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Paulette M. Gaynor, Ph.D.
Office of Food Additive Safety (HFS-200),
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

December 20, 2018

Re: GRAS Notice for acid prolyl endopeptidase produced by a genetically engineered strain of *Aspergillus niger*

Dear Dr. Gaynor:

On behalf of DSM Food Specialties (DSM), I am submitting under cover of this letter one paper copy and one digital copy of a generally recognized as safe (GRAS) notice for an acid prolyl endopeptidase enzyme produced by a pure culture of a strain of *Aspergillus niger* (GEP) genetically engineered for more efficient expression of the native gene encoding this enzyme.

The electronic copy is provided on a virus-free CD and is an exact copy of the paper submission.

DSM has determined through scientific procedures that this substance is GRAS when used in the production of beer and other fermented beverages. As such, the notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

If you have any questions regarding this notification, or require any additional information to aid in
the review of DSM' s conclusion, please do not hesitate to contact me.

Sincerely,

[redacted]

Katherine Vega, Ph.D.
Sr. Manager Regulatory Affairs

Summary of Information Supporting the Generally Recognized as Safe (GRAS) Status of Acid Prolyl Endopeptidase Produced by a Genetically Engineered Strain of *Aspergillus niger*

Prepared by: DSM Food Specialties B.V.
Alexander Fleminglaan 1
2613 AX Delft
Netherlands

December 20, 2018

Summary of Information Supporting the Generally Recognized as Safe (GRAS) Status of Acid Prolyl Endopeptidase Produced by a Genetically Engineered Strain of *Aspergillus niger*

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Part 1: Signed Statements and Certification

1.1 Submission of GRAS Notice

DSM Food Specialties (DSM) is hereby submitting a Generally Recognized as Safe (GRAS) notice in accordance with the provisions of 21 CFR part 170, subpart E.

1.2 Name and Address of Notifier

DSM Food Specialties B.V.
P.O. Box 1
2600 MA Delft
Netherlands

1.3 Name of the Substance

The notified substance consists of acid prolyl endopeptidase produced by a strain of *Aspergillus niger* (GEP) genetically engineered to overexpress *Aspergillus niger* acid prolyl endopeptidase.

1.4 Intended Conditions of Use

DSM's acid prolyl endopeptidase produced by *Aspergillus niger* GEP is marketed as an enzyme preparation for use in the production of beer and other fermented beverages. The enzyme is expected to be used at levels that do not exceed the amounts reasonably required to accomplish its intended effect in foods and in accordance with current Good Manufacturing Practices (cGMP).

1.5 Statutory Basis for the GRAS Conclusion

This GRAS conclusion is based upon scientific procedures in accordance with § 170.30(a) and (b).

1.6 Exclusion from Premarket Approval Requirements

The notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, based on the conclusion by DSM that the substance is GRAS under the conditions of its intended use.

1.7 Availability of Information

The complete data and information that are the basis of the GRAS conclusion will be made available to FDA. Upon request, DSM will provide access to review and copy the data during customary business hours at its facility in Parsippany, New Jersey, or, upon request, will provide copies in electronic format or on paper.

1.8 Freedom of Information Act (FOIA) Exemptions

Parts 2 through 7 of this notification do not contain data or information that are exempt from disclosure under the Freedom of Information Act.

1.9 Certification

To the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to DSM and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

Katherine Vega, PhD
Senior Manager, Regulatory Affairs

Part 2: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect of the Notified Substance

2.1 Identity of the Substance

Acid prolyl endopeptidase enzyme is produced by submerged fed-batch fermentation using a selected, pure culture of a (self-cloned) strain of *Aspergillus niger* (GEP) genetically engineered for more efficient expression of the gene encoding acid prolyl endopeptidase from *Aspergillus niger*. Additional details are provided below.

Table 2-1 Classification of the enzyme protein

Name:	Acid prolyl endopeptidase[1]
Synonyms:	Prolyl endoprotease; Proline-specific endoprotease; Proline endopeptidase; Endoprotease; Endo-protease; Protease; Brewers Clarex; Maxipro; AN-PEP; PEP2COMFORT; Tolerase G
IUBMB Enzyme Commission (EC) number[2]:	The classification of the enzyme according to the IUBMB is as follows: EC 3 Hydrolyases EC 3.4 Acting on peptide bonds (peptidase) EC 3.4.21 Serine endopeptidases EC 3.4.21.xx Acid prolyl endopeptidase
Enzyme function:	Hydrolysis of proteins and peptides in an endo-fashion, with a preference for cleavage of peptide bonds at the carboxyl site of proline residues and to a lesser extent alanine residues
Enzyme substrates:	Proteins and peptides
Source organism:	*Aspergillus niger* expressing multiple copies of the *Aspergillus niger* acid prolyl endopeptidase gene. The strain is referred to as *Aspergillus niger* GEP. For additional details about its development, see section 2.2.1.2.
Amino acid sequence: The peptide chain of acid prolyl endopeptidase from *Aspergillus niger* contains 526 amino acids, including a signal sequence of 22 (highlighted in yellow) and a pro-sequence of 19 (highlighted in blue) amino acids, which are cleaved off during secretion of the enzyme.	MRAFSAVAAAALALSWASLAQAARPRLVPKPVSRPASSKSAATTGEA YFEQLLDHHNPEKGTFSQRYWWSTEYWGGPGSPVVLFTPGEVSAD GYEGYLTNETLTGVYAQEIQGAVILIEHRYWGDSSPYEVLNAETLQYL TLDQAILDMTYFAETVKLQFDNSTRSNAQNAPWVMVGGSYSGALTA WTESVAPGTFWAYHATSAPVEAIYDYWQYFYPIQQGMAQNCSKDVS LVAEYVDKIGKNGTAKEQQALKELFGLGAVEHFDDFAAVLPNGPYLW QDNDFATGYSSFFQFCDAVEGVEAGAAVTPGPEGVGLEKALANYAN WFNSTILPDYCASYGYWTDEWSVACFDSYNASSPIYTDTSVGNAVDR QWEWFLCNEPFFYWQDGAPEGTSTIVPRLVSASYWQRQCPLYFPET NGYTYGSAKGKNAATVNSWTGGWDMTRNTTRLIWTNGQYDPWRDS GVSSTFRPGGPLASTANEPVQIIPGGFHCSDLYMADYYANEGVKKVV DNEVKQIKEWVEEYYA
Molecular mass	56 kDa (based on amino acid sequence without the highlighted sequences); about 66 kDa by SDS PAGE, due to glycosylation of the protein (as noted by Sebela *et al*., 2009).

