/day was determined for both males and females from the 13-week study in rats (Harazono *et al*., 2020), which results in a MoS of 703 when compared to the TMDI of 0.068 mg TOS/kg body weight/day, which exceeds the 100-fold MoS necessary to satisfy this question from Pariza and Johnson (2001).

With respect to Point 3c above, although the plasmid pIJ702 contains an antibiotic resistance gene (thiostrepton, *tsr*), it was reported by Kieser *et al.* (1982) that the plasmid is non-conjugative, non-transferable, and it maintains its autonomous form in the cell. Consequently, the frequency of the inserted vector's mobilization and the ability for the transfer of genetic material are estimated to be very low. Furthermore, the thiostrepton resistance protein can be easily digested by enzymes in the human digestive tract, and therefore, do not present a concern related to transferable antibiotic resistance gene DNA (see Section 2.2.3 for more details).

With respect to Point 3e above, the sequences introduced by the plasmid are well-characterized and come from strains that have been evaluated as safe. The promotor sequence, *samp-pro,* is the promotor sequence of the gene of metallo-endopeptidase from *S. avermitilis* ATCC 31267, which belongs to the Biosafety Level 1: not known to consistently cause diseases in immunocompetent adult humans. The terminator sequence, *pld-ter,* is the terminator sequence of the gene of phospholipase D from *S. cinnamoneus* NBRC 12852, which has been used previously for the construction of a self-cloned strain of *S. violaceoruber* pCol for the production of beta-glucanase (GRN 535). Therefore, the introduced DNA is well-characterized and free of attributes that render it unsafe for the construction of microorganisms for the production of food-grade products (see Section 2.1.2 for more details).

Finally, with respect to Point 4 above, modification of the host strain *S. violaceoruber* 1326 was performed by means of the protoplast method. This method has a long history of use in the introduction of plasmids into actinomycetes and has been used previously for the construction of a self-cloned strain of *S. violaceoruber* for the production of beta-glucanase (GRN 535).

Overall, based on the progression through the Pariza and Johnson (2001) Decision Tree, *S. violaceoruber* pCol is considered safe for use in the production of collagenase.

6.4 Other Studies

6.4.1 Allergenicity

Although Pariza and Foster (1983) have indicated that there have been no confirmed cases of allergies or primary irritations caused by enzymes used for food processing, collagenase from *S. violaceoruber* pCol was evaluated for potential allergenicity using the bioinformatics criteria recommended by FAO/WHO (FAO/WHO, 2001; Codex Alimentarius, 2009; JECFA, 2016). The amino acid sequence for the collagenase was compared to the amino acid sequences of known allergens in publicly available databases. No matches with greater than 35% percent identity were identified using a window of 80 amino acids, no exact matches were identified using a window of 8 amino acids, and no sequences were considered homologous with known allergens using a full sequence search with an E-value cut-off of 0.1. Therefore, dietary exposure to collagenase from *S. violaceoruber* pCol is not expected to pose a risk of allergenicity.

6.4.2 Toxigenic and Pathogenic Potential of Collagenase

The Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information (NCBI) was used to conduct a sequence alignment query of the collagenase

FASTA protein sequence against downloaded protein sequences obtained from a curated database of 7,235 animal venom proteins and toxins[1] maintained by UniProt. BLAST searches also were conducted against 9,832 putative virulence factors originating from 3 manually curated online databases: PATRIC VF[2], VFDB[3], and Victors[4] maintained by the Pathosystems Resource Integration Center (PATRIC)[5] (Mao *et al.*, 2015; Liu *et al.*, 2019; Sayers *et al.*, 2019). A sequence alignment of 35% identity and an E-value cut-off of 0.1 were used as thresholds for identification of a positive alignment. No significant similarity to any of the venom proteins and toxins were identified in the homology searches. Two sequences with significant alignment with collagenase were identified in the homology search for putative virulence factors. A summary of the significant sequence alignments is presented in Table 6.4.2-1 below.

The significant sequence alignment with collagenase ColT from *Clostridium tetani* E88 was not considered to be a positive alignment, as the percent identity of this alignment was below 35%. The significant sequence alignment with collagenase colA (kappa-toxin) was considered to be a positive alignment based on the percent identity of 40.86% and the E-value of 5×10^{-8}. The role of collagenase colA (kappa-toxin) in pathogenesis remains to be determined (Harrington, 1996; Rood, 1998); however, it has been shown to be an important virulence factor in *Leptospira interrogans* infection (Kassegne *et al.*, 2014) but was not an important virulence factor in *Clostridium perfringens* infection (Awad *et al.*, 2000). Furthermore, it has been reported that recombinant collagenases expressed in non-pathogenic strains would not result in undesired virulence factors for use in the food industry (Duarte *et al.*, 2016). Therefore, despite the positive sequence homology between the collagenase food enzyme and the collagenase colA (kappa-toxin), the potential for collagenase to increase infection and transmission as a virulence factor is limited if the host microorganism is non-pathogenic. Following a search of the publicly available scientific literature, no publications were identified that have indicated that strains of *S. violaceoruber* are pathogenic (see Section 6.3.2). As such, the potential for collagenase to cause pathogenicity is limited. In addition to the above, Pariza and Johnson (2001) do not consider pathogenicity to be a relevant consumer safety issue, provided the enzyme preparation does not contain live organisms. The production strain, *S. violaceoruber* pCol, is removed from the enzyme preparation during multiple filtration steps (see Section 2.2), and therefore, satisfies this stipulation by Pariza and Johnson (2001). Given the non-pathogenic nature of the production strain and the absence of the production strain from the commercial enzyme preparations, collagenase from *S. violaceoruber* pCol will not present a safety concern related to pathogenicity.

Table 6.4.2-1 Significant Sequence Alignments from Homology Search for Putative Virulence Factors

Virulence Factor	Native Strain	Query Cover	E-Value	Percent Identity
Collagenase ColT	*Clostridium tetani* E88	19%	4×10^{-8}	26.84%
(colA) Collagenase [kappa-toxin (VF0388)]	*Clostridium perfringens* str. 13	44%	5×10^{-8}	40.86%

6.5 GRAS Panel Evaluation

Nagase has concluded that the collagenase enzyme preparation from *S. violaceoruber* pCol (DENAZYME XPP-011F) is GRAS for direct use in meat processing such as beef jerky, marinated and injected meat, and sausage casings, as described in Section 1.3, on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of collagenase as discussed

[1] UniProt release Oct, 2017; available at: https://www.uniprot.org/program/Toxins.
[2] https://docs.patricbrc.org/user_guides/data/data_types/specialty_genes.html.
[3] http://www.mgc.ac.cn/VFs/main.htm.
[4] http://www.phidias.us/victors/.
[5] https://www.patricbrc.org/.

herein and on consensus among a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Professor Emeritus Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine); Professor David Brusick, (Toxicology Consultant); and Professor Emeritus Michael W. Pariza (University of Wisconsin-Madison).

The GRAS Panel, convened by Nagase, independently and critically evaluated all data and information presented herein, and also concluded that the collagenase enzyme preparation from *S. violaceoruber* pCol (DENAZYME XPP-011F) is GRAS for direct use in meat processing such as beef jerky, marinated and injected meat, and sausage casings, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and evaluation of such data as it pertains to the proposed GRAS uses of the collagenase enzyme preparation from *S. violaceoruber* pCol (DENAZYME XPP-011F) is presented in Appendix B.

6.6 Conclusion

Based on the above data and information presented herein, Nagase has concluded that the collagenase enzyme preparation from *S. violaceoruber* pCol (DENAZYME XPP-011F) is GRAS, on the basis of scientific procedures, for direct use in meat processing such as beef jerky, marinated and injected meat, and sausage casings, as described in Section 1.3. General recognition of Nagase's GRAS conclusion is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of the collagenase enzyme preparation from *S. violaceoruber* pCol (DENAZYME XPP-011F) in food, who similarly concluded that the proposed uses of the collagenase enzyme preparation from *S. violaceoruber* pCol (DENAZYME XPP-011F) are GRAS on the basis of scientific procedures.

The collagenase enzyme preparation from *S. violaceoruber* pCol (DENAZYME XPP-011F), therefore, may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the Code of Federal Regulations.

Part 7. §170.255 List of Supporting Data and Information

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APPENDIX A

Study Report for *In Vitro* Micronucleus Induction Assay

LABORATORY of PHARMACOLOGY and TOXICOLOGY

LPT

LPT Report No. 38051

IN VITRO ASSESSMENT OF pCOL PROTEASE IN THE MICRONUCLEUS TEST IN CULTURED HUMAN PERIPHERAL LYMPHOCYTES

- according to OECD guideline 487 and EC method B.49 -

Sponsor:

Nagase ChemteX Corporation
1-52 Osadano-cho Fukuchiyama
Kyoto 620-0853
Japan

Study conducted by:

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21147 Hamburg
Germany

Contact Person:

Dr. Koichi Harazono

Contact Person:

Dr. Jost Leuschner

27 May 2020

This report consists of 42 pages.

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STATEMENT OF COMPLIANCE

IN VITRO ASSESSMENT OF pCOL PROTEASE IN THE MICRONUCLEUS TEST IN CULTURED HUMAN PERIPHERAL LYMPHOCYTES

- according to OECD guideline 487 and EC method B.49 -

The study was performed in compliance with:

- 'Good Laboratory Practice' Regulations of the EC enacted in Germany in the 'Chemikaliengesetz' [Chemicals Act], current edition;
- 'OECD Principles of Good Laboratory Practice' Document No. 1 (ENV/MC/CHEM (98) 17) regulated in the Directive 2004/10/EC of the European Parliament and the Council of 11 February 2004;

These principles are compatible with 'Good Laboratory Practice' regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA) and Japan (MHLW, MAFF and METI).

There were no deviations from the 'Good Laboratory Practice' regulations. Raw data obtained during the performance of the study are accurately reflected.

[redacted]

Dr. rer. nat. B. Spruth
Study Director

27 May 2020

Date

QUALITY ASSURANCE STATEMENT

Based on a quality assurance review, it was concluded that this report accurately reflects the raw data for the study. Methods, procedures and observations are correctly and completely described in the report.

IN VITRO ASSESSMENT OF pCOL PROTEASE IN THE MICRONUCLEUS TEST IN CULTURED HUMAN PERIPHERAL LYMPHOCYTES

- according to OECD guideline 487 and EC method B.49 -

Study Plan dated 03 December 2019.

Text table 0-1 Inspections of **LPT**'s QAU

Date of inspection	Criteria	Date of report to the Study Director and the Management
03 Dec 2019	Study Plan	03 Dec 2019
27 Jan 2020 + 29 – 31 Jan 2020	Process-based inspection of mutagenicity tests in cultured human peripheral lymphocytes: seeding cells, test item identity, calculation of preparation, net weight of test item, preparations, addition of treatment medium, addition of S9-Mix, treatment, incubation, addition of chromosome culture medium, addition of cell cycle inhibitor, KCl treatment, fixation, spotting, documentation	31 Jan 2020
03 Apr 2020	Draft Report	03 Apr 2020
27 May 2020	Final Report	27 May 2020

In addition to the detailed study-based inspections series of routine facility inspections were also conducted and reported to the Management.

Approved and submitted by:

[redacted]

Dr. med. vet. habil. K. R. Sultan
Director of Quality
Assurance Unit (QAU)

27. May 2020
Date

1. SUMMARY

Test samples of pCol Protease were assayed in an *in vitro* micronucleus test using human peripheral lymphocytes both in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix) from Aroclor 1254 induced animals.

The test was carried out employing 2 exposure times without S9 mix: 4 and 24 hours, and 1 exposure time with S9 mix: 4 hours. The harvesting time was 20 hours after the end of exposure. The cytokinesis-block technique was applied.

pCol Protease was completely suspended in highly purified water. The vehicle highly purified water employed as the negative control.

The concentrations employed were chosen based on the results of a cytotoxicity study. In this preliminary experiment without and with metabolic activation concentrations of 125, 250, 500, 1000 and 2000 µg pCol Protease/mL medium were employed. No signs of cytotoxicity were noted. Test item precipitation was noted at concentrations of 1000 and 2000 µg pCol Protease/mL medium in the absence and presence of metabolic activation (24-hour or 4-hour exposure). No relevant changes in pH or osmolality of the test item formulations compared to the negative control were noted up to the top concentration of 2000 µg/mL medium.

Hence, 2000 µg pCol Protease/mL medium were employed as the top concentration for the genotoxicity tests without and with metabolic activation with a 4-hour exposure and for the 24-hour exposure experiment without S9 mix.

In the main study no signs of cytotoxicity were noted in the 4-hour exposure experiments without and with metabolic activation and in the 24-exposure experiment without S9-mix. Test item precipitation was noted at concentrations of 1000 and 2000 µg pCol Protease/mL medium in the absence and presence of metabolic activation (24-hour or 4-hour exposure).

