CHR HANSEN

Improving food & health

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July 12, 2018
USEMGR

GRAS Notification Submission

To Whom it May Concern,

Enclosed you will find one CD-ROM containing a GRAS dossier for a chymosin enzyme from *Camelius dromedarius* produced in *Aspergillus niger,* as well as an electronic copy of a signed expert panel report.

We appreciate your consideration of our submission.

Yours sincerely,

(b) (6)

Emily Gregoire
Regulatory Affairs Specialist

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July 11, 2018

Division of Biotechnology and GRAS Notice Review
Center for Food Safety & Applied Nutrition (HFS-255)
U.S. Food & Drug Administration
5100 Campus Drive, College Park, MD 20740

Reference: Chr. Hansen GRAS Notification for Chymosin enzyme from Camelus dromedarius produced in *Aspergillus niger*

To Whom it May Concern:

In accordance with the Federal Register [81 Fed. Reg. 159 (17 August 2016)] issuance on Generally Recognized as Safe (GRAS) notifications (21 CFR Part 170), Chr. Hansen is pleased to submit a notice that we have concluded, through scientific procedures, that Chr. Hansen's chymosin enzyme from Camelus dromedarius produced in *Aspergillus niger*, is generally recognized as safe and is not subject to the pre-market approval requirements for use as a processing aid in the production of cheese.

If there are any questions or concerns, please contact us.

Yours sincerely,

CHR. HANSEN, INC.

Chymosin enzyme from

Camelus dromedarius Produced in *Aspergillus niger*

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PART 1 §170.225 – SIGNED STATEMENTS AND CERTIFICATION

1.1 Submission of GRAS notice:

In accordance with 21 CFR §170.225, Chr. Hansen is hereby submitting a GRAS notice in accordance with subpart E of part 170.

1.2 The name and address of the notifier:

Chr. Hansen Holding A/S
Boege Alle 10-12
2970 Hoersholm, Denmark

Chr. Hansen, Inc.
9015 W Maple St.
Milwaukee, WI 53214

1.3 Name of notified substance:

Chymosin from *Camelus dromedarius* produced by *Aspergillus niger*.

1.4 Intended conditions of use:

This chymosin enzyme product is a processing aid intended for use in production of cheese. Chymosin is responsible for coagulation of milk. The chymosin is recommended to be used at the minimum dosage necessary to achieve the desired product.

1.5 Statutory basis for GRAS conclusion:

This GRAS determination is based on scientific procedures.

1.6 Premarket approval:

Pursuant to the GRAS rule [81 Fed. Reg. 159 (17 August 2016)], Chr. Hansen has concluded that chymosin from *Camelus dromedarius* produced by *Aspergillus niger* is GRAS through scientific procedures, in accordance with 21 CFR 170.30 (a) and (b).

1.7 Availability of information:

Chr. Hansen agrees to make our data and information that are the basis for our conclusion for GRAS status available, either during or after the approval of the Food and Drug Administration (FDA). In addition, upon the request of the FDA we will grant permission for reviewing and copying the data and information during customary business hours, at the address we specify for where these data and information will be available. We will also be responsible to provide the

FDA with a complete copy of the data and information either in an electronic format or on paper, which will be accessible for evaluation.

1.8 FOIA (Freedom of Information Act):

Parts 2 through 7 of this GRAS notice do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

1.9 Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notice is complete, representative and balanced. It contains both favorable and unfavorable information, known to Chr. Hansen and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

1.10 Signature:

(b) (6)

Katharine Urbain

Regional Regulatory Affairs Manager North America – Compliance
Chr. Hansen, Inc.

(b) (6)

Emily Gregoire

Regulatory Affairs Specialist – Food Cultures & Enzymes
Chr. Hansen, Inc.

PART 2 – IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

2.1 IDENTITY OF THE NOTIFIED SUBSTANCE

The topic of this GRAS notice is a chymosin enzyme product, obtained by a fermentation process of a genetically modified strain of *Aspergillus niger* carrying the gene encoding a protein engineered variant of the chymosin enzyme from *Camelus dromedarius*.

Scientific data and information that identifies the notified substrate are listed below:

Classification:	Protease, Hydrolase
IUBMB nomenclature:	Chymosin
EC No.:	3.4.23.4
CAS No.:	9001-98-3
Specificity:	Clots milk by cleavage of a single Phe105-Met106 bond of κ-casein
Amino acid sequence:	The amino acid sequence has been determined

2.2 IDENTITY OF THE SOURCE

2.2(a) Production Strain

The production strain used for this enzyme is *Aspergillus niger* Tiegh (previously *A. niger* var *awamori*) deposited as DSM 32805.

The subject of this dossier is a protein engineered (PE) variant of chymosin from dromedary produced in this *A. niger* strain.

The production strain is derived from a safe host strain lineage in Chr. Hansen which has been tested for safety and used for several years in the production of chymosin from dromedary, sold commercially as CHY-MAX® M, and chymosin from bovine (CHY-MAX®). The *A. niger* strain used in the production of chymosin (subject of this dossier) and for production of the commercial product CHY-MAX® M are constructed in a similar manner.

The term “safe strain lineage” refers to related strains that have all been derived by genetic modification from a single isolate that has been thoroughly characterized and shown to be non-toxigenic and non-pathogenic before the modifications were initiated to improve enzyme yield and/or function (Pariza & Cook, 2010).