[1] Name used in the *MEROPS* database (https://www.ebi.ac.uk/merops/cgi-bin/pepsum?id=S28.004;type=P) for peptidase S28.004: acid prolyl endopeptidase (*Aspergillus* sp.), also referred to as AN-PEP.

[2] Due to the fact that the name proline endopeptidase is also a synonym of prolyl oligopeptidase, EC (IUBMB) number 3.4.21.26, the enzyme protein described in this dossier has been classified as such in

various documents, including some premarket submissions to other countries. However, in contrast to oligopeptidases, the enzyme also acts on proteins (Edens *et al*., 2005; Kubota *et al*., 2005; Takahashi, 2013).

Acid prolyl endopeptidase is likely to belong to the so-called MEROPS S28 family of clan SC of serine proteases (Edens *et al.*, 2005; Kubota *et al*., 2005; Takahashi, 2013). Enzymes from this family have been identified in many sources, including plants, microorganisms, and animals, where they play a role in the protein processing pathway. These sources include plants such as soybeans, barley, rice, and corn (see Merops, 2014). Therefore, this enzyme occurs naturally in various organisms that are part of the human diet.

As a substance derived through fermentation, acid prolyl endopeptidase is a biological isolate containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process, which can vary.

2.1.1 Enzyme Activity

The acid prolyl endopeptidase described in this dossier hydrolyzes proteins in an endo-fashion, with a preference for cleavage of peptide bonds at the carboxyl side of proline residues and to a lesser extent alanine residues. Details on the enzyme activity have been described in the published literature (Edens *et al.*, 2005; Kubota *et al*., 2005; Takahashi, 2013).

DSM developed its own method to measure acid prolyl endopeptidase in order to standardize the activity in the final enzyme preparation. The method is described in Appendix 1. The enzyme activity described in this method is expressed in so-called Prolyl Peptidase Units (PPU/g). One PPU is defined as the quantity of enzyme that will liberate p-nitroanilide at a rate of 1 micromole per minute under the conditions of the assay[1].

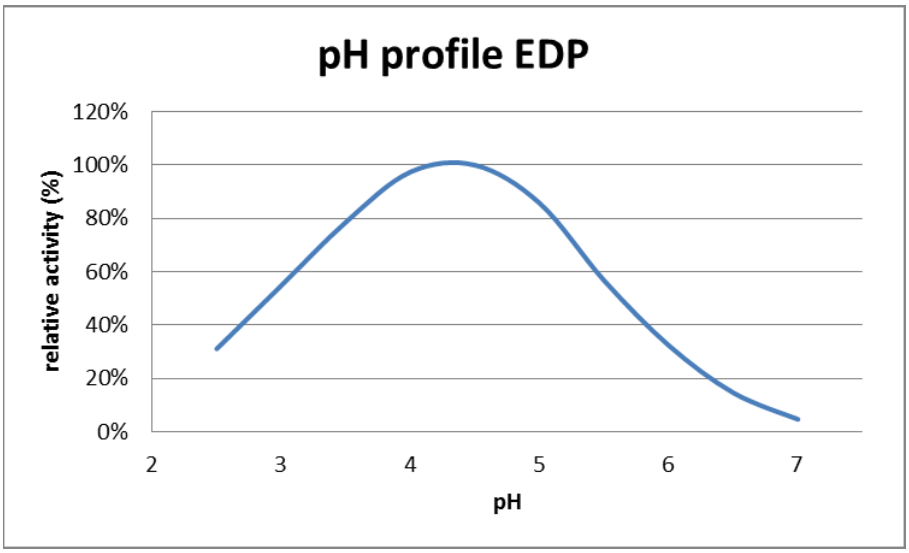
2.1.2 Activity under various conditions

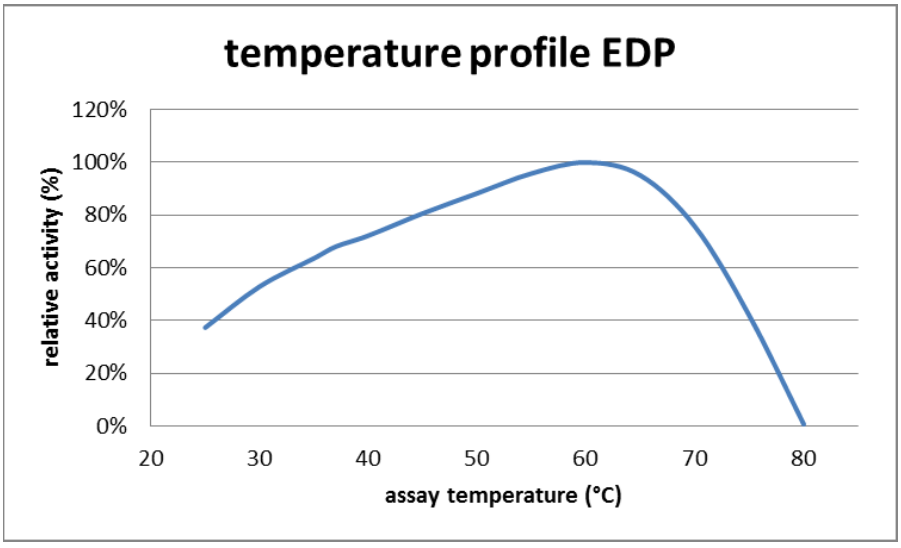
The activity of the acid prolyl endopeptidase produced by *Aspergillus niger* GEP was measured under various pH and temperature conditions, using the method described in Appendix 1 (4-minute incubation period).