Mitomycin C (at 0.2 µg/mL) and colchicine (at 0.02 µg/mL) were employed as positive controls in the absence and cyclophosphamide (at 20 µg/mL) in the presence of metabolic activation.

Tests without metabolic activation (4- and 24-hour exposure)
The mean micronucleus frequencies of cultures treated with the concentrations of 250, 500, 1000 and 2000 µg pCol Protease/mL medium in the absence of metabolic activation (4- and 24-hour exposure) ranged from 2.5 to 7.5 micronucleated cells per 1000 binucleate cells. There was no dose-related increase in micronuclei up to the top concentration of 2000 µg/mL medium (4- and 24-hour exposure). The frequency of micronucleated cells was within or slightly below the historical control range of the untreated and vehicle controls.

Vehicle controls should give reproducibly low and consistent micronucleus frequencies. In this test mean frequencies of 4.0 or 8.0 micronucleated cells per 1000 binucleate cells for the 4-hour and 24-hour exposure, respectively, were observed. The vehicle result was within the historical control ranges.

In the positive control cultures the mean micronucleus frequencies were increased to 19.0 or 25.0 micronucleated cells per 1000 binucleate cells for the 4-hour and 24-hour exposure, respectively. This demonstrated that Mitomycin C induced significant chromosomal damage and colchicine induced significant damage to the cell division apparatus.

Test with metabolic activation (4-hour exposure)

The mean micronucleus frequencies of cultures treated with the concentrations of 250, 500, 1000 and 2000 µg pCol Protease/mL medium (4-h exposure) in the presence of metabolic activation ranged from 5.0 to 8.5 micronucleated cells per 1000 binucleate cells. There was no dose-related increase in micronuclei up to the top concentration of 2000 µg/mL medium. The frequency of micronucleated cells was within or slightly above the historical control range of the untreated and vehicle controls.

Vehicle controls should give reproducibly low and consistent micronucleus frequencies. In this test a mean frequency of 8.0 micronucleated cells per 1000 binucleate cells was observed. The vehicle result was within the historical control ranges.

In the positive control culture, the mean micronucleus frequency was increased to 19.0 micronucleated cells per 1000 binucleate cells for the 4-hour exposure with metabolic activation. This demonstrated that cyclophosphamide induced significant chromosomal damage.

Conclusion

Under the present test conditions, pCol Protease tested up to the top concentration of 2000 µg pCol Protease/mL medium in the absence and in the presence of metabolic activation employing two exposure times without S9 mix and one exposure time with S9 mix revealed no indications of chromosomal damage in the *in vitro* micronucleus test.

The results for the vehicle controls were within the historical control range.

In the same test, Mitomycin C and cyclophosphamide induced significant chromosomal damage and colchicine induced significant damage to the cell division apparatus, respectively. Therefore, the test is considered valid.

Dr. rer. nat. B. Spruth
Study Director

27 May 2020
Date

2. GENERAL INFORMATION

2.1 Aim of experiment

The *in vitro* micronucleus assay is a genotoxic test system for the detection of chemicals which induce the formation of small membrane bound DNA fragments i.e. micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes, which are unable to migrate with the rest of the chromosomes during the anaphase of cell division.

The purpose of the micronucleus assay is to detect those agents, which modify chromosome structure and segregation in such a way as to lead to induction of micronuclei in interphase cells.

2.2 Sponsor / Test Facility / Responsible personnel

Sponsor	Nagase ChemteX Corporation 1-52 Osadano-cho Fukuchiyama Kyoto 620-0853 Japan
Contact Person and Monitor	Dr. Koichi Harazono Phone: +81 773 27 4371 Fax: +81 773 27 6450 E-mail: koichi.harazono@ncx.nagase.co.jp
Test Facility	**LPT** Laboratory of Pharmacology and Toxicology GmbH & Co. KG Redderweg 8 21147 Hamburg Germany Phone: +49 40 70 20 20 Fax: +49 40 70 20 22 60 E-mail: LPT@LPT-Hamburg.de
Study Director / Study conduct	Dr. rer. nat. Bastian Spruth **LPT**, Redderweg 8 21147 Hamburg Germany
Deputy Study Director	Dr. phil. Jost Leuschner
Test Facility Management	Dr. rer. nat. Andreas Winkler
Statistics	Dipl. Biol. Jens Köpcke
Quality Assurance Unit (QAU)	Dr. med. vet. habil. Karim R. Sultan
Code number of the study in the raw data	38051

2.3 Rules and regulations

The study was carried out according to:

- OECD Guideline for Testing of Chemicals No. 487: In Vitro Mammalian Cell Micronucleus Test (MNvit), adopted 29 July 2016;
- EC method B.49: In vitro Mammalian Cell Micronucleus Test, Commission Regulation (EU) No 2017/735 adopted 14 February 2017, published in the Official Journal of the European Union L 112/1, dated 28 April 2017.

In addition, the 'Good Laboratory Practice' regulations were considered (see the Statement of Compliance and the enclosed GLP Certificate of the Test Facility **LPT**).

2.4 Standard Operating Procedures / Staff safety

Standard Operating Procedures	All work was carried out according to Standard Operating Procedures, which were followed for all stages of the study; they may be inspected in those divisions, which were engaged in the study and in the Quality Assurance Unit (QAU).
Staff safety	The standard safety precautions operating within the department were applied to this study.

2.5 Archive

Archives of data and specimens	**During the study:** In the depot **LPT,** Redderweg 8 21147 Hamburg Germany **After reporting:** The final report will be archived by the Sponsor. A copy of the final report as well as all specimens, written raw data, and other study-related documents generated at **LPT** during the course of this study are stored in the **LPT** archives as required by the German 'Chemikaliengesetz' [Chemicals Act].
Duration of storage	According to the periods laid down in the German 'Chemikaliengesetz' [Chemicals Act] for at least 15 years. All archived study materials will be destroyed after the 15-year GLP storage period unless the Sponsor requests otherwise.

2.6 Study dates

Start of study

Date of the Study Plan	03 December 2019
Start of the experimental phase	04 December 2019
Period of treatment	January – March 2020

Study termination

Termination of the experimental phase	23 March 2020
Date of the final report	27 May 2020

2.7 Study Plan deviations

The study was conducted in accordance with the Study Plan agreed upon. There were no deviations from the Study Plan.

3. TEST ITEM

3.1 Identification of the test item

After receipt at **LPT**, the test item was inspected; batch number, amount, and characteristics (colour, consistency and form) were determined and compared with information given by the Sponsor. An identification sheet was then filed with the raw data.

Text table 3-1 Identification of the test item

Test item	Parameter	LPT Identification	Sponsor Identification#
pCol Protease	colour consistency form	beige solid powder	none none powder

According to the Study Plan.

No further identification was performed by **LPT**.

3.2 Description

Designation	pCol Protease
Product name	DENAZYME CO-BOC (Concentration)
IUBMB nomenclature	Microbial collagenase
IUBMB no.	EC 3.4.24.3
CAS no.	9001-12-1
EINECS no.	232-582-9
Batch no.	3T-1
Receipt no.	69238
Date of receipt	26 November 2019
Characteristics	Powder
Storage conditions	At -20°C, ±10% in a tightly closed container, stored in a cool, dry and ventilated place. Protected from light.
Stability/ Expiry date	At +4°C to +25°C for 1 month At -20°C for 48 months (28 October 2021)[1]
Activity	10,287 U/g For further information, see Appendix 1 ‘Certificate of Quality’.
Retention sample of the test item	No retention sample was taken due to the small amount of test item available.

[1] According to the ‘Certificate of Quality’

3.3 Preparation of the test item solution

pCol Protease was suspended in highly purified water[2] to the top concentration of 2000 µg pCol Protease/mL medium. The vehicle highly purified water was employed as the negative control. Fresh preparations of the test item were made on the day of the experiment and used for the treatment in all experimental parts.

[2] Lot no. 190528151; B. Braun Melsungen AG, 34212 Melsungen, Germany

4. METHODS

4.1 Initial considerations

The test requires the use of an exogenous source of metabolic activation unless the cells are metabolically competent with respect to the substances being tested. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions. Care is also taken to avoid conditions that would lead to artifactual positive results, which do not reflect intrinsic genotoxicity, and may arise from such factors as marked changes in pH or osmolality, or by high levels of cytotoxicity. If the test chemical would have cause a change in the pH of the medium at the time of addition, the pH would have been adjusted, preferably by buffering the stock solution so that all the volumes at all test concentrations, and for all controls, remain the same.

To analyse the induction of micronuclei, it is essential that mitosis has occurred in both treated and untreated cultures. The most informative stage for scoring micronuclei is in cells that have completed one mitosis during or after treatment with the test item.

4.2 Principle of the test method

Cell cultures (human peripheral lymphocytes) were exposed to the test item both with and without an exogenous source of metabolic activation. Concurrently the vehicle highly purified water and positive controls were included in all tests. During or after exposure to the test item, the cells were grown for a period sufficient to allow chromosome or spindle damage to lead to the formation of micronuclei in interphase cells. Harvested and stained interphase cells were analysed for the presence of micronuclei. Ideally, micronuclei are only scored in those cells that have completed mitosis during exposure to the test item. This is achieved by scoring only binucleate cells. It is important to demonstrate that cell proliferation has occurred in both the control and treated cultures, and the extent of test item-induced cytotoxicity or cytostasis is assessed in the cultures (or in parallel cultures) that are scored for micronuclei.

4.3 Culture establishment

Human peripheral blood was obtained by venipuncture from young, healthy, non-smoking individuals (18-35 years old) with no known recent exposures to genotoxic chemicals or radiation, and collected in heparinised vessels. Small innocula of whole blood (0.5 mL) were added to tubes containing 5 mL of Chromosome complete culture medium with Phytohemagglutinin[3] and 1% Penicillin/Streptomycin[4]. The tubes were sealed and incubated at 37°C, and shaken occasionally to prevent clumping.

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[3] Lot no. 2121979; GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany

[4] Lot no. 2076675; GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany

4.4 Cytokinesis blocker CytoB (Cytochalasin B)

One of the most important considerations in the performance of the assay is ensuring that the cells being scored have completed mitosis during the treatment incubation period. CytoB is the agent that has been most widely used to block cytokinesis because it inhibits actin assembly, and thus prevents separation of daughter cells after mitosis, leading to the formation of binucleated cells. Micronucleus scoring, therefore, can be limited to cells that have gone through mitosis during or after treatment. The effect of the test item on cell proliferation kinetics can be measured simultaneously. The appropriate concentration of CytoB was determined for human lymphocytes to achieve the optimal frequency of binucleated cells in the vehicle control cultures. The appropriate concentration of CytoB is usually between 3 and 6 µg/mL. The concentration used for this assay was 5 µg/mL.

Treatment of cultures with CytoB, and measurement of the relative frequencies of mononucleate, binucleate, and multi-nucleate cells in the culture, provides an accurate method of quantifying the effect on cell proliferation and the cytotoxic or cytostatic activity of a treatment and ensures that only cells that divided during or after treatment are scored.

4.5 Exposure concentrations

If no cytotoxicity or precipitate is observed, the highest test concentration corresponds to 0.01 M, 2 mg/mL or 2 µL/mL, whichever is the lowest. The concentrations selected for analysis will, in general, be separated by a spacing of no more than $\sqrt{10}$. If the test item exhibits a steep concentration-response curve, a closer spacing of test item concentration might be necessary for evaluation of concentrations of low and moderate cytotoxicity.

When solubility is a limiting factor, the maximum concentration, if not limited by cytotoxicity, would be the lowest concentration at which minimal precipitate is visible in cultures, providing that there is no interference with scoring. Evaluation of precipitation is evaluated by methods such as light microscopy, noting precipitate that persists, or appears during culture (by the end of treatment).

The highest concentration should aim to produce 55 ± 5% cytotoxicity using the cytotoxicity parameters (i.e. reduction in CBPI or RI to 45± 5% of the concurrent negative control). Higher levels may induce chromosome damage as a secondary effect of cytotoxicity. Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing 55 ± 5% cytotoxicity, to little or no cytotoxicity.

At least three analysable test concentrations were evaluated. In order to achieve this, a preliminary cytotoxicity test was performed to narrow the range of concentrations used for the definitive test. In this preliminary experiment without and with metabolic activation concentrations of 125, 250, 500, 1000 and 2000 µg pCol Protease/mL medium were employed. No signs of cytotoxicity were noted. Test item precipitation was noted at

concentrations of 1000 and 2000 µg pCol Protease/mL medium in the absence and presence of metabolic activation (24-hour or 4-hour exposure). No relevant changes in pH or osmolality of the test item formulations compared to the negative control were noted up to the top concentration of 2000 µg/mL medium.