The *A. niger* production strain complies with criteria for Good Industrial Large-Scale Practice (GILSP) set forth by the Organization for Economic Co-operation and Development (OECD)[1]. It also meets the criteria for a safe production strain as described by (Pariza & Foster, 1983) and later by (Pariza & Johnson, 2001).

2.2(b) Recipient Strain

An *Aspergillus niger* Tiegh (previously *A. niger* var *awamori*) strain with reduced proteolytic activity was chosen as recipient strain. Protease deficiency was obtained by spontaneous mutagenesis.

2.2(c) Chymosin Expression Plasmid

The gene coding for the chymosin enzyme with specific and known substitutions of amino acids was introduced into an expression plasmid. This plasmid comprises all genes needed for propagation and selection of the DNA construct in *E. coli* together with the pro-chymosin gene, glucoamylase gene (with promoter and terminator), and *pyr4* from *Neurospora crassa*. After removal of unneeded backbone plasmid DNA by restriction digestion, the desired fragment was transformed into the *A. niger* recipient strain. The DNA was inserted at a single genomic locus, the *glaA* locus. No antibiotic resistance markers were inserted in the genome.

Southern blot analysis and DNA sequencing confirmed the exclusive integration of all plasmid copies into the *glaA* locus and confirmed the absence of antibiotic resistance genes.

2.2(d) Construction of the Recombinant Microorganism

The production strain was constructed using the following steps:

- Site-directed mutagenesis was performed on the chymosin gene from *Camelus dromedarius* to substitute selected amino acids in the enzyme.
- The modified chymosin gene was inserted into a plasmid containing the *glaA* expression cassette of *A. niger*.
- The plasmid was digested and the relevant part transformed into the *A. niger* recipient strain. The selected DNA fragment was incorporated into the genome because of targeted homologous recombination.

Genomic integration of the chymosin expression cassette has been targeted exclusively to the glucoamylase locus (*glaA*) on chromosome 3 of *A. niger* by homologous DNA sequences. Using a DNA vector specifically designed for this purpose, only genes of the expression cassette, i.e. *glaA* promoter and terminator (derived from the host strain) as well as the glucoamylase-chymosin

[1] Organisation for Economic Cooperation and Development, Safety Evaluation of Foods Derived by Modern Biotechnology 1993

fusion construct and the *pyrG* transformation marker (derived from the fungus *N. crassa*), are inserted into the genome. No *E. coli* plasmid DNA or antibiotic resistance genes are inserted.

The integration locus has been analyzed by Southern Blot analysis and DNA sequencing.

2.2(e) Stability of the Introduced Genetic Sequences

Genetic stability of the chymosin expression cassette inside the genome of the production strain during production has been confirmed by Southern Blot analysis. Genomic DNA extracted from the production strain at the end of the fermentation showed the same correct integration of the chymosin expression cassette within the *glaA* locus as the originally constructed and deposited biological material of the production strain. Gene transfer is therefore not expected to occur during the chymosin production process, which includes preparation of inoculation material as well as seed and main fermentation.

2.2(f) Antibiotic Resistance Gene

No antibiotic resistance gene was introduced in the production strain during the genetic modification process. Absence of antibiotic resistance genes was confirmed by DNA sequencing and Southern Blot analysis.

2.2(g) Absence of Production Microorganism in Product

The production strain is removed during downstream processing. Absence of the producer strain is part of the specification (see table 2).

2.3 MANUFACTURING PROCESS

The enzyme is produced by submerged fed-batch pure culture fermentation of the *A. niger* production strain.

The enzyme manufacturing process follows standard industry practice (Kroschwitz, 1994) (Aunstrup, et al., 1979) (Aunstrup, 1979) and complies with current Good Manufacturing Practice for Food (cGMP) and the principals of Hazard Analysis of Critical Control Points (HACCP).

The manufacturing process comprises the following unit operations:
Preparation of inoculum

- Seed fermentation
- Main fermentation
- Recovery and purification (which includes a chromatographic separation of the enzyme)
- Sterile filtration
- Formulation and packaging

A. niger is grown in liquid nutrient medium until the desired activity of chymosin is reached. The broth is then treated with acid to interrupt the fermentation and kill the production organism cells. The acid treatment step also degrades any DNA which may be present in the broth. The

broth is filtered, removing the cell material from the filtrate, which contains the chymosin. Chymosin is then recovered from the filtrate by passing it through a chromatography column. The enzyme binds to the chromatographic resin while impurities pass through the column. Chymosin is then eluted from the column with an appropriate solution. Finally, chymosin is standardized to the desired strength and sterilized by filtration.

2.3(a) Raw Materials

The chymosin is produced using standard fermentation and formulation ingredients used in the enzyme industry (Aunstrup, 1979) (Aunstrup, et al., 1979) (Kroschwitz, 1994). They are all safe and suitable for use in human food and are permitted for the intended use in addition to meeting the specifications of the Food Chemical Codex[2].

Usage of antifoams or flocculants in the production processes are in accordance with the letter submitted by Enzyme Technical Association (ETA) to FDA dated September 11th, 2003. Hence, the maximum level of antifoams/flocculants added during the fermentation and recovery processes do not exceed 1%.

2.3(b) Identity and Purity of the Producing Microorganism

A stock culture vial of the production organism (as described in 2.2a) is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms and enzyme-generating ability before use.