[1] DSM's acid prolyl endopeptidase enzyme is also marketed as an ingredient for use in dietary supplements (see section 6.4.1), for which the enzyme activity is represented in slightly different units, Protease Picomol International (PPI) units. The principle of the assay to determine the activity of the enzyme underlying PPU and PPI is identical. The conversion factor between the units is: 1 PPU = 16,667 PPI (micromoles/minute converted to picomoles/second: 1,000,000/60 = 16,667).

As Figure 2-1 illustrates, the enzyme exhibited optimal activity between pH 4 and 5, and at a temperature of about 60 °C. Activity declined at temperatures higher than 60 °C, suggesting some denaturation of the enzyme protein. The absence of activity at temperatures above 80 °C, indicates complete denaturation of the enzyme.

Figure 2-1 Enzyme activity as a function of pH and temperature





2.2 Manufacturing

2.2.1 Production Organism

The strain used to produce the acid prolyl endopeptidase enzyme, *Aspergillus niger* GEP, was genetically engineered by DSM for more efficient expression of the *Aspergillus niger* gene encoding the enzyme protein. Integration of multiple copies of the acid prolyl endopeptidase expression cassette resulted in a strain that produces higher concentrations of acid prolyl endopeptidase enzyme.

The producing strain was taxonomically identified as *Aspergillus niger* by the internationally recognized CBS laboratory in Utrecht, The Netherlands (see Appendix 2)

2.2.1.1 Host and donor organism

Aspergillus niger represents both the host and donor organism, since the genes introduced encode the native acid prolyl endopeptidase.

Aspergillus niger is a fungus that produces large black or brown conidia by phialids [a bottle-shaped structure within or from which conidia (conidiospores) are formed]. The fungus is a saprophyte able to grow on a wide variety of complex substrates. It 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 also known to naturally occur in foods such as rice, seeds, nuts, olives, and dried fruits.

The formal classification of *Aspergillus niger* strain GEP is as follows:

Kingdom:	FUNGI
Division:	EUMYCOTA
Subdivision:	DEUTEROMYCOTINA
Class:	HYPHOMYCETES
Order:	Moniliales
Family:	Moniliaceae
Genus:	*Aspergillus*
Sub-genus:	Circumdati
Section:	Nigri (= *Aspergillus niger* group)
Species:	*Aspergillus niger*
Strain:	GEP

For several decades, *Aspergillus niger* has been safely used in the commercial production of food substances. For example, industrial production of citric acid by *Aspergillus niger* has taken

place since 1919 (Schuster *et al*., 2002). In the U.S. citric acid recovered from *Aspergillus niger* fermentation liquor is GRAS-affirmed under 21 CFR 184.1033.

Since the 1960s, *Aspergillus niger* has also been widely used in the food industry to produce many food enzymes (Bennett, 1985a, 1985b; Schuster *et al.*, 2002). These food enzymes, including those derived from recombinant *Aspergillus niger* strains, have been evaluated by JECFA and many countries that regulate the use of food enzymes, such as the U.S., France, Denmark, Australia, and Canada.

Proteases from *A. niger* are listed in the Adjunct Reference Manual (Beer Institute, 1998) as substances that may be employed in beer production.

Examples of enzymes derived from *Aspergillus niger* in the U.S. food regulations include carbohydrase and cellulase for use in clam and shrimp processing (21 CFR 173.120) and chymosin preparations to coagulate milk in cheeses and other dairy products (21 CFR 184.1685). As Table 2-2 illustrates, there are also several notices in the GRAS Notice Inventory for substances derived through use of classical *Aspergillus niger* strains (*e.g*., GRN Nos. 89, 111, 132, 750), along with others where *Aspergillus niger* was used as the host and/or donor organism in the development of genetically engineered strains. The U.S. FDA indicated it had no questions about the GRAS conclusion in these notices.

Table 2-2 Summary of GRAS notices submitted to and filed by U.S. FDA for substance derived through the use of *Aspergillus niger*

GRN No.	Substance	Date of closure	FDA Response
801	Chymosin enzyme from *Camelius dromedarius* produced in *Aspergillus niger*		Pending
783	*Triacylglycerol lipase* from *Rhizopus oryzae* produced in *Aspergillus niger*		Pending
750	Beta-glucosidase from *Aspergillus niger*		Pending
739	Mannanase enzyme from *Talaromyces leycettanus* produced in *Aspergillus niger*		Pending
703	Alpha-glucosidase from *Aspergillus niger* produced by *Trichoderma reesi*	Nov 9, 2017	FDA has no questions
699	Trehalase from *Myceliophthora sepedonium* produced by *Aspergillus niger*	Nov 13, 2017	FDA has no questions
657	Glucoamylase from *Penicillum oxalicum* produced in *Aspergillus niger*	Nov 23, 2016	FDA has no questions
651	Phospholipase A1 from *Talaromyces leycettanus* produced in *Aspergillus niger*	Nov 23, 2016	FDA has no questions
589	Xylanase from *Aspergillus niger* [carrying a endo-1,4-β-xylanase gene synthesized in vitro from a cDNA coding sequence obtained from *Rasamsonia emersonii*]	Sep 17, 2015	FDA has no questions
510	Acid lactase from *Aspergillus oryzae* expressed in *Aspergillus niger*	Sep 29, 2014	FDA has no questions