Hence, 2000 µg pCol Protease/mL medium were employed as the top concentration for the genotoxicity tests without and with metabolic activation with a 4-hour exposure and for the 24-hour exposure experiment without S9 mix.

The following concentration levels (concentrations in the medium) were established:

Text table 4-1 Concentrations in the medium

Culture number	Compound	Concentration (µg/mL medium)[#1]	S9 mix
4-h exposure			
	conduct: 27 January 2020 to 31 January 2020		
1, 9	highly purified water	0	-
6, 14[#2]	pCol Protease/mL	125	-
5, 13	pCol Protease/mL	250	-
4, 12	pCol Protease/mL	500	-
3, 11	pCol Protease/mL	1000	-
2, 10	pCol Protease/mL	2000	-
8, 16[#3]	Mitomycin C	0.1	-
7, 15	Mitomycin C	0.2	-
	conduct: 27 January 2020 to 31 January 2020		
1, 9	highly purified water	0	+
6, 14[#2]	pCol Protease/mL	125	+
5, 13	pCol Protease/mL	250	+
4, 12	pCol Protease/mL	500	+
3, 11	pCol Protease/mL	1000	+
2, 10	pCol Protease/mL	2000	+
8, 16[#3]	Cyclophosphamide	10	+
7, 15	Cyclophosphamide	20	+
24-h exposure			
	conduct: 27 January 2020 to 31 January 2020		
1, 9	highly purified water	0	-
6, 14[#2]	pCol Protease/mL	125	-
5, 13	pCol Protease/mL	250	-
4, 12	pCol Protease/mL	500	-
3, 11	pCol Protease/mL	1000	-
2, 10	pCol Protease/mL	2000	-
8, 16[#3]	Colchicine	0.01	-
7, 15	Colchicine	0.02	-

S9 mix: + with metabolic activation
- without metabolic activation

[#1] 5 mL treatment medium containing 50 µL of test item or control solution

[#2] not evaluated, as it was thought that this concentration would provide no further information

[#3] scoring of these slides was not necessary as the higher concentration revealed significant results

4.6 Preparation of S9 mix

Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254, prepared according to MARON and AMES (1983) was purchased from Trinova Biochem[5]. S9 was collected from male rats.

The protein content of the S9 fraction and the cytochrome activity P-450 is presented in Appendix 2.

The S9 fraction was stored at -80 °C. The S9 mix was freshly prepared on the day of the test according to MARON and AMES (1983):

Text table 4-2 Composition of S9 mix

Amount	Component
1.0 mL	Rat liver S9 (Aroclor 1254-induced)
0.2 mL	0.4 M $MgCl_2$ + 1.65 M KCl salt solution (sterile stock solution)
14 mg	Glucose-6-phosphate[6]
30.6 mg	NADP[7]
3.8 mL	20 mM Hepes buffer, pH 7.4 (sterile stock solution)
5 mL	Phosphate buffer[8]

Afterwards the S9 mix was filter-sterilised by using a 0.45 µm filter and kept on ice.

4.7 Vehicle and positive controls

Concurrent positive and vehicle controls both with and without metabolic activation were included in each experiment. Positive controls were needed to demonstrate the ability to identify clastogens and aneugens for the used human peripheral lymphocytes and to affirm the metabolic capability of the S9 preparation.

The positive controls should employ known inducers of micronucleus formation at concentrations expected to give small, but reproducible increases over background, and demonstrate the sensitivity of the test system.

Positive control concentrations were chosen so that the effects were clear but did not immediately reveal the identity of the coded slides to the reader. A clastogen that requires metabolic activation (e.g. cyclophosphamide) was used to demonstrate both the metabolic competence and the ability of the test system to detect clastogens.

At the present time, no aneugens are known that require metabolic activation for their genotoxic activity. A currently accepted positive control for aneugenic activity is colchicine.

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5 Lot no. 4120; tested by Moltox: Molecular Toxicology, Inc., Boone, NC 28607, USA and distributed by Trinova Biochem GmbH, 35394 Gießen, Germany

6 Lot no. SLBZ7814; SIGMA-ALDRICH Chemie GmbH, 82024 Taufkirchen, Germany

7 Lot no. 190284; Serva, 82152 Martinsried, Germany

8 Lot no. 2124939; GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany

Vehicle controls were included for every harvest time.

Text table 4-3 Positive control items

positive controls	without metabolic activation	with metabolic acitivation
clastogen	Mitomycin C[9] in highly purified water[10] (c = 0.1 µg/mL and c = 0.2 µg/mL)	cyclophosphamide[11] in highly purified water[10] (c = 10 µg/mL and c = 20 µg/mL)
aneugen	colchicine[12] in highly purified water[10] (c = 0.01 µg/mL and c = 0.02 µg/mL)	

The following concentrations were not evaluated, as it was thought that they would provide no further information: 0.1 µg Mitomycin C/mL and 0.01 µg colchicine/mL (without S9 mix) and 10 µg cyclophosphamide/mL in the experiments (with S9 mix).
The vehicle highly purified water was employed as the negative control.

4.8 Procedure / Treatment schedule

In order to maximise the probability of detecting an aneugen or clastogen acting at a specific stage in the cell cycle, it is important that sufficient numbers of cells are treated with the test item during all stages of their cell cycles. Hence, 0.5 mL of freshly prepared blood lymphocytes were seeded with 5 mL of Chromosome complete culture medium with Phytohemagglutinin[13] and 1% Penicillin/Streptomycin[14]. After initiation appropriate concentrations of the test item in the vehicle were added to the cell cultures for each target concentration of the test item in the test medium and each experiment. Precipitation of the test item was checked before and after each experiment. Evaluation of precipitation was done by light microscopy at the beginning and end of treatment. Theoretical considerations, together with published data, indicate that most aneugens and clastogens are detected by a short-term treatment period of 4 hours in the presence and absence of S9, followed by removal of the test item and a growth period of 1.5 cell cycles. Cells were sampled at a time equivalent to about 1.5 times the normal (i.e. untreated) cell cycle length either after the beginning or at the end of treatment. Sampling or recovery times would have been extended if it is known or suspected that the test item affects the cell cycling time (e.g. when testing nucleoside analogues). Because of the potential cytotoxicity of S9 preparations for cultured mammalian cells, an extended exposure treatment was used only in the absence of S9.

9 Lot no. SLBX1864; SIGMA-ALDRICH Chemie GmbH, 82024 Taufkirchen, Germany
10 Lot no. 190528151; B. Braun Melsungen AG, 34212 Melsungen, Germany
11 Lot no. MKC65464; SIGMA-A0LDRICH Chemie GmbH, 82024 Taufkirchen, Germany
12 Lot no. SLBX1712; SIGMA-ALDRICH Chemie GmbH, 82024 Taufkirchen, Germany
13 Lot no. 2121979; GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany
14 Lot no. 2076675; GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany

All treatments were conducted while the cells were growing exponentially.

Cell treatment and harvest times for the used human lymphocytes line:

Without S9 mix:

4-hour exposure:

Cultures were initiated and maintained as described in Section 4.3. After 48 hours the cultures were centrifuged (10 minutes at 800 – 900 rpm) and the medium was replaced by 4.95 mL of fresh Ham's F10 medium [15] with fetal calf serum (FCS) [16]. Five concentrations of 125, 250, 500, 1000 and 2000 µg pCol Protease/mL were employed. The test item treatments, the vehicle control and the positive control were added at a volume of 50 µL to obtain the corresponding target concentrations. The cultures were then incubated for 4 hours at +37°C. Afterwards the medium was removed and the cultures were washed twice with Ham's F10 medium with FCS. After addition of 5 mL Chromosome complete medium containing 5 µg/mL Cytochalasin B[17] the cultures were incubated for further 20 hours at 37°C.

24-hour exposure:

Cultures were initiated and maintained as described in Section 4.3. After 48 hours the cultures were centrifuged (10 minutes at 800 – 900 rpm) and the medium was replaced by 4.95 mL of fresh Ham's F10 medium with FCS. Five concentrations of 125, 250, 500, 1000 and 2000 µg pCol Protease/mL were employed. The test item treatments, the vehicle control and the positive control were added at a volume of 50 µL to obtain the corresponding target concentrations. The cultures were then incubated for 24 hours at +37°C. Afterwards the medium was removed and the cultures were washed twice with Ham's F10 medium with FCS. After addition of 5 mL Chromosome complete medium containing 5 µg/mL Cytochalasin B the cultures were incubated for 20 hours at 37°C.

With S9 mix:

4-hour exposure:

Cultures were initiated and maintained as described in Section 4.3. After 48 hours the cultures were centrifuged (10 minutes at 800 – 900 rpm) and the medium was carefully removed and replaced by 4.45 mL Ham's F10 medium with FCS and 0.5 mL S9 Mix. Five concentrations of 125, 250, 500, 1000 and 2000 µg pCol Protease/mL were employed. The test item treatments, the vehicle control and the positive control were added at a volume of 50 µL to obtain the corresponding target concentrations. The cultures were then incubated for 4 hours at +37°C. Afterwards the medium was removed and the cultures were washed twice with Ham's F10 medium with FCS. After addition of 5 mL Chromosome complete medium containing 5 µg/mL Cytochalasin B the cultures were

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15 Lot no. 2091540; GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany

16 Lot no. 08F1394K; GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany

17 Lot no. 8B011910; AppliChem GmbH 64291 Darmstadt, Germany

incubated for further 20 hours at 37°C.

Experiment 1:

Text table 4-4 Treatments without and with S9 mix

Hours	**Absence of S9** **4-h exposure / 24-h sampling**	**Presence of S9** **4-h exposure / 24-h sampling**
0	Commence treatment	Commence treatment
4	Remove treatment medium, wash and add fresh medium, add CytoB	Remove treatment medium, wash and add fresh medium, add CytoB
24	Harvest, prepare slides	Harvest, prepare slides

In experiment 2: A 24-h continuous treatment without metabolic activation was conducted.

Experiment 2:

Text table 4-5: Treatment without S9 mix

Hours	**Absence of S9** **24-h exposure / 44-h sampling**
0	Commence treatment
24	Remove treatment medium, wash and add fresh medium, add CytoB
44	Harvest, prepare slides

Two replicate cultures were used for each test item concentration and for the vehicle and positive control cultures.

4.9 Culture harvesting and slide preparation

Each culture was harvested and processed separately. After the test item incubation, mitotic activity was arrested by the addition of CytoB to each culture at a final concentration of 5 µg/mL. After an additional incubation of 20 hours the cultures were centrifuged for 10 minutes at 800 rpm, the supernatant was discarded and the cells resuspended in KCl (0.56%). After incubation for 17 minutes at 37°C, the cell suspensions were centrifuged for 10 minutes at 800 rpm. The supernatant was discarded and 5 mL of freshly prepared fixative (3 parts methanol[18] : 1 part glacial acetic acid[19] v/v) was added. The cells were left in fixative for 30 minutes followed by centrifugation at 800 rpm. The supernatant was carefully removed and discarded, and the cell pellet was resuspended in about 0.5 mL of fresh fixative and 30% glacial acetic acid by repeated aspiration through a Pasteur pipette. Two drops of this cell suspension were dropped onto a prewarmed, pre-cleaned microscope slide. The slides were then stained using 10% Giemsa[20] and left to air-dry at room temperature.

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18 Lot no. I186BS; Honeywell Specialty Chemicals Seelze GmbH, 30926 Seelze Germany
19 Lot no. STBH9751; SIGMA-ALDRICH Chemie GmbH, 82024 Taufkirchen, Germany
20 Lot no. HX72892604; Merck KGaA, 64293 Darmstadt, Germany

4.10 pH values and osmolality measurements

The pH and osmolality of the negative control and all test item formulations in the medium of the preliminary experiment were determined employing the methods given below:

Text table 4-6: pH values and osmolality measurement

Parameter	Units	Method
- pH	non-dimensional	Digital pH meter type SevenCompact S210 Mettler-Toledo GmbH, 35396 Gießen, Germany
- Osmolality	mOsmol/kg	Semi-micro osmometer Typ ML A0299 Knauer GmbH, 14163 Berlin, Germany

5. ANALYSIS

At least 500 cells per replicate cell culture (two cultures per concentration in the main study, one culture per concentration in the preliminary test) were scored and classified as mononucleates, binucleates or multinucleates to estimate the proliferation index as a measure of toxicity. The evaluation of cytotoxicity was based on the Cytokinesis-Block Proliferation Index (CBPI) or the Replicative Index (RI).

The CBPI indicates the average number of cell cycles per cell during the period of exposure to CytoB, and is used to calculate cell proliferation.