2.3(c) Microbiological Hygiene and Chemical Contaminants

Measures to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are:

- Hygienic design of equipment
- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the process area and equipment
    - Sterilization of all fermentation media
    - Use of sterile air for aeration of the fermenter
- Hygienic processing:
    - Aseptic transfer of the contents of the inoculation material, inoculum flask or seed fermenter
    - Maintaining a positive pressure in the fermenter
- Germ filtration

[2] United States Pharmacopeial Convention. Food Chemical Codex. Edition 9. Monograph: Enzyme Preparations. United States Pharmacopeial Convention, Board of Trustees, 2014. Pg 410-415.

2.3(d) In-process testing and monitoring

In addition to the above-mentioned control measures, in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high-quality product (GMP).

These in-process controls include:

- Microbial controls
    - Absence of significant microbial contamination is analysed by microscopy or plate counts before inoculation of both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.
- Monitoring of fermentation parameters, like
    - pH
    - Temperature
    - Dissolved oxygen content
    - CO_2
- Enzyme activity and other relevant analyses (such as dry matter, refraction index or viscosity)
    - This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

2.4 COMPOSITION AND SPECIFICATIONS

2.4(a) Quantitative Composition

The chymosin is sold in liquid stocks. Total Organic Solids (TOS) is the sum of all organic compounds present in the enzyme product after the manufacturing process. A typical composition of a sample shows the fraction of water, diluents, preservatives and stabilizers present in the enzyme product (Table 1).

The commercial products will be available in different concentrations. The typical composition of a 1000 IMCU[3]/g product is shown below.

Table 1 Typical composition for a commercial product standardized to 1000 IMCU/ml

Substrate	Typical composition
TOS*	1-2%
NaCl	11%
Na-benzoate	0.4 %
Water	86 %

*TOS = 100%-water-ash

[3] International Milk Clotting Units

2.4(b) Specifications

The chymosin conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications[4] (Table 2). In addition; it also complies with the recommended purity specification criteria for "Enzyme Preparations" as described in *Food Chemicals Codex*[5].

Table 2 Analytical data for three unstandardized enzyme concentrates

Parameter	Specification	Batch 171025F8	Batch 171114F8	Batch 171010F8
Chymosin activity	IMCU/ml	5408	5918	5237
Total viable count	< 100 CFU/ml	< 1	< 1	< 1
Lead	Not more than 5 mg/kg	<0,05	<0,05	<0,05
Salmonella sp.	Absent in 25 g of sample	ND	ND	ND
Total coliforms	Not more than 30 per gram	ND	ND	ND
Escherichia coli	Absent in 25 g of sample	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND
Ochratoxin A	No significant levels µg/kg	<0,2	<0,2	<0,2
Fumonisin B2	No significant levels µg/kg	<20	<20	<20
Producer strain	Absent in 1 ml of sample	ND	ND	ND

The Ochratoxin A and Fumonisin B2 are mycotoxins that are relevant for the production organism *A. niger*.

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Use in food and mode of Action

In nature, chymosin is produced in the stomachs of mammals for the purpose of digesting milk proteins. Specifically, the milk protein substrate for chymosin is κ-casein which is found naturally in milk-based products. In cheesemaking, chymosin is used as a processing aid. Chymosin can be extracted from mammalian stomachs, or produced on an industrial scale via fermentation.

The function of chymosin in cheese making is to clot milk by removing a highly charged peptide fragment from κ-casein on the surface of micellar casein, which constitutes most of milk protein. Destabilized casein micelles aggregate and form the structure of the milk clot that will

[4] JECFA (Joint FAO/WHO Expert Committee on Food Additives. General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of Food Additive Specifications, FAO FNP (Food and Nutrition Paper) 52, Add. 9, FAO, Rome 2001 and FAO JECFA Monographs 3 (2006).

[5] United States Pharmacopeial Convention. Food Chemical Codex. Edition 9. Monograph: Enzyme Preparations. United States Pharmacopeial Convention, Board of Trustees, 2014. Pg 410-415.

subsequently be acidified by lactic acid cultures to make cheese curd. The formed cheese curd is cut and drained for whey and further processed to cheese.

Fate of the enzyme in whey

Chymosin is a water-soluble enzyme. A large fraction of the enzyme protein is expected to be carried over into the whey when it is separated from the cheese curd. Whey can be further processed and used as an ingredient in food. In whey, proteolytic activity is highly undesirable. Residual enzymatic activity in whey protein products is highly unwanted because the chymosin could cause unintended effects during processing of foods in which the whey is used, or affect the organoleptic properties of the final foods. It is therefore in the interest of the food manufacturer that the enzymatic activity be reduced as much as possible.

Whey is heat treated to stabilize it microbiologically before further processing. This pasteurisation is normally done at high temperature for a short time (72°C-74°C for 15-20 seconds). Whey as a by-product of the manufacture of cheese has a pH of 5.9 to 6.6. It has been shown that at that pH range, over 98% of the activity is lost after pasteurisation at 72°C for 15-20 seconds at a dosage of 30-60 IMCU/L milk (Harboe, 2010).

Most, if not all, of the enzyme present in the whey should therefore be destroyed during this step.

Fate of the enzyme in cheese

A minor fraction of the enzyme will stay in the cheese curd. Many cheese types are pasteurized which may largely inactivate the enzyme.

If pasteurization is not applied or not enough to fully inactivate the enzyme, the cheese may contain residues of active enzyme. As the substrate, the casein is depleted, the chymosin can no longer function as a clotting enzyme. Any residual activity would be unspecific protease activity. It is expected that the chymosin will be digested (hydrolyzed) by the proteases released by the starter cultures and any additional ripening cultures added to the cheese. In the final cheese, the residual activity of chymosin is therefore reduced and indistinguishable from the activity of enzymes produced by the microbial cultures.