GRN No.	Substance	Date of closure	FDA Response
428	Asparaginase enzyme preparation from genetically modified *Aspergillus niger*	Nov 26, 2012	FDA has no questions
412	Chitin-glucan from *Aspergillus niger*	Jun 18, 2012	FDA has no questions
402	Peroxidase enzyme preparation derived from a genetically modified strain of *Aspergillus niger*	Nov 23, 2012	FDA has no questions
397	Chitosan from *Aspergillus niger*	Dec 19, 2011	FDA has no questions
345	Caboxypeptidase enzyme preparation from modified *Aspergillus niger*	Dec 22, 2010	FDA has no questions
315	Transglucosidase enzyme preparation from *Trichoderma reesei* expressing the gene encoding transglucosidase from *Aspergillus niger*	May 5, 2010	FDA has no questions
296	Lipase enzyme preparation from a genetically modified strain of *Aspergillus niger*	Oct 1, 2009	FDA has no questions
214	Asparaginase enzyme preparation from *Aspergillus niger* expressing the asparaginase gene from *Aspergillus niger*	Mar 13, 2007	FDA has no questions
183	Phospholipase A2 enzyme preparation from *Aspergillus niger* expressing a gene encoding a porcine phospholipase A2	May 11, 2006	FDA has no questions
158	Lipase preparation from *Aspergillus niger* expressing a gene encoding a lipase from *Candida antartica*	Mar 16, 2005	FDA has no questions
132	Lactase enzyme preparation from *Aspergillus niger*	Dec 12, 2003	FDA has no questions
111	Lipase enzyme preparation from *Aspergillus niger*	Dec 20, 2002	FDA has no questions
106	Glucose oxidase enzyme preparation from Aspergillus oryzae carrying a gene encoding a glucose oxidase from *Aspergillus niger*	Oct 3, 2002	FDA has no questions
89	Five enzyme preparations from *Aspergillus niger*: Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation	Apr 3, 2002	FDA has no questions (additional correspondence available)
32	Pectin lyase derived from *Trichoderma reesei* carrying a gene encoding pectin lyase from *Aspergillus niger*	Apr 20, 2000	FDA has no questions
Page Last Updated: 10/18/2018 Accessed online in November 2018 through: https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices			

The long experience of industrial use has resulted in extensive knowledge of the characteristics of *Aspergillus niger* and an understanding of its metabolic reactions. The nonpathogenic nature of the organism has been confirmed by several experimental studies (Schuster *et al*., 2002). *Aspergillus niger* is therefore generally accepted as a nonpathogenic organism, as supported by the following:

- *Aspergillus niger* can be used under the lowest containment level at Good Industrial Large Scale Practice (GILSP), as defined by the Organisation for Economic Co-operation and Development (OECD, 1992);

- In the U.S., *Aspergillus niger* is not listed as a Class 2 or higher Containment Agent under the National Institutes of Health (NIH) Guidelines for Recombinant DNA Molecules (USA, 2013);

- The U.S. Environmental Protection Agency (EPA) has exempted *Aspergillus niger* from review by the agency, due to its extensive history of safe use (USA, 1997);

- *Aspergillus niger* is classified as a low-risk-class microorganism, as exemplified by the listing as Risk Group 1[2] in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BauA) (Germany, 2016), the German Central Commission for Biological Safety (ZKBS) (Germany, 2018), and the Dutch Commission on Genetic Modification (Netherlands, 2011).

- *Aspergillus niger* does not appear on the list of pathogens in the Belgian Biosafety Server (Belgium, 2010), or the list of pathogens in Annex III of Directive 2000/54/EC (EU, 2000) on the protection of workers from risks related to exposure to biological agents at work, as it is globally regarded as a safe microorganism.

Although *Aspergillus niger* is known to produce ochratoxins and fumonisins (Palencia *et al*., 2010; Frisvad *et al*., 2011; Blumenthal, 2004), toxin production by industrial strains under the routine conditions of industrial submerged fermentations has not been reported, despite the long history of use.

In 1988, JECFA allocated a numerical Acceptable Daily Intake (ADI) to enzyme preparations produced by *Aspergillus niger*, based on the concern that some strains may produce unknown toxins (JECFA, 1988). However, in 1990, JECFA revised the ADI to “not specified,” based on the long history of use of the organism for enzyme production, numerous toxicological studies, and two expert reports that concluded that the production of toxins was highly unlikely (JECFA, 1990).

[2] In the German lists, *Aspergillus niger* is listed as Risk Group 2, with the exception of well-defined production strains with a long history of use, which are classified as Risk Group 1.

DSM has established specifications to ensure the absence of mycotoxins in the enzyme preparation (see section 2.2.3).

2.2.1.2 Development of the Production Strain

The parental strain *Aspergillus niger* NRRL 3122 was obtained from the Culture Collection Unit of the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, USA. The parental strain is one of the most common industrial *Aspergillus niger* strains used (Frisvad, 2011).

For increased production of a required protein (such as an enzyme), it is necessary that the genome of the production organism contain more than one copy of the gene encoding the protein. In classical production strains, such 'gene multiplication' is achieved by classical mutation-selection techniques. Starting from the parental strain, these classical techniques were used to obtain mutants with an enhanced production capacity for the enzyme glucoamylase (GAM). When the molecular biological techniques were developed for *Aspergillus niger*, it was shown that one of these classical mutants, deposited as DS 03043, contains 7 copies of the gene encoding glucoamylase (the *gla*A gene).

Subsequently, *Aspergillus niger* strain DS 03043 was used as parental strain in a series of genetic modifications, including the deletion of the seven *gla*A loci (*i.e.*, the promoter and the coding sequences) and the inactivation of a major protease (*pep*A), resulting in the recipient strain DS 38556. The purpose of the genetic modifications was to create a safe standard recipient strain in which any desired gene can be integrated into predefined loci of the genome, resulting in a genetically well-characterized engineered production strain for any protein of interest (van Dijck *et al*., 2003).

In the recipient strain DS 38556, multiple expression cassettes encoding the *Aspergillus niger* acid prolyl endopeptidase encoding *gep*A gene were inserted, resulting in the *Aspergillus niger* strain GEP, which produces higher concentrations of the native (*Aspergillus niger)* acid prolyl endopeptidase. The gene encoding the *Aspergillus niger* acid prolyl endopeptidase was amplified by PCR from genomic DNA isolated from *Aspergillus niger* strain DS 37449. Correct integration of the expression cassettes in the *Aspergillus niger* genome was verified *via* Southern blotting and hybridization.