The RI indicates the relative number of nuclei in treated cultures compared to control cultures and can be used to calculate the % cytostasis:

$$CBPI = \frac{((\text{No. mononucleate cells})+(2\times\text{No. binucleate cells})+(3\times\text{No. multinucleate cells}))}{(\text{Total number of cells})}$$

Thus, a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

Cytostasis = 100 - RI

$$RI = \frac{((\text{No. binucleated cells})+(2\times\text{No. multinucleate cells}))\div(\text{Total number of cells})_T}{((\text{No. binucleated cells})+(2\times\text{No. multinucleate cells}))\div(\text{Total number of cells})_C} \times 100$$

T= treated cultures
C= control cultures

Thus, an RI of 53% means that, compared to the numbers of cells that have divided to binucleate and multinucleate cells in the control culture, only 53% of this number divided in the treated culture, i.e. 47% cytostasis.

All slides, including those of the solvent controls, were independently coded before the microscopic analysis.

The micronucleus frequencies were analysed in at least 2000 binucleated cells per concentration (at least 1000 binucleated cells per culture; two cultures per concentration). If substantially fewer than 1000 binucleate cells per culture are available for scoring at each concentration, and if a significant increase in micronuclei is not detected, the test would be repeated using more cells, or at less toxic concentrations, whichever is appropriate. Care was taken not to score binucleate cells with irregular shapes or where the two nuclei differ greatly in size; neither would binucleate cells be confused with poorly spread multi-nucleate cells. Cells containing more than two main nuclei were not analysed for micronuclei, as the baseline micronucleus frequency might be higher in these cells. Scoring of mononucleate cells is acceptable if the test item is shown to interfere with CytoB activity.

6. ACCEPTABILITY CRITERIA

The assay demonstrates its ability to reliably and accurately detect substances of known aneugenic and clastogenic activity, with and without metabolic activation, as well as known negative substances.

Acceptance of a test is based on the following criteria:

- The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database (Poisson-based 95% control limits). Where concurrent negative control data fall outside the 95% control limits, they may be acceptable for inclusion in the historical control data as long these data are not extreme outliers.
- Concurrent positive controls induce responses that are compatible with those generated in the laboratory's historical positive control data base and produce a statistically significant increase compared with the concurrent negative control.
- Adequate number of cells, cell proliferation criteria and concentrations are analysable and are consistent with those described in paragraph 4.5.

Vehicle control and untreated cultures give reproducibly low and consistent micronucleus frequencies. Data from vehicle and positive controls are used to establish historical control ranges (see Appendix 3). These values are used in deciding the adequacy of the concurrent vehicle controls or positive controls for an experiment.

7. STATISTICAL EVALUATION AND INTERPRETATION OF RESULTS

Only the frequencies of binucleate cells with micronuclei (independent of the number of micronuclei per cell) were used in the evaluation of micronucleus induction. Concurrent measures of cytotoxicity and/or cytostasis for all treated and vehicle control cultures were determined. Individual culture data were provided.

Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined:

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control
- the increase is dose-related in at least one experimental condition when evaluated with an appropriate trend test
- any of the results are outside the distribution of the historical negative control data (Poisson-based 95% control limits)

When all of these criteria are met, the test chemical is then considered able to induce chromosome breaks and/or gain or loss in this test system.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

- none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- there is no concentration-related increase when evaluated with an appropriate trend test,
- all results are inside the distribution of the historical negative control data (Poisson-based 95% control limits).

The test chemical is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification by additional testing of a clear positive or negative response.

Equivocal results may be clarified by analysis of another 1000 cells from all the cultures to avoid loss of blinding. If this approach does not resolve the result, further testing would be necessary. Modification of study parameters over an extended or narrowed range of conditions, as appropriate, would be considered in follow-up experiments. Study parameters that might be modified include the test concentration spacing, the timing of treatment and cell harvest, and/or the metabolic activation conditions.

Although most experiments give clearly positive or negative results, in some cases the data set would preclude making a definite judgement about the activity of the test item. These equivocal or questionable responses may occur regardless of the number of times the experiment is repeated.

8. REFERENCES

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3. MARON, D. M. and B. N. AMES: Revised methods for the Salmonella mutagenicity test. *Mutation Research* 113, 173-215 (1983).

4. OECD (2016), In Vitro Mammalian Cell Micronucleus Test, Test Guideline No. 487, OECD Guidelines for Testing of Chemicals, OECD, Paris.

5. WAKATA, A., MATSUOKA, A., YAMAKAGE, K., YOSHIDA, J., KUBO, K., KOBAYASHI, K., SENJYU, N., ITOH, S., MIYAJIMA, H., HAMADA, S., NISHIDA, S., ARAKI, H., YAMAMURA, E., MATSUI, A., THYBAUD, V., LORENZON, G., MARZIN, D. and LORGE, E.: SFTG International collaborative study on the in vitro micronucleus test, IV. Using CHO/IU cells, Mutation Res., 607, 88-124 (2006).

9. RESULTS AND DISCUSSION

The concentrations employed were chosen based on the results of a cytotoxicity study. In this preliminary experiment without and with metabolic activation concentrations of 125, 250, 500, 1000 and 2000 µg pCol Protease/mL medium were employed. No signs of cytotoxicity were noted. Test item precipitation was noted at concentrations of 1000 and 2000 µg pCol Protease/mL medium in the absence and presence of metabolic activation (24-hour or 4-hour exposure). For details see Table 1. No relevant changes in pH or osmolality of the test item formulations compared to the negative control were noted up to the top concentration of 2000 µg pCol Protease/mL medium (see Text table 9-1).

Hence, 2000 µg pCol Protease/mL medium were employed as the top concentration for the genotoxicity tests without and with metabolic activation with a 4-hour exposure and for the 24-hour exposure experiment without S9 mix.

In the main study no signs of cytotoxicity were noted in the 4-hour exposure experiments without and with metabolic activation and in the 24-exposure experiment without S9-mix. Test item precipitation was noted at concentrations of 1000 and 2000 µg pCol Protease/mL medium in the absence and presence of metabolic activation (24-hour or 4-hour exposure).

Mitomycin C (at 0.2 µg/mL) and colchicine (at 0.02 µg/mL) were employed as positive controls in the absence and cyclophosphamide (at 20 µg/mL) in the presence of metabolic activation.

Tests without metabolic activation (4- and 24-hour exposure)

The mean micronucleus frequencies of cultures treated with the concentrations of 250, 500, 1000 and 2000 µg pCol Protease/mL medium in the absence of metabolic activation (4- and 24-hour exposure) ranged from 2.5 to 7.5 micronucleated cells per 1000 binucleate cells. There was no dose-related increase in micronuclei up to the top concentration of 2000 µg/mL medium (4- and 24-hour exposure). The frequency of micronucleated cells was within or slightly below the historical control range of the untreated and vehicle controls (see Appendix 3).

Vehicle controls should give reproducibly low and consistent micronucleus frequencies. In this test mean frequencies of 4.0 or 8.0 micronucleated cells per 1000 binucleate cells for the 4-hour and 24-hour exposure, respectively, were observed. The vehicle result was within the historical control ranges (see Appendix 3).

In the positive control cultures the mean micronucleus frequencies were increased to 19.0 or 25.0 micronucleated cells per 1000 binucleate cells for the 4-hour and 24-hour exposure, respectively. This demonstrated that Mitomycin C induced significant chromosomal damage and colchicine induced significant damage to the cell division apparatus

Test with metabolic activation (4-hour exposure)

The mean micronucleus frequencies of cultures treated with the concentrations of 250, 500, 1000 and 2000 µg pCol Protease/mL medium (4-h exposure) in the presence of metabolic activation ranged from 5.0 to 8.5 micronucleated cells per 1000 binucleate cells. There was no dose-related increase in micronuclei up to the top concentration of 2000 µg/mL medium. The frequency of micronucleated cells was within or slightly above the historical control range of the untreated and vehicle controls (see Appendix 3).

Vehicle controls should give reproducibly low and consistent micronucleus frequencies. In this test a mean frequency of 8.0 micronucleated cells per 1000 binucleate cells was observed. The vehicle result was within the historical control ranges.

In the positive control culture, the mean micronucleus frequency was increased to 19.0 micronucleated cells per 1000 binucleate cells for the 4-hour exposure with metabolic activation. This demonstrated that cyclophosphamide induced significant chromosomal damage.

A summary of the results (means of the two replicate cultures) of this study is listed in Tables 2 and 3. Individual values of the experiment without metabolic activation (4- and 24-hour exposure) can be taken from Tables 4 (4-hour) and 5 (24-hour exposure). Individual values of the experiment with metabolic activation (4-hour exposure) can be taken from Table 6.

The following pH and osmolality data of the vehicle control and of all test item formulations in the medium were determined in a preliminary test:

Text table 9-1: pH values and osmolality

Concentration of pCol Protease [µg/mL medium]	**pH value**	**osmolality [mOsmol/kg]**
Medium	7.51	295
0, vehicle control	7.59	300
125	7.62	290
250	7.60	295
500	7.60	290
1000	7.57	295
2000	7.62	295

Conclusion

Under the present test conditions, pCol Protease tested up to the top concentration of 2000 µg pCol Protease/mL medium in the absence and in the presence of metabolic activation employing two exposure times without S9 mix and one exposure time with S9 mix revealed no indications of chromosomal damage in the in vitro micronucleus test.
The results for the vehicle controls were within the historical control range.
In the same test, Mitomycin C and cyclophosphamide induced significant chromosomal damage and colchicine induced significant damage to the cell division apparatus, respectively. Therefore, the test is considered valid.

Preliminary test

TABLE 1 Estimation of the cytotoxicity

Concentration of test item [µg/mL medium]	S9 mix	Number of mononucleate	binucleate cells#1	multinucleate	CBPI	RI [%]
pCol Protease						
24-hour exposure						
0 (vehicle control)	-	362	129	9	1.29	71
125	-	395	102	3	1.22	54
250	-	364	128	8	1.29	71
500	-	347	142	11	1.33	80
1000#2	-	348	147	5	1.31	76
2000#2	-	371	124	5	1.27	66
4-hour exposure						
0 (vehicle control)	+	313	169	18	1.41	100
125	+	331	157	12	1.36	88
250	+	315	168	17	1.40	98
500	+	309	176	15	1.41	100
1000#2	+	329	159	12	1.37	90
2000#2	+	336	155	9	1.35	85

CBPI	Cytokinesis block proliferation index
RI	Replicative Index
+	with metabolic activation
-	without metabolic activation
#1	at least a total of 500 cells have to be scored
#2	test item precipitation

Summary tables

Table 2 Experiments without metabolic activation (-S9 mix)

4-h exposure					24-h exposure				
Concentration of test item [µg/mL medium]	CBPI	RI [%]	Number of binucleate cells scored	Number of micronucleated cells per 1000 binucleate cells	Concentration of test item [µg/mL medium]	CBPI	RI [%]	Number of binucleate cells scored	Number of micronucleated cells per 1000 binucleate cells
Highly purified water					Highly purified water				
0	1.32	100	2000	4.0	0	1.42	100	2000	8.0
				pCol Protease					
250	1.34	105	2000	7.5	250	1.39	96	2000	6.0
500	1.37	114	2000	6.5	500	1.42	102	2000	6.5
1000#	1.38	119	2000	6.5	1000#	1.34	82	2000	5.5
2000#	1.34	107	2000	6.5	2000#	1.35	82	2000	2.5
Mitomycin C					Colchicine				
0.2	1.29	89	2000	19.0 s.	0.02	1.25	60	2000	25.0 s.

CBPI = Cytokinesis block proliferation index
RI = Replicative Index
s. = significantly different from negative control ($p \leq 0.05$)
\# = test item precipitation

Summary tables

Table 3 Experiment with metabolic activation (+S9 mix)

Concentration of test item [µg/mL medium]	CBPI	RI [%]	Number of binucleate cells scored	Number of micronucleated cells per 1000 binucleate cells
		4-h exposure		
Highly purified water				
0	1.27	100	2000	8.0
pCol Protease				
250	1.32	118	2000	8.5
500	1.37	135	2000	8.0
1000#	1.34	128	2000	5.0
2000#	1.34	125	2000	5.5
Cyclophosphamide				
20	1.24	90	2000	19.0 s.