2.5(b) Use Levels

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practice.

The amount of active enzyme added to the raw material by the individual food manufacturer should be determined case by case, based on the desired effect needed and based on process conditions. A food producer who would add much higher doses than the recommended ones would experience high costs as well as negative technological consequences.

The recommended levels of use for the final chymosin product is 60 IMCU per liter of milk.

PART 3 – DIETARY EXPOSURE

As mentioned before, chymosin is used in cheese making for coagulation of milk producing cheese curd. According to the explanation given above **2.5(c)**, it could be concluded that only a small fraction of the active enzyme is present in any final food product, if at all.

However, to calculate the possible daily human exposure to the enzyme, a worst-case scenario would be to assume that all enzyme added to the process is retained and is not inactivated in the final food product. The calculations are based on the per capita/day consumption of cheese/whey products of the American population in 2016[6].

3(a) Assumptions in Dietary Exposure

Basis for calculations in the next section:

Conservative daily intake estimation can be calculated by making the following assumptions:

- All milk used to produce cheese is treated with chymosin, at its maximum dosage.
- The full amount of enzyme protein is still present in the final product, with no loss or denaturation during production.
- 10 liters of milk are needed to produce 1 kg of cheese.

3(b) Food Consumption Data

The TOS value provided can help calculating the cheese and whey consumption.

TOS (%)	Activity (IMCU/mL)	IMCU/mg TOS	mg TOS/ liter milk
1-2	1000	50-100	1.2

The typical composition provided shows that the TOS (Total Organic Solids) is 1-2%.

For the calculation, the maximum dosage of 60 IMCU per liter of milk is used. This corresponds to 0.6 - 1.2 mg TOS per liter of milk. Calculations will be made using 1.2 mg TOS/liter.

[6] Numbers are taken from the United States Department of Agriculture (USDA), Dairy products: Per capita consumption, United States (Annual), last updated 9/5/2017

Cheese and whey:

According to USDA (United States Department of Agriculture[7]) the average cheese and whey powder consumption per capita/day was 38.5g and 2.9g, respectively

The numbers are taken from the latest available (2016) overview of the dairy product per capita consumption, United States (in pounds per person) and converted from pounds per year to grams per day.

Numbers:
For dry whey and whey protein concentrate (WPC), the 2016 numbers give 2.3 pounds per person per year. This is equivalent to 1.04 kg per year, which is rounded up to 2.9 grams per day.

For cheese, the 2016 numbers are 14.3 pounds for American cheese, 22.0 for other and 2.2 for cottage. This is 38.5 pounds of cheese, or 17.46 kilos per year, equivalent to 47.8 grams per day.

To take high consumers into account, the amount of cheese consumed is calculated by multiplying the average intake by three (which is a very exaggerated assumption):

$47.8\ g/day \cdot 3 = 143.4\ g/day$
$2.9\ g/day \cdot 3 = 9.7\ g/day$

For a person weighing 60 kg, then cheese and whey intake per capita/ kg body weight/ day would therefore be:

$0.1434\ kg/day \div 60\ kg = 0.00239\ kg\ cheese/kg\ bw/day$
$0.0097\ kg/day \div 60\ kg = 0.000162\ kg\ whey/kg\ bw/day$

If 10 litres of milk are used for production of 1.0 kg of cheese, and every liter of milk has been treated with the highest amount recommended of TOS (1.2mg TOS/ liter), this will mean a daily enzyme intake of:

$0.00239\ kg\ cheese/kg\ bw/day \cdot 1.2\ mg \cdot 10\ litre\ (milk)$: <u>0.029 mg TOS/kg bw/day</u>

9 litres of whey are obtained after production of 1 kg of cheese. Therefore, in our assumption the enzyme would be concentrated in whey with a factor of 10/9:

[7] United States Department of Agriculture (USDA) Economic Research Service. Dairy Data.
Available online: https://www.ers. usda.gov/data-products/dairy-data/

1.2mg · 10/9 = 1.3 mg

Whey consists of approximately 93% water. 14.3 litres of whey are needed to produce 1.0 kg of dry whey powder, therefore:

0.000162 kg whey/kg bw/day · 14.3 liters/kg · 1.3mg/liter = <u>0.003 mg TOS/kg bw/day</u>

The sum of enzyme consumed (according to all the exaggerated assumptions mentioned above) would therefore be:

0.029 + 0.003 = 0.032 mg TOS/kg bw/day.

Margin of Safety for Dietary Intake

The safety margin is calculated as the No Observed Adverse Effect Level (NOAEL) divided by the estimated human consumption.

The NOAEL value used in this GRAS dossier is based on an earlier 13-week gavage study (Huntingdon Life Sciences study no CHH0001) conducted for the non-PE variant of the same enzyme produced in the same safe strain lineage (i.e. CHY-MAX®M). It was concluded that the oral administration of chymosin to CD rats for 13 weeks at doses of 0.967, 4.84 and 24.2 mg/kg/day did not produce any adverse treatment related findings at any dose and the NOAEL was 24.2 mg enzyme protein/kg bw/day, the highest dose tested.