Due to the targeted integration of the expression cassettes, there is no risk of disturbing other parts of the genome that might lead to the accidental activation of *e.g.,* mycotoxin genes.

The final production strain, *Aspergillus niger* GEP, does not contain any selection markers or heterologous DNA.

2.2.1.3 Stability of the genetic traits

The genotypic and phenotypic stability of the *Aspergillus niger* GEP production strain was tested. The phenotypic stability of the strain is proven by its capacity to produce a constant level of the acid prolyl endopeptidase enzyme. This was assessed by measuring the enzyme activity in relation to the Total Organic Solids (TOS) in three independent batches of the food enzyme (also see Appendix 3):

Batch no	[redacted]	[redacted]	[redacted]	**Mean**
Ash (%)	0.25	0.5	0.5	0.41
Water (%)	78.9	72.3	72.5	74.6
TOS (%)	20.85	27.2	27.0	25.0
Activity (Units/g)	16	18.5	18.7	17.7
Units/mg TOS	0.076	0.068	0.069	0.071

The genotypic stability of the GEP strain was tested by comparing the genotype of the strain at the end of the fermentation with the strain of the original working cell bank (WCB) that was used as inoculation material for the fermentation, as well as with the recipient strain DS 38556. For three independent 'end of fermentation' batches, the DNA from the biomass at the end of the fermentation was isolated and used as template for the PCR amplification of the characteristic Δ*gla*A loci and the P*gla*A-*gep*A promoter-gene insert.

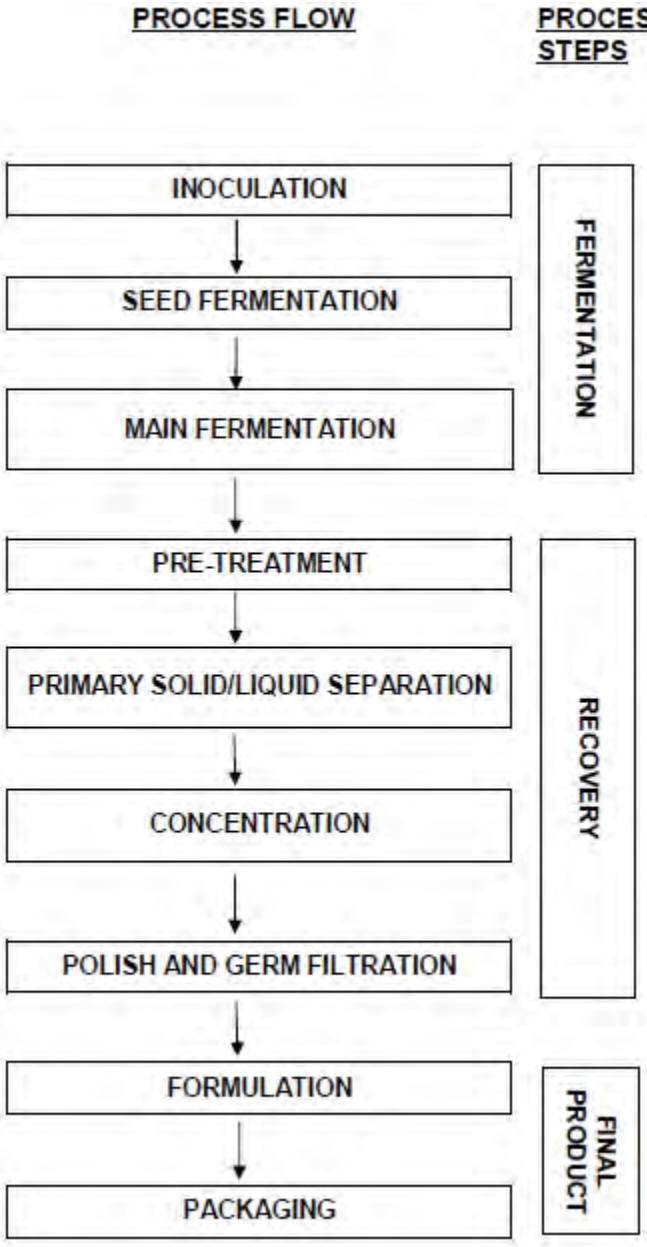
The results showed no changes in the genetic structure of the acid prolyl endopeptidase expression cassettes between the WCB (start of fermentation) and the *Aspergillus niger* GEP strain at the end of the fermentation. These data thus confirm the genetic stability of the strain.

2.2.2 Manufacturing Process

DSM's acid prolyl endopeptidase is produced in a controlled submerged fed-batch fermentation of a selected, pure culture of *Aspergillus niger* strain GEP. The production process includes fermentation, recovery (downstream processing), and formulation of the product. Production is in accordance with current Good Manufacturing Practices (GMP) for human food, with adequate controls.

The manufacturing process comprises multiple steps. An overview is provided in Figure 2-2.

Figure 2-2 Overview of the manufacturing process



Raw materials used in the fermentation include carbon and nitrogen sources, vitamins, salts, and minerals. The raw materials used for the media are of food-grade quality and meet predefined quality standards that are strictly monitored and controlled by the Quality Assurance Department of DSM Food Specialties. The same applies to all substances used as processing aids (*e.g.*, pH and foam control agents, filter aids).

Preparation of the inoculum is realized by transferring aseptically the production microorganism from culture vials into an inoculum flask containing fermentation medium.

After sufficient growth, the biomass is transferred to a seed fermentor, where further growth takes place under agitation and aeration.

Finally, the contents of the seed fermentor are transferred into the main fermentor, where enzyme production will take place. The main fermentation is conducted under specified pH, temperature, and aeration conditions, until sufficient enzyme production has taken place.

When the main fermentation is complete, the downstream processing can begin, with the killing and efficient removal of the microorganism from the culture broth.

The broth is then subjected to a series of separation and concentration steps by physical separation techniques such as filtration and diafiltration. The enzyme concentrate, devoid of production microorganisms, is then formulated into a commercial preparation (solid and/or liquid) that will be used in food processing[3].