CBPI = Cytokinesis block proliferation index
RI = Replicative Index
s. = significantly different from negative control ($p \leq 0.05$)
# = test item precipitation

Individual data

Table 4 Experiment without metabolic activation (-S9 mix) - 4-h exposure

Culture number	Concentration [µg/mL medium]	mononucleate	Number of binucleate cells#1	multinucleate	CBPI	RI [%]	Number of binucleate cells scored	Number of micronucleated cells per 1000 binucleate cells	Significance chi^2-test
Highly purified water									
1	0	340	154	6	1.33	100	1000	3	-
9	0	353	140	7	1.31	100	1000	5	-
pCol Protease									
5	250	361	130	9	1.30	91	1000	6	n.s.
13	250	330	156	14	1.37	119	1000	9	n.s.
4	500	320	161	19	1.40	121	1000	8	n.s.
12	500	342	150	8	1.33	106	1000	5	n.s.
3	1000#2	304	181	15	1.42	127	1000	5	n.s.
11	1000#2	346	140	14	1.34	110	1000	8	n.s.
2	2000#2	343	152	5	1.32	97	1000	5	n.s.
10	2000#2	333	152	15	1.36	116	1000	8	n.s.
Mitomycin C									
7	0.2	356	139	5	1.30	91	1000	17	s.
15	0.2	366	133	1	1.27	87	1000	21	s.

n.s. = not significantly different from negative control ($p \leq 0.05$)
s. = significantly different from negative control ($p \leq 0.05$)
#1 = at least a total of 500 cells have to be scored
#2 = test item precipitation

CBPI = Cytokinesis block proliferation index
RI = Replicative Index

Individual data

Table 5 Experiment without metabolic activation (-S9 mix) - 24-h exposure

Culture number	Concentration [µg/mL medium]	mononucleate	Number of binucleate cells#1	multinucleate	CBPI	RI [%]	Number of binucleate cells scored	Number of micronucleated cells per 1000 binucleate cells	Significance chi^2-test
Highly purified water									
1	0	331	156	13	1.36	100	1000	8	-
9	0	277	207	16	1.48	100	1000	8	-
pCol Protease									
5	250	299	186	15	1.43	119	1000	7	n.s.
13	250	335	154	11	1.35	73	1000	5	n.s.
4	500	295	189	16	1.44	122	1000	7	n.s.
12	500	323	158	19	1.39	81	1000	6	n.s.
3	1000#2	331	162	7	1.35	97	1000	4	n.s.
11	1000#2	349	144	7	1.32	67	1000	7	n.s.
2	2000#2	354	141	5	1.30	83	1000	3	n.s.
10	2000#2	311	182	7	1.39	81	1000	2	n.s.
Colchicine									
7	0.02	380	110	10	1.26	72	1000	24	s.
15	0.02	391	101	8	1.23	48	1000	26	s.

n.s. = not significantly different from negative control ($p \leq 0.05$)
s. = significantly different from negative control ($p \leq 0.05$)
#1 = at least a total of 500 cells have to be scored
#2 = test item precipitation

CBPI = Cytokinesis block proliferation index
RI = Replicative Index

Individual data

Table 6 Experiment with metabolic activation (+S9 mix) - 4-h exposure

Culture number	Concentration [µg/mL medium]	Number of mononucleate	binucleate cells#1	multinucleate	CBPI	RI [%]	Number of binucleate cells scored	Number of micronucleated cells per 1000 binucleate cells	Significance chi^2-test
Highly purified water									
1	0	369	119	12	1.29	100	1000	8	-
9	0	390	96	14	1.25	100	1000	8	-
pCol Protease									
5	250	365	126	9	1.29	100	1000	11	n.s.
13	250	343	145	12	1.34	136	1000	6	n.s.
4	500	306	181	13	1.41	141	1000	6	n.s.
12	500	357	128	15	1.32	128	1000	10	n.s.
3	1000#2	359	132	9	1.30	103	1000	6	n.s.
11	1000#2	332	147	21	1.38	152	1000	4	n.s.
2	2000#2	346	144	10	1.33	114	1000	5	n.s.
10	2000#2	346	139	15	1.34	136	1000	6	n.s.
Cyclophosphamide									
7	20	392	106	2	1.22	76	1000	19	s.
15	20	374	121	5	1.26	104	1000	19	s.

n.s. = not significantly different from negative control ($p \leq 0.05$)
s. = significantly different from negative control ($p \leq 0.05$)
#1 = at least a total of 500 cells have to be scored
#2 = test item precipitation

CBPI= Cytokinesis block proliferation index
RI = Replicative Index

A P P E N D I X 1

Certificate of Quality

and

Analytical Report

by Nagase ChemteX Corporation

Nagase ChemteX
NAGASE Group

October 30, 2019

To whom it may concern,

Certificate of Quality

The Quality of pCol protease (DENAZYME CO-BOC (Concentration)), which was used in the in vitro micronucleus test (OECD), was confirmed by the re-evaluation on October 28, 2019 in the following;

Product name	DENAZYME CO-BOC (Concentration)
Form	Powder
Batch No.	3T-1
Activity	10,287 U/g
TOS	93.96%
Expiry date	October 28, 2021

Naoki Shirasaka

Manager,
Development Division
Bio Chemicals Department

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1-52 Osadanocho Fukuchiyama Kyoto 620-0853 JAPAN
Tel: +81-773-27-4371 Fax: +81-773-27-6450
http://www.nagasechemtex.co.jp/en/

Nagase ChemteX

Analytical Report

Date: 01/20/15

Product name: DENAZYME CO-BOC (Concentration)

Lot # 3T-1

Item	Specification	Results	LD	Method	Organization
Appearance	Light Brown liquid	Light Brown liquid	-	By eye	In-house
Activity	-	10,287U/g	-	1	In-house
Lead	Less than 5mg/kg	0.48 mg/kg	0.1mg/kg	2	In-house
Arsenic(as AsSO3)	Less than 4mg/kg	< 1 mg/kg	1mg/kg	3	In-house
Cadmium	Less than 0.5mg/kg	0.09 mg/kg	0.01mg/kg	2	JFRL
Mercury	Less than 0.5mg/kg	< 0.01mg/kg	0.01 mg/kg	4	JFRL
Salmonella	Negative in 25g	Negative	-	3	In-house
Total coliforms	Less than 30 CFU in 1g	< 30 CFU in 1g	30CFU/g	5	In-house
E. coli	Negative in 25g	Negative	-	5	JFRL
Sulphur-reducing anaerobe	Less than 30 CFU/g	< 30 CFU in 1g	-	5	Falco
Staphylococcus aureus	Negative in 1g	Negative	-	3	In-house
Antibiotic activity	Negative	Negative	-	6	JFRL
Total viable bacteria count	Less than 10,000CFU/g	< 100cfu/g	100CFU/g	3	In-house
Ash %	-	1.65	-	3	In-house
moisture %	-	4.39	-	3	In-house
D	-	0.00	-	3	In-house
Aflatoxin B1	Negative	< 1 µg/kg	1.0 µg/kg	7	JFRL
Aflatoxin B2	Negative	< 1 µg/kg	1.0 µg/kg	7	JFRL
Aflatoxin G1	Negative	< 1 µg/kg	1.0 µg/kg	7	JFRL
Aflatoxin G2	Negative	< 1 µg/kg	1.0 µg/kg	7	JFRL

Nagase ChemteX Corporation

Methods

1. in-house method in Nagase ChemteX Corporation
2. Atomic absorption spectrometry
3. The Japanese Pharmacopoeia
4. Cold vapor atomic absorption spectrometry
5. Standards methods of analysis in food safety regulation (Japan)
6. APPENDIX "DETERMINATION of ANTIBIOTICS ACTIVITY to ANNEX A in joint FAO/WHO Expert Committee on Food Additive (FAO FOOD AND NUTRITION PAPER 49) "
7. HPLC

Organization

- JFRL : Japan Food Research Laboratories
- Falco : FALCO LIFE SCIENCE Ltd.

Daisuke Ishiyama
Manager, Quality Assurance Section 3
Quality Assurance Division
Nagase ChemteX Corporation

Nagase ChemteX Corporation

A P P E N D I X 2

Post-mitochondrial fraction (S9 fraction)

TRINOVABIOCHEM

MOLTOX®
Molecular Toxicology, Inc.

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information
SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
AGE: 5 – 6 weeks
WEIGHT: 175 – 199 g
TISSUE: Liver

Part Number Information
LOT NO.: 4120
PART NO.: 11-101
VOLUME: 1 mL
BUFFER: 0.15 M KCl
STORAGE: At or below -70°C

PREP: July 03, 2019
EXPIRY: July 03, 2021
INDUCING AGENT: Aroclor 1254, (Monsanto KL615), 500 mg/kg i.p.

REFERENCE: Maron, D & Ames, B., *Mutat Res*, **113**: 173, 1983. **For Research Purposes Only**

BIOCHEMISTRY:
- PROTEIN: 36.2 mg/ml — Assayed according to the method of Lowry et al., *JBC* **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
BROD	2B1, 2B2	164.2
EROD	1A1, 1A2	425.9
MROD	1A1, 1A2	152.4
PROD	2B1, 2B2	68.0

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 95.2, 56.8, 39.5, & 34.7 for BROD, EROD, MROD and PROD, respectively.

BIOASSAY:
- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microorganisms by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Duplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
TA98	TA1535
60.8	576

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**: 299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 – 10% in S9 mix, were tested for their ability to activate benzo(α)pyrene (BP) and 2-aminoanthracene (2-AA) to metabolites mutagenic to TA100. Assays were conducted as described by Maron & Ames, (*Mutat Res* **113**: 173, 1983).

	µl S9 per plate/number *his*⁺ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	175	164	226	287	341	545
2-AA (2.5 µg)	104	179	499	1429	1642	1417

Approved: [redacted] 07/08/19

MOLECULAR TOXICOLOGY, INC. www.moltox.com (828) 264-9099

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A P P E N D I X 3

Historical Background Data

Historical Background Data
***in vitro* Micronucleus Test**
in cultured human peripheral lymphocytes

The micronucleus frequencies of the vehicle controls and positive controls without and with metabolic activation for the last 25 studies (most recent background data of the years 2017 to 2019, not audited by the QAU-department) are given as follows:

Micronucleus frequency per 1000 cells						
Vehicle controls						
	Without metabolic activation				**With metabolic activation**	
	Untreated control[#1]		**Vehicle controls**		**Untreated control[#1]**	**Vehicle control**
	exposure:		**exposure:**		**4-hour exposure**	
	4-hour	**24-hour**	**4-hour**	**24-hour**		
Mean	6.3	6.8	5.1	4.4	6.4	4.0
SD	2.2	3.4	2.5	1.8	2.6	1.9
Range 95%[#2]	2.7 – 10.0	3.0 – 16.4	1.2 – 10.0	2.0 – 8.8	3.0 - 12.4	1.2 – 8.0
MLE[#2]	6.3	6.8	5.1	4.4	6.4	4.0
95% Confidence limits[#2]	5.4 - 7.3	5.9 - 7.9	4.5 - 5.8	3.8 - 5.0	5.7 - 7.1	3.5 - 4.6

MLE = Maximum Likelihood Estimation
[#1] = Data of the years 2013 to 2015 (n=21)
[#2] = Poisson-based

Positive controls			
	Mitomycin C	**Colchicine**	**Cyclophosphamide**
Mean	22.6	22.5	18.6
SD	7.4	6.4	3.9
Range	12 - 47	12 - 38	12 - 31

SD = Standard deviation

A P P E N D I X 4

GLP Certificate of the Test Facility LPT

FREIE UND HANSESTADT HAMBURG
Behörde für Gesundheit und Verbraucherschutz

Gute Laborpraxis / Good Laboratory Practice

GLP – Bescheinigung / Statement of GLP Compliance

(gemäß/according to § 19b (1) Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/ EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/ EEC at:

[x] Prüfeinrichtung/ Test facility [ ] Prüfstandort/ Test site

LPT Laboratory of Pharmacology and Toxicology GmbH & Co. KG
Redderweg 8
21147 Hamburg
(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/ Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

Kategorie 2, 3, 4 und 9 (Sicherheitspharmakologie und Auftragsarchiv)

Datum der Inspektion (Abschluss)
Datum der Inspektion/ Date of Inspection:

07. Juli 2016

Die/Der genannte Prüfeinrichtung /Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.
Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/ diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that this test facility/ test site is able to conduct the aforementioned studies in compliance with the Principles of GLP

Hamburg, den 8. Mai 2017

Amt für Arbeitsschutz - Arbeitsschutzverwaltung 1 Hamburg

[redacted]
Dr. Volker Kregel
Direktor des Amtes für Arbeitsschutz

Behörde für Gesundheit und Verbraucherschutz
Marckmannstraße 129b, 20539 Hamburg

APPENDIX B

GRAS Panel Statement

GRAS Panel Statement Concerning the Generally Recognized as Safe (GRAS) Status of the Proposed Uses of a Collagenase Enzyme Preparation (DENAZYME XPP-011F) from *Streptomyces violaceoruber* pCol as a Processing Aid in Food Production

15 March 2021

INTRODUCTION

At the request of NAGASE & CO., LTD (Nagase), a panel of independent scientists (the "GRAS Panel"), qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information related to Nagase's collagenase enzyme preparation derived from a genetically-modified strain of *Streptomyces violaceoruber* (strain pCol) to determine whether the intended use as a processing aid in food production would be Generally Recognized as Safe (GRAS) based on scientific procedures. For purposes of the GRAS Panel's evaluation, "safe" or "safety" indicates that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2020).