The estimated human consumption is usually given in TOS/kg bw/day, which is what we have calculated in section 3(a). The enzyme protein has been measured to be approximately 10% of the TOS, therefore the maximum enzyme intake calculated in 3(a) would be 0.0032 mg enzyme protein/kg bw/day. This is relevant for the calculation of the safety margin since the NOAEL of the 90 sub-chronic study was reported on the enzyme protein and not the TOS.

Margin of Safety: 24 mg enzyme protein/kg bw/day ÷ 0.0032 mg enzyme/kg bw/day = **<u>7562.</u>**

PART 4 – SELF-LIMITING LEVELS OF USE

This part does not apply

PART 5 – COMMON USE IN FOOD BEFORE 1958

The basis for the GRAS conclusion chymosin from *Camelus dromedarius* produced by *Aspergillus niger* is based on scientific procedures and not common use in food before 1958.

PART 6 – NARRATIVE ON THE DETERMINATION OF THE GRAS STATUS OF THE PROPOSED USES OF THE CHYMOSIN PRODUCT

In conducting its determination, Chr. Hansen critically evaluated the available information on the safety of the chymosin product and on chymosin, and applied the decision tree of Pariza and Johnson (2001). The decision tree is internationally recognized by regulators and experts in food ingredient safety as the definitive tool in assessing the safety of microbially produced enzymes used in food processing.

6(a) Safety of the Production Organism

The safety of the production strain should be the primary consideration in the safety evaluation of an enzyme as concluded by (Pariza & Foster, 1983) and later by Pariza & Johnson (2001), Olempska-Beer, at al (2006) and Pariza & Cook (2010).

The history of safe use for *A. niger* is primarily based on its use since the 1960s in the food industry to produce a large number of food enzymes (Schuster, et al., 2002). These food enzymes, including those derived from recombinant *A. niger* strains, were evaluated by JECFA and by countries which regulate the use of food enzymes including the USA, France, Denmark, Australia, and Canada, resulting in the approval of the use of food enzymes from *A. niger* in the production of various foods and dairy products (Harboe, 2010). In addition to chymosin, *A. niger* is used in the industry as a production organism for a variety of enzymes used in food processing including carbohydrates, proteases, phosphatases and lipases (Pariza & Johnson, 2001).

According to Pariza & Foster (1983), a non-toxigenic organism is "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a non-pathogenic organism is "one that is very unlikely to produce disease under ordinary circumstances". It is safe to consume food ingredients derived from nontoxigenic and nonpathogenic organisms when these food ingredients are produced consistent with current Good Manufacturing Practices (cGMP)[8].

A. niger is generally regarded as a nonpathogenic fungus widely distributed in nature. Humans are exposed to its spores every day without suffering any apparent adverse health effects. *A. niger* is ubiquitous in soil and is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles and other decaying vegetation. Consequently, it is commonly present as a contaminant in foods, such as rice, seeds, nuts, olives and dried fruits (Sharma, 2012).

[8] IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. 1990, Vol. 12, pp. S1-S196.)

A. *niger* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC[9] on the protection of workers from risks related to exposure to biological agents at work, as it is globally regarded as a safe microorganism.
In the USA, *Aspergillus niger* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules (USA, 1989). Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *A. niger* for human and animal consumption demonstrate that the enzymes are nontoxic. The US Environmental Protection Agency (EPA) has exempted *A. niger* from review by the Agency, due to its extensive history of safe use (EPA, 1997).
In Europe, *A. niger* is classified as a low-risk-class microorganism, as exemplified in the listing as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA) (BAuA, 2010) and the Federal Office of Consumer Protection and Food Safety (BVL) (BVL, 2013). It is not mentioned on the list of pathogens in Belgium (Belgium, 2010).

6(b) Safety of the Donor Organism

The donor for the chymosin gene is a mammal, the dromedary (*Camelus dromedarius*).

6(c) Safety of the Chymosin Enzyme

The safety of milk coagulants, including chymosin, is well established. They have been used for centuries in the production of cheese (Harboe, 2010).

The chymosin subject of this GRAS notification is a protein engineered (PE) variant of the chymosin from *Camelus dromedarius* – sold by Chr. Hansen as CHY-MAX® M.

The variant was generated by introducing specific amino acid substitutions into the protein using site-directed mutagenesis. This slight change in the amino acid sequence contributes to improved function and activity. The PE modification does not affect the strain itself nor does it alter the toxigenic potential of the enzyme product (see 6d).

The parent enzyme (CHY-MAX® M) has been safely used as processing aid for cheese manufacture in the last decade and is approved for the intended application in several countries; e.g. Australia/New Zealand[10], Canada[11], Denmark[12], and France. [13].

As mentioned in 2.2(a), the variant is expressed in the same safe *A. niger* strain lineage as used for the dromedary chymosin (CHY-MAX® M) and the bovine chymosin (CHY-MAX®) and fulfils the

[9] Annex III of Directive 2000/54/EC, https://eur-lex.europa.eu/homepage.html
[10] Standard 1.3.3 processing aids
[11] Interim market authorization published on August the 19th, 2010
[12] The approval is company-specific and not publicly available.
[13] Arrêté du 19 Octobre 2006

criteria of a safe strain lineage in accordance with the decision tree published by Pariza & Johnson (2001), see PART 7.

6(d) Allergenic/Toxigenic Potential of the Chymosin Enzyme (Decision Tree Analysis)

This Chymosin enzyme from *Camelus dromedarius* Produced in *Aspergillus niger* was evaluated according to the decision tree published in (Pariza & Johnson, 2001).
The evaluation is presented below.