The enzyme preparation is formulated with food ingredients, GRAS substances, or additives otherwise permitted for use in human food.

2.2.3 Specifications

The common starting material for all formulations is the ultra-filtration (UF) concentrate. Typically, its composition falls within the following ranges:

Enzyme activity	15-20 PPU/g
Water (%)	70-80
Ash (%)	0.2-0.6

To obtain a final enzyme preparation, the stabilized food enzyme is formulated either as a dry or a liquid preparation, depending on the intended food processing application. As mentioned previously, the enzyme is formulated with food ingredients or substances otherwise permitted for use in human food.

[3] The enzyme is also formulated for use as an ingredient in dietary supplements (see section 6.4.1).

As Table 2-3 illustrates, DSM has established purity specifications consistent with those of JECFA for chemical and microbiological purity of food enzymes (FAO/WHO, 2006), and the USP Food Chemicals Codex (FCC) monograph for Enzyme Preparations.

Table 2-3 Specifications for purity of DSM's acid prolyl endopeptidase

Parameter	Specification
Lead	≤ 5 mg/kg
Coliform	≤ 30 CFU/g
Salmonella	0 per 25 g
Escherichia coli	0 per 25 g
Antimicrobial activity	Not detected
Mycotoxins	No significant levels[1]

[1] See JECFA specifications (http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/enzymes/en/). Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

DSM has established specifications to ensure the absence of mycotoxins in the enzyme preparation. The acceptance limit is set as "absent by test" (below the limit of detection). The analyses are performed by accredited external laboratories using validated methods.

2.2.4 Total Organic Solids (TOS)

Microbial food enzyme preparations used in food processing are generally mixtures of an enzyme protein that has a specific technological purpose in the food, along with substances derived from the production microorganism and the fermentation medium. The latter are constituents consisting of organic material (proteins, peptides, amino acids, carbohydrates, lipids) and inorganic salts.

The organic materials present in an enzyme preparation are normally expressed as Total Organic Solids (TOS). The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes. TOS is defined as the sum of the organic components in the final preparation, excluding diluents and other ingredients. It is derived experimentally as follows:

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

where:

A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

For enzymes that are not yet formulated, the TOS is the same as dry matter minus ash. The amount of ash (*e.g.*, mineral salts used in the fermentation) is generally a small percentage. Therefore, the ratio between the enzyme activity and TOS is a good indication of the relative purity of the enzyme.

Table 2-4 provides TOS, protein content, and relative purity values based on analysis of 3 representative batches of acid prolyl endopeptidase produced by *Aspergillus niger* GEP (also see Appendix 3).

Table 2-4 Results based on analysis of 3 batches of DSM's acid prolyl endopeptidase

	Batch No.			
	[redacted]	[redacted]	[redacted]	Mean
Ash (%)	0.25	0.5	0.5	0.41
Water (%)	78.9	72.3	72.5	74.6
TOS (%)	20.85	27.2	27.0	25.0
Activity (Units/g)	16	18.5	18.7	17.7
Units/mg TOS	0.076	0.068	0.069	0.071
Protein (%)	14.31	18.87	18.59	17.3

2.2.5 Stability

Food enzymes are not sold as such, but are formulated into various enzyme preparations in order to obtain standardized and stable products. The stability therefore depends on the type of formulation, not on the enzyme as such.

Information about special conditions of storage and/or use appears on DSM's product label and/or other accompanying documentation.

Part 3: Dietary Exposure

3.1 Proposed Food Uses

DSM's acid prolyl endopeptidase enzyme is intended for use in the degradation (hydrolysis) of proteins and peptides. In the production of beer and other fermented beverages, including distilled spirits, it is intended to accomplish the following functional/technical effects:

- Degradation of cereal storage proteins such as gluten/gliadins, C hordein, and glutelin (zein)
- Increased flexibility in the choice of raw materials (raw grain/malt ratio)
- Creation of reaction products (smaller peptides) for optimal development of the fermentation
- Consistent beer stability, prevention of chill haze without loss of foam properties

The enzyme preparation is intended for use during food production and is not expected to perform any technological function in the final food product[4].

In the production of distilled spirits, the food enzyme is added during the presaccharification and fermentation steps. During the distillation process, the enzyme proteins are completely removed. Consequently, no acid prolyl endopeptidase will be present in the final potable alcohol. Therefore, distilled spirits do not result in any consumer exposure, and this use was excluded from Table 3-3, which shows the highest possible levels that might be present in the final food (as mg TOS/L) based on its use as the highest recommended level.

For the purposes of assessing consumer exposure (see section 3.2), beer was used as the representative final food because: (1) experience from use of the enzyme in other countries suggests this is the most common application; and (2) beer is widely consumed in the U.S.

[4] As noted elsewhere in this GRAS notice, DSM's acid prolyl endopeptidase enzyme is also formulated for use as an ingredient in dietary supplements. However, such uses are considered outside the scope of this section of the notice, which describes uses in food processing and the resulting dietary exposures from such uses.

Table 3-1 Summary of proposed food uses and use levels

Application	Raw material (RM)	Recommended use level (mg TOS/kg RM)	Example Final Food (FF) or ingredient	Ratio RM/FF	Levels in FF (mg TOS/L food)
Production of beer and other fermented beverages	Wort	0.7-6.3	Beer	1[1]	0.7-6.3

[1] For beer production, it is assumed as worst-case situation that 1L beer is obtained from 1L wort.

3.2 Anticipated Consumer Intakes

Based on the proposed food uses and use levels described above, DSM estimated consumer intakes, which are summarized in Table 3-2.

DSM used a worst-case exposure scenario that assumed the following:

- A 90th percentile of 17 drinks/week, as reported for men (*vs*. 7 drinks/week for women) based on 2015 National Alcohol Survey[5] data, or 4 drinks on any day per the NIAAA[6] guidelines for "heavy drinking" among men (*vs*. 3 drinks on any day for females).
- All drinks would consist of beer.
- A standard beer consists of 12 fl oz, approximately 355 mL (1 fl oz = 29.5735 mL).
- A body weight of 60 kg.