The GRAS Panel consisted of the below-signed qualified scientific experts: Professor Emeritus Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine); Professor David Brusick, (Toxicology Consultant); and Professor Emeritus Michael W. Pariza (University of Wisconsin-Madison). The GRAS Panel was selected and convened in accordance with the United States (U.S.) Food and Drug Administration's (FDA's) guidance for industry on *Best Practices for Convening a GRAS Panel* (U.S., FDA 2017). Nagase ensured that all reasonable efforts were made to identify and select a balanced GRAS Panel with expertise in food safety, toxicology, and microbiology. Efforts were placed on identifying conflicts of interest or relevant "appearance issues" that could potentially bias the outcome of the deliberations of the GRAS Panel; no such conflicts of interest or appearance issues were identified. The GRAS Panel received an honorarium as compensation for their time; the honorarium provided to the GRAS Panel was not contingent upon the outcome of their deliberations.

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of all publicly available scientific data and information compiled from a comprehensive search of the scientific literature conducted by Nagase, which included all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food uses of the enzyme preparation. The data evaluated by the GRAS Panel included information on the identity and purity of the enzyme preparation and production organism, method of manufacture, product specifications, batch analyses and analytical data, stability data, intended conditions of use, estimated intakes based on intended conditions of use, and a summary of the available scientific information and data pertinent to the safety of the enzyme preparation, including the safety of the production organism. The GRAS Panel also evaluated other publicly available information, as considered appropriate.

Following its independent and collaborative critical evaluation of the data and information, the GRAS Panel convened *via* teleconference on 17 December 2020 and unanimously concluded that the intended uses of the enzyme preparation as described herein, meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice (cGMP), are GRAS based on scientific procedures. A summary of the information critically evaluated by the GRAS Panel and serving as the basis for the GRAS Panel's conclusion is presented below.

SUMMARY AND BASIS FOR GRAS

The collagenase enzyme catalyzes the hydrolysis of peptide bonds in collagen to produce collagen fragments. The collagenase enzyme preparation is marketed in a dry (powdered) form under the trade name DENAZYME XPP-011F. The collagenase enzyme preparation is intended for direct use in the processing of meat for beef jerky, marinated and injected meat, and sausage casings.

The GRAS Panel reviewed data related to the characterization of the source organism. The food enzyme is produced by the self-cloned *S. violaceoruber* pCol. The plasmid pIJ702, which is extracted from *S. violaceoruber* ATCC 35287, is genetically modified to plasmid pCol, which is subsequently transfected to the host strain, *S. violaceoruber* 1326, to produce the production organism, *S. violaceoruber* pCol. The host microorganism, which was previously named *Streptomyces lividans* or *Streptomyces coelicolor*, has been reported not to be pathogenic or toxigenic (Korn-Wendisch and Kutzner, 1992; Bergey's Manual, 1994). The plasmid pCol contains:

- A promoter sequence which is the promotor sequence of the gene for metallo-endopeptidase from *Streptomyces avermitilis* ATCC 31267;

- The structural gene of collagenase obtained from *S. violaceoruber* NBRC 15146 (or *S. coelicolor* A3(2)) after identification and amplification of the structural gene by polymerase chain reaction; and

- A terminator sequence which is the terminator sequence of the gene for phospholipase D from *Streptomyces cinnamoneus* NBRC 12852. This terminator sequence has been used previously for the construction of a self-cloned strain of *S. violaceoruber* to produce beta-glucanase.

The tyrosinase genes (*melC1* and *melC2*) were also removed from plasmid pIJ702 during the modification process to plasmid pCol. The plasmid pIJ702 contains a gene, *tsr*, encoding for a thiostrepton-resistance protein. Thiostrepton is a cyclic oligopeptide antibiotic of the thiopeptide class and its resistance gene, *tsr*, is naturally present in *Streptomyces azereus;* however*,* the presence of this antibiotic resistance gene is only considered to be a concern if the gene is transferable; the plasmid pCol, along with the nucleotide sequences of which it is comprised, is described as non-conjugative and non-transferable.

The collagenase preparations are produced under Quality Assurance Certification and cGMP and comply with the guidelines for the safe handling of microbial enzyme preparations by the Association of Manufacturers of Fermentation Enzyme Products (AMFEP). All raw materials and processing aids are food-grade and are permitted for use in the U.S. for such purposes. The food enzyme is produced by *S. violaceoruber* pCol, which is cultivated in 3 different phases: seed culture, pre-culture, and main culture. At each phase, *S. violaceoruber* pCol is grown in culture medium containing nutrients and antifoam material and controlled for temperature, pH, and microscopic observations. The first step in the production process involves isolation of *S. violaceoruber* pCol from a pure culture, which is then cultivated in culture medium to produce the main culture. Subsequently, the main culture undergoes 3 separate phases of filtration. The

permeate is then concentrated, and the protein is precipitated from the solution by addition of ammonium sulfate. The solution containing the protein sediments undergoes further filtration and dispersion to obtain the enzyme concentrate. Prior to release for further processing and for the formulation of the final enzyme preparations, the food enzyme is assessed for microbiological contamination. The final powdered product is prepared by freeze-drying and standardization with dextrin. The final liquid product is prepared by standardization with glycerin and water and undergoes a final filtration step. The final enzyme preparations are then analyzed to ensure that the product meets its specifications. Nagase has conducted studies to assess the removal of the production strain, the results of which demonstrated the absence of the production strain in the food enzyme concentrate and collagen peptides treated with a powdered collagenase enzyme preparation characterized by a TOS content of 21.1%.

The GRAS Panel reviewed the product specifications established for identity and purity of Nagase's enzyme preparations, which comply with the purity specifications established for enzyme preparations by Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex (JECFA, 2006 and FCC, 2018). Nagase has also provided analytical data on 3 representative batches of the DENAZYME XPP-011F (powder) collagenase enzyme preparation from *S. violaceoruber* pCol, which demonstrated that the manufacturing process consistently yields a product that complies with the established specifications for each enzyme preparation. Three (3) batches were demonstrated to be free from recognized mycotoxins (aflatoxins B1, B2, G1, and G2) and lacking significant amylase or lipase subsidiary activity that would raise a safety concern.

Data related to the storage stability of the DENAZYME XPP-011F (powder) collagenase enzyme preparation were reviewed by the Panel indicating that powder preparations were stable for 12 months at 5°C, when stored in aluminum bags as no significant losses in collagenase activity were reported during storage under these conditions. The GRAS Panel also reviewed data related to the stability and activity of the collagenase food enzyme produced by *S. violaceoruber* pCol, as a function of temperature and pH. The data demonstrated that the optimal pH and temperature conditions for the collagenase food enzyme enzyme preparations were 8.0 and 50°C, respectively, and that the food enzyme is inactivated at temperatures above 50°C.

The collagenase enzyme preparations are intended for direct use in meat processing, such as beef jerky, marinated and injected meat, and sausage casings.

Technological effects of the food enzyme on the final foods are limited by denaturation of the food enzyme by a heating step during the manufacturing processes of meat products and collagen hydrolysates to inactivate the enzyme. The maximum recommended use level of collagenase from the DENAZYME XPP-011F powder preparation for processing of meat for beef jerky, meat (marinated or injection), or sausage casing is 10.9 mg total organic solids (TOS)/kg food.

The potential human exposure to collagenase has been estimated using the Budget Method, which is a widely accepted preliminary screening tool used to assess the intake of chemicals such as food additives (FAO/WHO, 2009). The Budget Method allows for the calculation of a theoretical maximum daily intake (TMDI) based on assumptions regarding the maximum human physiological levels of daily food and beverage consumption, rather than on food consumption data collected from dietary surveys. Specifically, the Budget Method relies on conservative assumptions made regarding (i) the level of consumption of solid foods (0.05 kg/kg body weight/day); (ii) the level of presence of the substance in solid foods (10.9 mg TOS/kg body weight); and (iii) the proportion of solid foods that may contain the substance (12.5%) (FAO/WHO, 2009). The levels of anticipated exposure to food enzymes that are derived using the Budget Method are thus considered to be conservative estimates (FAO/WHO, 2009). Based on these assumptions, the overall TMDI of collagenase from *S. violaceoruber* pCol from all dietary sources (foods and dietary

supplements) was calculated to be 0.068 mg TOS/kg body weight/day. The GRAS Panel noted that the estimated intake values obtained are gross overestimations of the exposure to the food enzyme based upon the use of the conservative Budget Method (FAO/WHO, 2009). The GRAS Panel also noted that the food enzyme would be heat-denatured and inactivated during the final stages of processing for the food ingredient and/or final food products that undergo treatment at high temperatures, such as those that occur during pasteurization or sterilization.

The GRAS Panel critically evaluated the data and information characterizing the safety of the food enzyme. The safety of the food enzyme for use in foods was assessed according to the safety paradigm developed by Pariza and Foster (1983) and Pariza and Johnson (2001) for assessing the safety of microbial enzyme preparations used in foods. The safety of collagenase from *S. violaceoruber* pCol was evaluated based on the safety of the production strain lineage and data and information supporting the safety of the enzyme preparation under its conditions of intended use in foods.

S. violaceoruber is a Gram-positive bacterium that occurs naturally in soil (Duangmal *et al.,* 2005). Species of bacteria from this genus have been widely used in the production of industrially important enzymes, including those intended for use in food processing (Mukhtar *et al.,* 2017). The GRAS status of several food enzymes derived from *S. violaceoruber*, including Genencor's phospholipase A2 enzyme preparation from *S. violaceoruber*, Nagase's phospholipase A2 enzyme preparation from *S. violaceoruber* expressing a gene encoding phospholipase A2, and beta-glucanase from *S. violaceoruber*, has been notified to the U.S. FDA in 2004, 2007, and 2015, respectively, and all received letters of "no questions" from the Agency for use in food processing (Genencor International, Inc., 2004; U.S. FDA, 2004, 2007, 2015; Nagase ChemteX Corporation, 2006; Nagase America Corporation, 2014). A review of the publicly available scientific literature through August 2020 was conducted to identify the relevant literature on the pathogenicity of strains of *S. violaceoruber.* The pathogenicity of species of the genus *Streptomyces* in humans has been reviewed (Kieser *et al.,* 2000), the results of which indicated that infections caused by actinomycetes can mostly be attributed to species *Mycobacterium*, *Actinodura*, *Nocardia*, and *actinomyces* rather than *Streptomyces*. Although *Streptomyces somaliensis*, *Streptomyces madurae*, and *Streptomyces sudanenisis* have been identified as pathogens (Boiron *et al.,* 1998; Khatri *et al.,* 2002; Dieng *et al.,* 2003, 2005; Quintana *et al.,* 2008), no publications were identified from the search for relevant literature that have indicated that strains of *S. violaceoruber* are pathogenic. The GRAS Panel concluded that the production strain was derived from an organism that has an established history of safe use in the production of food enzymes and is considered to be a non-pathogenic and non-toxigenic. The production strain, *S. violaceoruber* pCol, was determined to be safe for use in the production of the collagenase food enzyme based on the Pariza and Johnson (2001) decision tree.

The GRAS Panel further reviewed the publicly available information on the safety of the food enzyme, which was supported by the results of an acute oral toxicity study in rats, a subchronic 13-week oral toxicity study in rats, bacterial reverse mutation assays, *in vitro* mammalian cell gene mutation assays, an *in vivo* micronucleus induction assay in rats (Harazono *et al.,* 2020), and in an *in vitro* micronucleus assay (Laboratory of Pharmacology and Toxicology, 2020 [unpublished]). All toxicology tests of collagenase from *S. violaceoruber* pCol have been conducted using a powdered collagenase concentrate (Lot No. 3T-1, characterized by 93.96% TOS and enzyme activity of 10,287 U/g).

The acute oral toxicity study was conducted in accordance with the OECD Test Guideline No. 423 (OECD, 2001) and Principles of GLP (OECD, 1998a; Harazono *et al.,* 2020). Female Sprague-Dawley SPF rats (2 groups of 3 each) were given a single oral (gavage) administration of the powdered collagenase concentrate dissolved in water to a concentration of 200 mg/mL at a dose volume of 10 mL/kg body weight providing doses of 2,000 mg/kg body weight or 1,879.2 mg TOS/kg body weight. This dose is the upper limit level described in the test guideline and was selected because enzymes are known to have low acute toxicity. No deaths or abnormalities were reported throughout the study. No significant changes related to administration of the collagenase enzyme were reported in the general condition, body weight, body weight gain, or gross pathology compared to controls. The median lethal dose of collagenase from *S. violaceoruber* pCol *via* gavage was reported to be >1,879.2 mg TOS/kg body weight (Harazono *et al.,* 2020).