Decision Tree Analysis:

1. Is the production strain genetically modified?
   YES
   If yes, go to 2.

2. Is the production strain modified using rDNA techniques?
   YES
   If yes, go to 3.

3. Issues relating to the introduced DNA are addressed in 3a-3e.

   3a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
   YES, *go to 3c*

   3c. Is the test article free of transferable antibiotic resistance gene DNA?
   YES, *go to 3e*

   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 products?
   YES, *go to 4*

4. Is the introduced DNA randomly integrated into the chromosome?
   NO, *go to 6*

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?
   YES
   The test article is ACCEPTED.

According to Pariza & Cook (2010), a safe strain lineage is a lineage of strains that have all been derived by genetic modification from a single isolate that was thoroughly characterized and shown to be non-toxigenic and non-pathogenic before the modifications to improve enzyme function were initiated. The genetic improvement could be to increase enzyme yield as is the case with the enzyme in this notification. The method (site-directed mutagenesis) used for constructing our production strain was also mentioned in the paper as a classical approach for amino acid substitutions (Pariza & Cook, 2010).

The safety of protein engineered enzymes has been reported (Pariza & Johnson, 2001 and Pariza & Cook, 2010), based on the findings that evolving natural variation within enzyme families is bigger than variation in two identical proteins with some amino acid changes. It has been shown that enzymes (from same protein family) obtained from microorganisms found in diverse habitats retain significant sequence similarity, enzymatic activity and similar tertiary structure (Conrad, et al., 1995) (Siezen & Leunissen, 1997) (Janecek, et al., 1999) (Todd, et al., 1999) but could differ in certain functional characteristics such as stability and substrate specificity (Pariza & Johnson, 2001).

According to Pariza & Johnson (2001) there has been "no instance in which such natural variation within enzyme families has resulted in the generation of a toxin active via the oral route. This also follows from the observation that toxicity is an unusual property among proteins". They add, "Extensive studies on engineered enzymes have also demonstrated that enzymes within families/super-families (e.g., subtilases) that are altered by these techniques still retain their characteristic three-dimensional structure and catalytic activities" (Bott, et al., 1992). Hence, engineered enzymes exhibit variation that is like that observed in nature. An examination of enzyme structure and function indicates that it is unlikely that changes which improve upon desired enzyme function will result in the creation of a toxic protein (Pariza & Johnson, 2001).

A PSI-BLAST (Position-Specific Iterated Basic Local Alignment Search Tool) search was performed on the NCBI (National Center for Biotechnology Information) web page[14] to find the closest sequence homologs of bovine and camel chymosin. The search revealed that sequence identities of mammalian chymosin can be as low as 70%. Bovine chymosin, the classical coagulant for industrial cheese manufacture, shows sequence identity to dromedary chymosin of 85% (proenzymes). Both enzymes reveal very similar protein folds and catalytic activities. The proenzymes of the chymosin subject of this dossier and its parent have 97.2% identical sequences. The engineered variant is therefore more like dromedary chymosin than most of the structurally homologous chymosin variants found in other organisms.

A sequence homology search to known toxins and allergens was performed using the protein sequence of the chymosin proenzyme (designated cy593).

[14] Ref. web page: https://blast.ncbi.nlm.nih.gov/Blast.cgi; Ref. scientific publication: Nucleic Acids Res. 2017, 45 (D1): D12-D17

Toxigenic Potential of Chymosin:

Public protein sequence databases[15] were screened for toxic sequence homologs to cy593. A PSI-BLAST search was performed with the *cy593* coding the proenzyme against all proteins in non-redundant databases that contain the word "toxin" in their descriptions. Highest similarities found were 35% and 22% identity covering 13% and 41% of the protein sequence, respectively. The query hits are non-similar and non-homologous to cy593.

Allergenic Potential of Chymosin:

The sequence of the chymosin proenzyme was queried against the "Allergen Online" database[16]. The summary of this safety evaluation is shown below.

Full sequence alignment: The allergen showing highest similarity to cy593 was aspartyl endopeptidase from *Rhizopus oryzae* with a total sequence identity of 37.9% (far below the threshold of 50%, the threshold defined by the database). The same protein was identified as most similar allergen to dromedary (CHY-MAX® M) and bovine chymosin (CHY-MAX®) with very similar identities of 38.2 and 37.5%, respectively.

Identity of consecutive 8-aa stretches: The proenzyme cy593 shared 10 identical amino acid sequences with the above-mentioned *R. oryzae* aspartyl endopeptidase (both enzymes reveal three overlapping identical stretches of 8-aa). This exact stretch of 10 amino acids is found in wild-type dromedary chymosin (proenzyme), which has a long history of being a safe commercial cheese coagulant (CHY-MAX® M).

Identity of consecutive 80-aa stretches: The cy593 proenzyme sequence contains regions of similarity with the aspartyl endopeptidase from *R. oryzae*. The highest 80-aa stretch identity was found to be 56.3%, while exceeding the 35% threshold. Similar sequence homologies were obtained for bovine and dromedary chymosin proenzymes. No food allergenic reactions to chymosin enzymes (sold as CHY-MAX® and CHY-MAX® M) have been reported, so there is no evidence for potential allergenicity of this food enzyme.