Table 3-2 Expected consumer exposures to acid prolyl endopeptidase (on TOS basis) based on proposed uses in food processing

Representative Final Food (FF)	Amounts of TOS in the final food (mg TOS/L FF)	FF intake level per person	Estimated daily intake of TOS
Beer	0.7-6.3	6.0 L/week[1]	4.2-37.8 mg TOS/person/week (0.60-5.40 mg TOS/person/day) 0.07-0.63 mg TOS/kg bw[3]/week (0.010-0.090 mg TOS/kg bw/day)
		1.4 L/day[2]	0.98-8.82 mg TOS/person/day 0.016-0.147 mg TOS/kg bw[3]/day

[1] 17 drinks/week x 355 mL/drink = 6033 mL/week.
[2] 4 drinks on any day x 355 mL/drink = 1420 mL on any day.
[3] Assuming a body weight of 60 kg.

[5] As reported in the Alcohol Research Group website (percentile summary tables at http://arg.org/news/drinking-norms-in-the-us/) based on an analysis by Thomas K. Greenfield, Katherine Karriker-Jaffe, and Yu Ye of the 2015 National Alcohol Survey (NAS) of 7,071 individuals, residing in 50 states and Washington DC.
[6] National Institute on Alcohol Abuse and Alcoholism. Information accessed through https://www.rethinkingdrinking.niaaa.nih.gov/.

Part 4: Self-Limiting Level of Use

The amount of an enzyme required to accomplish a specific technical or functional effect will depend in part on the type and quality of the raw material being treated. DSM's enzyme preparation containing acid prolyl endopeptidase produced by *Aspergillus niger* strain GEP is intended to be used in a manner consistent with current GMP. Use of excessive amounts would likely be associated with undesirable technological effects.

Part 5: Common Use in Food Prior to 1958

The elements of this section do not apply because DSM's GRAS conclusion is based on scientific procedures. However, it is important to note that proteases (including those with high specificity for proline residues) occur naturally in various organisms that are part of the human diet.

Part 6: Narrative of the Basis for the GRAS Conclusion

6.1 Overview

DSM has determined that its acid prolyl endopeptidase enzyme from a genetically engineered strain of *Aspergillus niger* (GEP) is GRAS when used in the production of beer and other fermented beverages. DSM's GRAS conclusion is based on the totality of available information, discussed below and elsewhere in this document.

In particular, DSM considered that:

- The acid prolyl endopeptidase enzyme is produced *via* controlled submerged fed-batch fermentation using a strain of *Aspergillus niger* (GEP) developed by DSM Food Specialties. At the end of fermentation, the cell material is separated from the enzyme by means of filtration, followed by concentration through ultrafiltration (UF), and formulation using ingredients adequate for use in human foods.

- *Aspergillus niger* represents the host and donor organism. This microorganism has a long history of use in the production of food ingredients, including food enzymes.

- The genetic modifications made to *Aspergillus niger* to make the production strain have been well characterized and do not lead to production of harmful or toxic substances. Multiple copies of the acid prolyl endopeptidase expression cassette results in a strain that produces higher concentrations of the native (*Aspergillus niger*) acid prolyl endopeptidase enzyme. The strain does not contain antibiotic resistance markers or heterologous DNA, and lacks the genes encoding for glucoamylase and a pepsin-like protease (see section 2.2.1.2).

- The enzyme is manufactured under current GMP, using food-grade materials and under adequate controls. The acid prolyl endopeptidase preparation meets the specifications established by DSM, including JECFA and USP FCC purity criteria (see sections 2.2.3).

- Acid prolyl endopeptidase is likely to belong to the so-called MEROPS S28 family of clan SC of serine proteases, which have been identified in many sources, including various organisms (*e.g.*, soybeans, barley, rice, corn) that are part of the human diet (see section 2.1).

- DSM's acid prolyl endopeptidase has been used in food processing for several years across the European Union and in other markets worldwide. Its safety has been evaluated by Australia/New Zealand, Denmark, and France.

- A version of the enzyme formulated for use as a dietary supplement ingredient was the subject of a New Dietary Ingredient Notification (NDIN) submitted by DSM to U.S. FDA in November 2009. Dietary supplement products containing the enzyme have been marketed in the U.S. and Canada since 2015, with no evidence of any adverse health

effects linked to the enzyme. Estimated intakes of the enzyme (as TOS) from dietary supplement products range from 163-656 mg TOS/day (see Table 6-1).

- Multiple human studies of the enzyme formulated for use in dietary supplements have been published (see section 6.4.2). The studies involved 12-16 subjects receiving the enzyme as a single administration at levels equivalent to up to 1.5 g TOS/day on 4 separate occasions, or at 2.77 g TOS/day for up to 4 weeks (see Table 6-2). No serious adverse events were observed in any of the studies. Complaints were generally limited to mild and transient gastrointestinal discomfort.

- Toxicity studies of the enzyme concentrate (genotoxicity and 90-day rat oral toxicity) did not reveal any significant findings (see section 6.5).

- A comparison of the amino acid sequence to that of known food allergens did not reveal any significant matches (see section 6.6).

- The greatest anticipated consumer exposure from use of the enzyme in the production of beer and other fermented beverages was 8.82 mg TOS/person/day or 0.147 mg TOS/kg bw/day, assuming a body weight of 60 kg (Table 3-2). This level of exposure was developed using a worst-case approach (described in Part 3 of this notice) and is:

    - At least 18 times lower than the highest level of intake (on a TOS basis) from use of the enzyme in dietary supplements currently marketed in North America (163-656 mg TOS/day), as shown in Table 6-1;

    - 314 times below the highest level of enzyme administered to human volunteers for up to 4 weeks (2.77 g TOS/person/day), as reported in published studies summarized in Table 6-2; and

    - More than 34,000 times lower than the NOAEL of 5040 mg TOS/kg bw/day from a 90-day rat oral toxicity study (section 6.5.3).