The subchronic oral toxicity study was conducted in accordance with the OECD Test Guideline No. 408 (OECD, 1998b) and Principles of GLP (OECD, 1998a; Harazono *et al.,* 2020). Sprague-Dawley SPF rats (10/sex/group) were given oral (gavage) administrations of the powdered collagenase concentrate dissolved in water to provide doses of 0 (distilled water), 58.7, 234.9, and 939.6 mg TOS/kg body weight/day for 13 weeks. The dose levels were selected based on the results of a 14-day dose-range finding repeated-dose oral toxicity study, in which animals were administered 0 (distilled water), 58.7, 234.9, or 939.6 mg TOS/kg body weight/day *via* gavage (Harazono *et al.,* 2020). No compound-related adverse effects were reported in any of the dose groups; therefore, the high-dose level for the 13-week study was set at 939.6 mg TOS/kg body weight/day, the highest tested. The study parameters monitored during the 13-week study were consistent with OECD Test Guideline No. 408 (OECD, 1998).

In the 13-week study, no mortality was reported. There were no significant and treatment-related clinical signs of toxicity or differences in body weight, body weight gain, food consumption, measured ophthalmological, hematology, urinalysis, or coagulation parameters, or histopathological findings between control and treatment groups. A statistically significant increase in food consumption was recorded on Day 21 in females of the low dose group (58.7 mg TOS/kg body weight/day) compared to controls, and a statistically significant reduction in food consumption was reported on Day 42 in males of the low-dose group compared to the control group. The study authors considered these effects not to be toxicologically relevant as there was no clear dose-response relationship and there were no reported effects on body weight gain in these groups. A significantly decreased value for blood chloride was reported in males of the high-dose group. Abnormally high values in aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic acid dehydrogenase (LDH), total cholesterol, triglyceride, and phospholipids were reported in 1 female of the high-dose group, but no significant changes were reported in this group compared to the controls. A statistically significant increase in the relative kidney weight in males of the high-dose group compared to the controls was reported. Based on the results of this study, administration of 939.6 mg TOS/kg body weight/day, the highest dose tested, resulted in a statistically significant reduction in blood chloride levels and a statistically significant increase in relative kidney weights in males and abnormally high values AST, ALT, LDH, total cholesterol, triglycerides, phospholipids, and total bilirubin in 1 female. The study authors reported that the no-observed-adverse-effect level (NOAEL) was 234.9 mg TOS/kg body weight/day, the mid-dose group, providing a margin of safety of 3,454 compared to the TMDI of 0.068 mg TOS/kg body weight/day. However, considering that the reported effects were isolated, only seen in 1 female, were not dose-related, and were not accompanied by any histopathological findings, the effects were not considered to be of toxicological significance. A revised NOAEL of 939.6 mg TOS/kg body weight/day, the highest dose tested, was therefore considered by the GRAS Panel for collagenase from *S. violaceoruber* pCol, which results in a margin of safety of 13,818 compared to the TMDI of 0.068 mg TOS/kg body weight/day.

The mutagenic potential of the powder concentrate was evaluated in a bacterial reverse mutation assay conducted in 5 tester strains: *Salmonella typhimurium* TA100, TA98, TA1535, and TA1537 and *Escherichia coli* WP2uvrA using the preincubation method at concentrations up to 4,698 µg/plate (Harazono *et al.,* 2020). The study was conducted in accordance with the OECD Test Guideline No. 471 (OECD, 1997a) and the Principles of GLP (OECD, 1998a). The number of revertant colonies exceeded twice that of the negative control group at concentrations of 2,349 and 4,698 µg TOS/plate in *S. typhimurium* strain TA1513 in the presence of metabolic activation; however, growth inhibition was also reported at 4,698 µg TOS/plate in *S. typhimurium* strain TA1535 in the presence of metabolic activation. A dose-dependent increase in the number of revertant colonies was also reported in *S. typhimurium* strain TA100 in the absence of metabolic activation and in strains TA100 and TA1535 in the presence of metabolic activation. The number of revertant colonies in all other tester strains was less than twice that of the negative control at all tested concentrations in the presence and absence of metabolic activation and precipitation of the test article was not reported. A second bacterial reverse mutation assay was then conducted using the preincubation and treat-and-wash methods in *S. typhimurium* strains TA100 and TA1535 at concentrations up to 4,698 µg TOS/plate to determine if the findings from the first study were due to free amino acids. The number of revertant colonies was similar to the negative controls in all of the tester strains up to the highest tested concentration (4,698 µg TOS/plate) using the preincubation or treat-and-wash methods in the presence and absence of metabolic activation. The study authors concluded that the positive results reported in the first study were likely due to the presence of free amino acids and that the collagenase from *S. violaceoruber* pCol is non-mutagenic under the conditions of this assay (Harazono *et al.,* 2020).

The potential mutagenicity of the powder concentrate of collagenase was further evaluated in an *in vitro* mammalian cell gene mutation assay (mouse lymphoma TK assay) using mouse lymphoma cultured cells (L5178Y) under short-term and continuous exposure conditions at concentrations up to 4,698 µg TOS/mL (Harazono *et al.,* 2020). The study was based on OECD Test Guideline Nos. 476 (*in vitro* mammalian cell gene mutation test) (OECD, 1997b) and 490 (*in vitro* mammalian cell gene mutation tests using the thymidine kinase gene) (OECD, 2016a) and conducted in accordance with the Principles of GLP (OECD, 1998a). No precipitation was reported and the total mutant frequency (TMF) was less than twice that of the negative control group and less than 200×10^{-6} up to the highest tested concentration of 4,698 µg TOS/mL with and without metabolic activation under short-term exposure conditions. Under continuous treatment conditions, the TMF exceeded 200×10^{-6} (299.75×10^{-6}) at the highest tested concentration of 4,698 µg TOS/mL without metabolic activation. Due to a lack of a clear dose-response relationship, a confirmatory test was conducted to further investigate the results obtained at the highest tested dose in the main test. In the confirmatory test, no precipitation was reported and the TMF was similar to the negative controls and did not exceed 200×10^{-6} up to the highest tested concentration of 4,698 µg TOS/mL. In addition, the percentage of small colonies was 83% at concentrations of 4,698 µg TOS/mL, which is higher than the negative controls (36%) (statistical significance not reported). The study authors suggested this finding may have been caused by chromosomal aberrations (Harazono *et al.,* 2020). In order to elucidate the potential induction of chromosomal aberrations, *in vitro* and *in vivo* micronucleus tests were conducted.

The genotoxic potential of the powder collagenase concentrate was evaluated in an *in vitro* micronucleus induction assay in cultured human peripheral lymphocytes at concentrations up to 2,000 µg/mL for 4 or 24 hours (Laboratory of Pharmacology and Toxicology, 2020 [unpublished]). The study was conducted in accordance with OECD Test Guideline No. 487 (OECD, 2016b) and the Principles of GLP (OECD, 1998a). No cytotoxicity was reported in the 4-hour exposure conditions in the presence and absence of metabolic activation or the 24-hour exposure conditions in the absence of metabolic activation up to the highest tested concentration of 2,000 µg/mL. Test item precipitation was reported at concentrations of 1,000 and

2,000 µg/mL under all treatment conditions. No significant differences were reported in the mean micronucleus frequencies in any treated group under all treatment conditions compared to the negative controls. The study authors conclude that collagenase from *S. violaceoruber* pCol did not cause chromosomal damage under the conditions of this assay (Laboratory of Pharmacology and Toxicology, 2020 [unpublished]).

The genotoxic potential of the powder collagenase concentrate was further evaluated in an *in vivo* micronucleus induction assay conducted in accordance with OECD Test Guideline No. 474 (OECD, 1997c; Harazono *et al.,* 2020). Male Sprague-Dawley SPF rats (5/group) were administered doses of 0 (water), 234.9, 469.8, 939.6, or 1,879 mg TOS/kg body weight/day *via* oral gavage for 2 consecutive days. No clinical signs of toxicity or significant differences in body weight gain were reported in animals up to the highest tested dose (1,879 mg TOS/kg body weight/day) compared to the negative controls. Furthermore, no significant differences in the proportion of micronucleated polychromatic erythrocytes (MNPCE) and polychromatic erythrocytes were reported between treatment and negative control groups and the positive control resulted in a significant increase in the proportion of MNPCE compared to the negative control. The study was therefore considered to have been conducted appropriately and study authors concluded that collagenase from *S. violaceoruber* pCol is non-genotoxic under the conditions of this assay (Harazono *et al.,* 2020). Based on the available evidence, the GRAS Panel concluded that the collagenase from *S. violaceoruber* pCol was not mutagenic or genotoxic.

The allergenicity potential of the collagenase food enzyme was evaluated using the bioinformatics criteria recommended by FAO/WHO (FAO/WHO, 2001; Codex Alimentarius, 2009; JECFA, 2016). The amino acid sequence for the collagenase was compared to the amino acid sequences of known allergens in publicly available databases (see Appendix E). No matches with greater than 35% percent identity were identified using a window of 80 amino acids, no exact matches were identified using a window of 8 amino acids, and no sequences were considered homologous with known allergens using a full sequence search with an E-value cut-off of 0.1. Therefore, dietary exposure to collagenase from *S. violaceoruber* pCol is not expected to pose a risk of allergenicity.

The Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information (NCBI) was used to conduct a sequence alignment query of the collagenase FASTA protein sequence against downloaded protein sequences obtained from a curated databases of 7,235 animal venom proteins and toxins[1] maintained by UniProt. A sequence alignment of 35% identity and an E-value cutoff of 0.1 were used as thresholds for identification of a positive alignment. No matches with a sequence alignment ≥35% or an E-value ≤0.001 were identified. A review of the publicly available scientific literature was conducted through August 2020 to identify the relevant literature on the pathogenicity of strains of *S. violaceoruber*. The pathogenicity of species of the genus *Streptomyces* in humans has been reviewed (Kieser *et al.,* 2000), the results of which indicated that infections caused by actinomycetes can mostly be attributed to species *Mycobacterium*, *Actinodura*, *Nocardia*, and *actinomyces* rather than *Streptomyces*. No publications were identified indicating that strains of *S. violaceoruber* are pathogenic. Given the non-pathogenic nature of the production strain and the absence of the production strain from the commercial enzyme preparations, collagenase from *S. violaceoruber* pCol will not present a safety concern related to pathogenicity.

Following its independent and collective critical evaluation of the available information related to collagenase) from *S. violaceoruber* pCol, the GRAS Panel unanimously concluded that the available information supports the conclusion presented on the following page.

[1] UniProt release Oct, 2017; available at: https://www.uniprot.org/program/Toxins.

CONCLUSION

We, the undersigned independent qualified members of the GRAS Panel, have individually and collectively critically evaluated the data and information summarized above, and other data and information that we deemed pertinent to the safety of the intended conditions of use for the collagenase enzyme preparation from *Streptomyes violaceoruber* pCol, DENAZYME XPP-011F. We unanimously conclude that the proposed use of collagenase enzyme preparation from *Streptomyces violaceoruber* pCol (DENAZYME XPP-011F), as a processing aid in food production, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting the appropriate food-grade specifications presented in the supporting dossier, is safe and suitable.

We further unanimously conclude that the proposed use of collagenase enzyme preparation from *Streptomyces violaceoruber* pCol (DENAZYME XPP-011F), as a processing aid in food production, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications presented in the supporting dossier, is Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

[redacted]

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Viebrock, Lauren

From: Srinivasan, Jannavi
Sent: Wednesday, August 3, 2022 10:30 AM
To: Viebrock, Lauren
Subject: FW: [EXTERNAL] RE: Filing of GRAS Notice 001046

(b) (5) [redacted]

[redacted]

From: koichiro.kojima@nagase.co.jp <koichiro.kojima@nagase.co.jp>
Sent: Friday, May 27, 2022 7:00 AM
To: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: [EXTERNAL] RE: Filing of GRAS Notice 001046

Dear Dr. Jannavi R. Srinivasan

We would like to inform you our trade secret parts in the attached submission document (PDF: Collagenase XPP-011F GRAS NOTICE-Confidential 002).
The parts that we want you to be undisclosed in the public are marked with orange color, please find them in the attached document, thank you.

For your information, they are shown below.

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- On page 11
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- On page 21
- On page 22
- Bottom of page 25
- Bottom of page 27
- On page 30
- On page 43

Best regards,

Koichiro Kojima
General Manager, Food Ingredients Department, Nagase & Co., Ltd.
Tel: +81-3-3665-3384 Fax: +81-3-3665-3976
Mobile: +81-80-7712-8982

From: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Sent: Monday, April 25, 2022 9:14 PM
To: Kojima Koichiro 小嶋　宏一郎<koichiro.kojima@nagase.co.jp>
Subject: Filing of GRAS Notice 001046

Dear Mr. Kojima,

Find attached the Filing Letter for the notice that you submitted to FDA for our review; this is for collagenase enzyme preparation for its intended uses.