Since aspartyl endopeptidase from *R. oryzae* is described to be an aeroallergen (Sicar G., 2012), consumption of proteins containing similar sequences in cheese is unlikely to cause an allergenic response. Further similarities of 80-mer windows of the cy593 sequence with potential pathogens exceed the 35% identity threshold only slightly (aspartic acid precursor and Bla g2 from *Blattella germanica* with 36.2% identity each). Similar results were obtained with the natural dromedary chymosin, where the protein had 40% sequence homology with Bla g2. However, the homology was spread evenly over the sequence, meaning that no allergenic 'hotspots' could be identified.

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[15] Ref. web page: https://blast.ncbi.nlm.nih.gov/Blast.cgi; Ref. scientific publication: Nucleic Acids Res. 2017, 45 (D1): D12-D17

[16] Ref. web page: http://www.allergenonline.org/; Ref. scientific publication: references cited on http://www.allergenonline.org/

Based on the information and data provided in this subsection, it is concluded that oral intake of Chr. Hansen's chymosin product does not pose any toxigenic or allergenic concerns.

6(e) Safety of the Manufacturing Process

The manufacturing process of chymosin is in consistent with current Good Manufacturing Practice for Food (GMP) and the principals of Hazard Analysis of Critical Control Points (HACCP). The production is carried on a site with compliance with ISO 9001 and ISO 22000 standards. Compliance with Food Hygiene Regulation is regularly controlled by relevant food inspection services.

The food enzyme product is tested by Quality Control for all quality related aspects, such as expected enzyme activity and the general JECFA Specifications for Food Enzyme Preparations, before released by Quality Assurance.

6(f) Safety Studies

The following toxicological studies were performed on an unstandardized enzyme concentrate, batch 171010F8, representative of the final product.

- Bacterial revers mutation test (Ames test)
- *In vitro* micronucleus assay
- Fourteen Day Repeated Dose Oral (Gavage) Range-Finding Toxicity Study in the Rat

Bacterial reverse mutation test

Envigo study no KS68RH

To investigate the potential of chymosin product batch 171010F8 to induce gene mutations, an AMES test was performed according to the OECD Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP). Four strains of Salmonella Typhimurium (TA1535, TA1537, TA98 and TA100,) as well as E. coli WP2 *uvr*A (pKM101) were tested in the presence and absence of metabolic activation (S9-mix). The mutation test used a treat-and-wash method performed in the presence and absence of liver preparations (S9 mix) from rats treated with phenobarbital and 5,6-benzoflavone. Concentrations of chymosin batch 171010F8 (reported in terms of Total Organic Solids (TOS)) were tested up to 5000 µg TOS /plate, in line with recommendations from applicable regulatory guidelines on standard limit concentrations. Other concentrations used were a series of *ca* half-$\log_{10}$ dilutions of the highest concentration.

Two experiments were performed and showed:

- In the first experiment, no signs of toxicity towards the tester strains were reported following exposure to chymosin. Results obtained with strain TA100 in the absence of S9 mix, and strains TA1535 and TA1537 in the absence and presence of S9 mix were from a

repeat test due to positive controls not producing sufficient increases in revertant colony numbers in the original test. A maximum exposure concentration of 5000 µg TOS/plate was selected for use in the second test. No substantial increases in revertant colony numbers over control counts were reported with any of the tester strains following exposure to chymosin at any concentration up to and including 5000 µg TOS / plate in either the presence or absence of S9 mix.

- In the second test, no signs of toxicity towards the tester strains were reported following exposure to chymosin. Results obtained with strains TA1537 and WP2 uvrA (pKM101) in the absence and presence of S9 mix were from a repeat test due to positive controls not producing sufficient increases in revertant colony numbers in the original test. No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to chymosin at any concentration up to and including 5000 µg TOS / plate in either the presence or absence of S9 mix.

It was concluded that chymosin product batch 171010F8 showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

***In vitro* micronucleus assay**

Envigo study number DC45YP

The *in vitro* study was designed to assess the potential of chymosin product batch 171010F8 to cause an increase in the induction of micronuclei in cultured human peripheral blood lymphocytes. The study consisted of a preliminary toxicity test and a main micronucleus test. Human lymphocytes in whole blood culture were exposed to chymosin enzyme for 3 hours in both the absence and presence of exogenous metabolic activation (S9 mix) and for 20 hours in the absence of S9 mix. The maximum final concentration to which the cells were exposed was 5000 µg total organic solids (TOS)/mL, dosed at 10% v/v, in order to test up to the maximum concentration as recommended in the current European Food Safety Authority Guidance (2014). Vehicle (water) and positive control cultures were included in all appropriate test conditions.

Three chymosin concentrations, 1250, 2500 and 5000 µg TOS/mL, were assessed for the induction of micronuclei. The following results were reported.

- Following the 3-hour treatment in the absence of S9 mix, chymosin caused no significant reductions in the cytokinesis-block proliferative index (CBPI) at any concentration tested. Concentrations of chymosin selected for micronucleus analysis were 1250, 2500 and 5000 µg TOS/mL. Chymosin caused no statistically significant increases in the number of binucleate cells containing micronuclei and there was no evidence of a linear dose-concentration relationship. The mean micronucleus frequencies for the vehicle and test item treated cultures were within the laboratory historical 95% confidence limits.
- Following the 3-hour treatment in the presence of S9 mix, chymosin caused no reductions in the CBPI at any concentration tested. Concentrations of chymosin selected for

micronucleus analysis were 1250, 2500 and 5000 µg TOS/mL. Chymosin caused no statistically significant increases in the number of binucleate cells containing micronuclei and there was no evidence of a linear dose-concentration relationship. The mean micronucleus frequencies for the vehicle and test item treated cultures were within the laboratory historical 95% confidence limits.