Please let me know if you have any questions.

Thank you,

Jannavi

Jannavi R. Srinivasan
Team Lead, Chemistry Review Branch
DFI/OFAS/CFSAN/FDA
College Park, MD
Ph: 240 402 1199

From: koichiro.kojima@nagase.co.jp <koichiro.kojima@nagase.co.jp>
Sent: Tuesday, September 13, 2022 7:58 PM
To: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Dear Dr. Jannavi Srinicasan,

Thank you for your waiting.
The attached is the revised document.
Please check it.
Even I hope you accept it, but if it is not enough unfortunately, could you please let me know.

Best regards,
Koichiro Kojima

From: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Sent: Friday, September 9, 2022 8:17 AM
To: Kojima Koichiro 小嶋　宏一郎 <koichiro.kojima@nagase.co.jp>
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Yes, that is fine, Dr. Kojima.
Thank you,
Jannavi

From: koichiro.kojima@nagase.co.jp <koichiro.kojima@nagase.co.jp>
Sent: Friday, September 9, 2022 3:21 AM
To: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Dear Dr. Jannavi Srinicasan,

Thank you for below text.

We could identify what we didn't understand, and will submit revised documents by 14th September 2022.
Could you please accept the schedule.

Best regards,
Koichiro Kojima

From: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Sent: Thursday, September 8, 2022 2:39 PM
To: Kojima Koichiro 小嶋　宏一郎 <koichiro.kojima@nagase.co.jp>
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Sure. I have copied the text with the details from my previous email here:

Figure 2.5.3-7 identifies "**Beef** chuck from JAPAN" as the initial ingredient for a marinated **chicken** product. This typographical error is also present under the Result section of the same attachment.

Figure 2.5.3-8 contains a similar typographical error (i.e., **beef** listed instead of **pork**) under the Results section of the attachment.

I hope this helps.

Thank you,

Jannavi

From: koichiro.kojima@nagase.co.jp <koichiro.kojima@nagase.co.jp>
Sent: Thursday, September 8, 2022 12:36 AM
To: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Dear Dr. Jannavi Srinivasan,

Thank you for your reply below.

Even though I am discussing and trying to identifying “two of the figures and the corresponding texts” with my team, we cannot identify it, and we don’t know the cause. Could you please let me know what are “two of the figures and the corresponding texts” specifically?
I’ll bother you, but please.

Best regards,
Koichiro Kojima

From: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Sent: Wednesday, September 7, 2022 4:54 PM
To: Kojima Koichiro 小嶋　宏一郎 <koichiro.kojima@nagase.co.jp>
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Dr. Kojima,
I just realized that there are some requested corrections for two of the figures and the corresponding texts in the GRAS Notice, that are still missing. Please let me know when you will you be sending them to us so we may continue with our review.
Thank you,
Jannavi

From: koichiro.kojima@nagase.co.jp <koichiro.kojima@nagase.co.jp>
Sent: Friday, September 2, 2022 10:55 AM
To: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: [WARNING : MESSAGE ENCRYPTED] [WARNING : MESSAGE ENCRYPTED] RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Dear Dr. Jannavi Srinivasan,

I’m sorry I missed to submit SDS on 11th Aug., 2022.
The attached is SDS and SDS-PAGE data.
I believe I have submitted everything that this instructed me to do.
If not enough, please let me know.

Best regards,
Koichiro Kojima

From: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Sent: Friday, August 26, 2022 9:59 AM
To: Kojima Koichiro 小嶋　宏一郎 <koichiro.kojima@nagase.co.jp>
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Dr. Kojima,
Please find additional questions from USDA/FSIS; this includes a previous question requesting SDS PAGE data that we did not receive in your responses on 8/11/22.
I would appreciate if you acknowledge receipt of this email, and a response to the questions within 10 business days. I will be in touch as we continue with our review.
Thank you,
Jannavi

FSIS' technical review team identified what appears to be typographical errors in two of the revised figures.

Figure 2.5.3-7 identifies "**Beef** chuck from JAPAN" as the initial ingredient for a marinated **chicken** product. This typographical error is also present under the Result section of the same attachment.

Figure 2.5.3-8 contains a similar typographical error (i.e., **beef** listed instead of **pork**) under the Results section of the attachment.

The technical review team at FSIS should be able to make a suitability determination once the submitter has provided an SDS sheet and corrected versions of Figure 2.5.3-7 and Figure 2.5.3-8.

From: Srinivasan, Jannavi
Sent: Thursday, August 11, 2022 9:43 AM
To: koichiro.kojima@nagase.co.jp
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Thank you for sending your responses, Dr. Kojima. I will be in touch if we seek further clarifications to the responses and/or from our continued review of GRN 1046.
Regards,
Jannavi

From: koichiro.kojima@nagase.co.jp <koichiro.kojima@nagase.co.jp>
Sent: Thursday, August 11, 2022 9:06 AM
To: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: RE: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Dear Dr. Jannavi Srinivasan,

The attached file is the answers to the inquiry I received on 1st August 2022.
If they are not appropriate against your request or you will have further questions, please let me know.

Thank you for your work for us.

Best regards,
Koichiro Kojima

From: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Sent: Monday, August 1, 2022 2:43 PM
To: Kojima Koichiro 小嶋　宏一郎 <koichiro.kojima@nagase.co.jp>
Subject: FW: [EXTERNAL] GRAS Notice No. 001046 - Collagenase from genetically modified Streptomyces violaceoruber

Please find below the questions from **USDA FSIS** regarding the safety of the collagenase enzyme preparation. We would appreciate your responses to these questions within ten business days, and request that you please send them to me. Also, this GRAS Notice is still under review by FDA; we will send any additional questions, if any, upon completion of our review.
Thank you,
Jannavi

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****
In accordance with the procedures outlined in the Memorandum of Understanding between the Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS), you requested comments from FSIS regarding restrictions, conditions of use, or prohibitions that FSIS may have regarding the use of collagenase enzyme preparation (GRAS Notice No. (GRN) 001046) from genetically modified *Streptomyces violaceoruber* expressing a collagenase gene for use as an enzyme in processing beef jerky, marinated and injected meat, and sausage casings at a level up to 10.9 mg Total Organic Solids (TOS)/kg.

In general, FSIS requires additional information from the submitter before the Agency can comment on suitability of this ingredient for use in meat and poultry products.

Section 2.1 - Identity

FSIS requests that the submitter provide an SDS for the collagenase enzyme preparation that includes the preparation's composition and the concentration of all components.

The submitter indicates in Section 2.1.3 that dextrin comprises 96% of the collagenase enzyme preparation when distributed in dry form. FSIS requests that the submitter clarify what concentration (e.g., ppm) the dextrin will be present at in meat and poultry products if the collagenase component is present at the maximum use level proposed. As indicated in Table 2.5.2-1, the maximum proposed collagenase use level in meat and poultry products is:

- 7.25 ppm for beef jerky and meat (marinated);
- 10.9 ppm for meat (injected); and
- 3.63 ppm for sausage casings.

NOTE: If the submitter were to pursue listing this ingredient within FSIS Directive 7120.1 after FDA completes its GRAS review, these would be the maximum use levels indicated.

Section 2.5 – Intended Technical Effects

The submitter indicates in Section 2.5.1 that use of this collagenase enzyme preparation will involve a heating step which inactivates the enzyme. Low heat-treatment (e.g., 50°F-130°F or 10°C-54°C) steps are common when producing fermented and dried sausages. FSIS recommends the submitter clarify whether heat treatment conditions outlined in Section 2.5.1 will be further reflected in the ingredient's intended use or provide additional commentary on the ingredient's technological function in partially heat-treated meat and poultry products.

The submitter indicates in Section 2.5.2 that "Collagenase from *S. violaceoruber* pCol is intended for direct use in meat processing such as beef jerky, marinated and injected meat, and sausage casings." Ultimately, it is unclear whether the collagenase enzyme preparation is intended for use in both meat and poultry products. Given that suitability data is provided for chicken (i.e., poultry), the submitter may be unaware that the term "meat" is not necessarily inclusive of poultry within the context of FSIS regulation. FSIS recommends the submitter explicitly state whether this ingredient is intended for direct use in both meat and poultry processing.

The submitter provides suitability data in Section 2.5.3. With respect to marinated products (beef jerky, beef, chicken, and pork), "Brine (Control)" results are reported for marinated beef jerky [Figure 2.5.3-5] and marinated pork [Figure 2.5.3-8]. "Brine (Control)" results are not provided for marinated beef [Figure 2.5.3-6] and marinated chicken [Figure 2.5.3-7]. While the suitability data for marinated pork would support suitability in other red-meat products, it would not support suitability in poultry. If the submitter wishes for FSIS to evaluate suitability for poultry products, they should provide the "Brine (Control)" results for this product or comment on why the data is not included in Figure 2.5.3-7. Additionally, FSIS recommends the submitter comment on why the "Brine (Control)" may have increased toughness in marinated pork [Figure 2.5.3-8] relative to untreated product.

General Labeling

Regarding labeling, tenderizing agents are not considered processing aids and, as such, both the collagenase and dextrin components of this enzyme preparation would need to be declared on the label.

Viebrock, Lauren

From: koichiro.kojima@nagase.co.jp
Sent: Wednesday, April 12, 2023 11:41 AM
To: Viebrock, Lauren
Subject: RE: [EXTERNAL] RE: Filing of GRAS Notice 001046
Attachments: Collagenase XPP-011F GRAS Notice - Confidential .pdf

Dear Dr. Lauren VieBrock,

Thank you for your reply, and we found out that it was incorrectly designated confidential/trade secret.
We apology it and we would like to apply by the original GRN submission document as attached.
If it not enough, please let me know and we would like to have discuss by phone.
We really appreciate your kind confirmation.

Best regards,

Koichiro Kojima
General Manager, Food Ingredients Department
Nagase & Co., Ltd.
Mobile: +81-80-7712-8982（USA+1-224-288-7096）
E-mail: koichiro.kojima@nagase.co.jp
https://www.nagase-foods.com/jp/

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Monday, April 3, 2023 1:06 PM
To: Kojima Koichiro 小嶋　宏一郎<koichiro.kojima@nagase.co.jp>
Subject: RE: [EXTERNAL] RE: Filing of GRAS Notice 001046

Dear Koichiro Kojima,

During our review of GRN 1046, we noted an issue that needs to be addressed. On May 27, 2022, you submitted a revised GRAS notice that designated information previously submitted in the original GRN as trade secret and confidential. The role of an amendment submitted during an evaluation of a GRAS notice is to clarify questions we have about your GRAS conclusion rather than to substantively amend the GRAS notice.

Data and information used to support a GRAS conclusion must be generally available and generally recognized and therefore cannot be claimed as confidential and/or trade secret. Please clarify whether that information is considered confidential/trade secret or if it was incorrectly designated confidential/trade secret. If it is considered confidential, please provide a narrative to explain how experts could get to a GRAS conclusion of safety without the confidential information.

Please let me know if you have any questions. I am happy to discuss this matter by phone as well.

Regards,
Lauren

Lauren VieBrock, Ph.D.
Regulatory Review Scientist/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

From: koichiro.kojima@nagase.co.jp <koichiro.kojima@nagase.co.jp>
Sent: Friday, May 27, 2022 7:00 AM
To: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: [EXTERNAL] RE: Filing of GRAS Notice 001046

Dear Dr. Jannavi R. Srinivasan

We would like to inform you our trade secret parts in the attached submission document (PDF: Collagenase XPP-011F GRAS NOTICE-Confidential 002).
The parts that we want you to be undisclosed in the public are marked with orange color, please find them in the attached document, thank you.

For your information, they are shown below.

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- On page 11
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- Bottom of page 25
- Bottom of page 27
- On page 30
- On page 43

Best regards,

Koichiro Kojima
General Manager, Food Ingredients Department, Nagase & Co., Ltd.
Tel: +81-3-3665-3384 Fax: +81-3-3665-3976
Mobile: +81-80-7712-8982

From: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Sent: Monday, April 25, 2022 9:14 PM
To: Kojima Koichiro 小嶋　宏一郎<koichiro.kojima@nagase.co.jp>
Subject: Filing of GRAS Notice 001046

Dear Mr. Kojima,

Find attached the Filing Letter for the notice that you submitted to FDA for our review; this is for collagenase enzyme preparation for its intended uses.

Please let me know if you have any questions.

Thank you,

Jannavi

Jannavi R. Srinivasan
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