- Following the 20-hour exposure in the absence of S9 mix, a reduction in the CBPI equivalent to 34.9% cytotoxicity was obtained with chymosin at 5000 µg TOS/mL. Concentrations of chymosin selected for micronucleus analysis were 625, 2500 and 5000 µg TOS/mL. Chymosin caused no statistically significant increases in the number of binucleate cells containing micronuclei and there was no evidence of a linear dose-concentration relationship. The mean micronucleus frequencies for the vehicle and test item treated cultures were within the laboratory historical 95% confidence limits.
- The positive control compounds (mitomycin C, colchicine and cyclophosphamide) caused statistically significant increases in the number of binucleate cells containing micronuclei under appropriate conditions, demonstrating the efficacy of the S9 mix and the sensitivity of the test system.

It was concluded that chymosin batch 171010F8 did not show any evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes, in this *in vitro* test system under the experimental conditions described.

Fourteen Day Repeated Dose Oral (Gavage) Range-Finding Toxicity Study in the Rat
Envigo study no VV93HH

The test item, chymosin batch 171010F8, was administered by gavage to three groups, each of three male and three female Wistar Han™:RccHan™:WIST strain young adult rats, for fourteen consecutive days, at doses of 250, 500 and 1000 mg TOS/kg bw/day. The top dose chosen is the highest dose that was technically achievable, i.e. the limit dose. The test item was provided by the Sponsor with a defined concentration. Doses were adjusted by using a different dose volume, as necessary, for each dose group. The total volume administered considered the Total Organic Solids (TOS) of the sample (6.8 % (w/w)) in order to adjust for water content of the test item. A control group of three males and three females was dosed with vehicle alone (Chlorine Free Distilled Water). Clinical signs, body weight change, dietary intake and water consumption were monitored during the study. All animals were subjected to gross necropsy examination.

No treatment-related deaths or effects on clinical signs, changes in body weight, body weight gain, food consumption and macroscopic or microscopic pathology were reported.

It was concluded that treatment up to 1000 mg TOS /kg bw/day was well tolerated and was not associated with any adverse effects and is the NOAEL.

6(g) Summary and Conclusion

Based on its critical evaluation of the data and information summarized in this GRAS Notification, Chr. Hansen concludes that the intended uses of its chymosin product are GRAS based on scientific procedures. GRAS status is corroborated by the long safe history of *A. niger* as a safe production organism and the long safe history of commercially produced chymosin.

PART 7 – SUPPORTING DATA AND INFORMATION

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The Report of an Expert Panel on the GRAS status of Chr. Hansen's Chymosin Enzyme from *Camelus dromedarius* Produced by *Aspergillus niger* for Use as a Processing Aid in Cheese Production

We, the undersigned members of the Expert Panel, are qualified by scientific education and experience to evaluate the use of enzymes as food ingredients. We individually and collectively critically evaluated the materials summarized in the attached monograph entitled, "Chymosin enzyme from *Camelus dromedarius* Produced in *Aspergillus niger*," prepared by Katharine Urbain, Regional Regulatory Affairs Manager North America – Compliance, and Emily Gregoire, Regulatory Affairs Specialist, Chr. Hansen, Inc. and other materials deemed appropriate. We then discussed our findings during a teleconference,and reached a unanimous conclusion.

In evaluating Chr. Hansen's chymosin enzyme from *Camelus dromedarius* produced by *Aspergillus niger* for use a processing aid in cheese production, we considered the biology of *A. niger*, relevant information available in the peer-reviewed scientific literature, and information that Chr. Hansen provided in its monograph on the production organism, *A. niger* Tiegh (previously *A. niger* var *awamori*) deposited as DSM 32805.

Aspergillus niger is a mold that is commonly found in soil and on plants. It is an opportunistic pathogen that only rarely infects humans, typically those with compromised immune systems. The species does not possess the genetic elements required to produce aflatoxin, but some strains produce ochratoxin and the genome of one strain of *A. niger* contains a gene cluster that encodes for fumonisin (HJ Pel et al., Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nature Biotechnology 25 (2) 221-231, 2007). Nontoxigenic strains of *A. niger* are widely utilized by food ingredient manufacturers for numerous applications including the production of enzyme preparations for use in human food and animal feed.

The production strain, *A. niger* Tiegh (previously *A. niger* var *awamori*) deposited as DSM 32805, was derived from a safe strain lineage that Chr. Hansen has tested for safety and used for several years to produce chymosin from dromedary (sold commercially as CHY-MAX® M) and chymosin from bovine (sold commercially as CHY-MAX®). Under test conditions, the production and parental strains did not produce mycotoxins, and the chymosin specifications include routine testing for Ochratoxin A and Fumonisin B2.

The chymosin product produced by *A. niger* Tiegh is a variant of native dromedary chymosin which is sold by Chr. Hansen as CHY-MAX® M. The variant gene was produced by modifying the native dromedary gene using site-directed mutagenesis, resulting in improvements in enzyme

Conclusion

We conclude that Chr. Hansen's *Aspergillus niger* Tiegh, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is safe to use in the production of food grade chymosin.

We further conclude that Chr. Hansen's chymosin enzyme from *Camelus dromedarius* produced by *Aspergillus niger* Tiegh, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is *GRAS* (Generally Recognized As Safe) based on scientific procedures for use as a processing aid in cheese production.

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

Signature: (b) (6) Date: 11 July 2018

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