DSM also searched the published scientific literature in November 2018[7]. Any information relevant to the safety of this enzyme is included herein.

6.2. Safety of the Host and Donor Organism

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the International Food Biotechnology Council, food or food ingredients are safe to consume if they have been produced according to current Good Manufacturing Practices from a nontoxigenic and nonpathogenic organism (IFBC, 1990). A nontoxigenic organism is defined as “one which does

[7] Terms used to search the published literature were “(acid) prolyl endopeptidase/prolyl oligopeptidase/proline endopeptidase/proline endoprotease/prolyl endoprotease/serine endopeptidase” and “safe/safety/toxicity”

not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances" (Pariza and Foster, 1983).

As discussed previously, *Aspergillus niger* is widely distributed in nature and is generally considered as nonpathogenic (Schuster *et al*., 2002).

Some *Aspergillus niger* strains are capable of mycotoxin production, with ochratoxin A and fumonisin B2 being of most concern in terms of human and animal safety (Nielsen *et al*., 2009; Frisvad *et al*., 2018). However, ochratoxin A and fumonisin production by *Aspergillus niger* was not observed under standard industrial submerged fermentation conditions.

The safety of *Aspergillus niger* as a production organism for food enzymes and as a host for recombinant strains is well-documented. Therefore, *Aspergillus niger* would be considered a safe and appropriate host and donor organism, further supported by its classification as a low-risk organism in the U.S. and other countries (see section 2.2.1.1).

6.3. Safety of the Production Strain

The GEP production strain was derived from DSM's safe strain lineage of *Aspergillus niger* recipient and production strains (van Dijck *et al*., 2003). DSM has used *Aspergillus niger* strains from this lineage for more than 30 years for food enzyme production, and has performed a number of safety studies on different enzyme products produced by strains from this lineage. A series of classical strain improvements and selection steps were employed, along with genetic modifications that integrated multiple expression cassettes of the *Aspergillus niger* acid prolyl endopeptidase gene at defined locations, and deleted genes coding for a glucoamylase and a protease.

Southern blot analysis and PCR analysis of the final production strain confirmed the integration of twenty copies of the acid prolyl endopeptidase gene at the targeted integration sites, as well as the deletion of the glucoamylase and protease genes. In the final production strain, no selection markers and vector sequences are present.

The genotypic stability of *Aspergillus niger* strain GEP was confirmed by comparing the genetic structure of (i) the acid prolyl endopeptidase expression cassettes of the working cell bank (WCB) at the start of fermentation with (ii) samples at the end of fermentation. The ability of *Aspergillus niger* strain GEP to produce a constant level of the acid prolyl endopeptidase enzyme provides evidence of its phenotypic stability (see section 2.2.1.3).

6.4 History of Use of the Enzyme

Acid prolyl endopeptidase produced by *Aspergillus niger* GEP has been used in food processing for several years across the European Union and in other markets worldwide. Its safety has been evaluated by Australia/New Zealand, Denmark, and France. It has also been used as an ingredient in dietary supplements in the U.S. and Canada.

6.4.1 Dietary Supplement Use

A version of DSM's acid prolyl endopeptidase has been studied and marketed as an ingredient in dietary supplements for the past few years. In this form, the enzyme is known as AN-PEP and Tolerase™ G (formerly PEP2COMFORT™). The production organism and enzyme used for each the food processing and dietary supplement preparations are identical; aside from the formulation, the differences are limited to the filtration method (germ microfiltration *vs*. membrane filter press) used to remove the production organism biomass. The activity/TOS of the resulting ultra-filtrate concentrates are comparable[8].

The enzyme (as PEP2COMFORT™) was the subject of a New Dietary Ingredient Notification (NDIN) submitted by DSM to U.S. FDA in November 2009. The notice was subsequently listed in U.S. FDA Docket FDA-1995S-0039 (formerly docket number 95S-036).

As Tolerase™ G, it was the subject of a Natural Health Product Master File filed by the Health Canada Natural and Non-Prescription Health Products Directorate (NNHPD) in 2015.

Table 6-1 provides some examples of dietary supplements marketed in the U.S. and Canada that contain Tolerase™ G.

Tolerase™ G has been sold for use as an ingredient in dietary supplements since April 2015. DSM is not aware of any adverse events linked to Tolerase™ G.

[8] Analyses of 5 ccUF batches revealed an average of 64.0 PPU/g TOS for the dietary supplement version of the enzyme (Tolerase™ G) *vs*. an average of 69.0 PPU/g TOS for the food processing version of the enzyme (Brewers Clarex).

Table 6-1 Subset of dietary supplements containing Tolerase™ G marketed in North America

Brand Name	Producer	Recommended use	Amount of Tolerase™ G per tablet/capsule[1]	Maximum daily intake based on recommended use[2]
Spectrazyme	Metagenics	1-2 capsules with each meal	116,000 PPI	PPI: 696,000 PPU[3]: 42 [equivalent to 656 mg TOS/day]
Gluten Rid	Swanson Health Products	1 capsule with each meal	58,000 PPI	PPI: 174,000 PPU[3]: 10.4 [equivalent to 163 mg TOS/day]
GlutnGo™	Bricker Labs	1 capsule with each meal	58,000 PPI (100 mg)	PPI: 174,000 PPU[3]: 10.4 [equivalent to 163 mg TOS/day]
Gluterase	Biotics Research Corporation	2 tablets with each meal	78,300 PPI (135 mg)	PPI: 469,800 PPU[3]: 28 [equivalent to 438 mg TOS/day]

Tolerase™ G is standardized to 580,000 PPI/g. PPI: Protease Picomol International units; PPU: Prolyl Peptidase Units. TOS calculated based on an average of 64.0 PPU/g TOS for Tolerase™ G from analyses of 5 ccUF batches.

[1] Some marketed products present the enzyme units as Food Chemicals Codex (FCC) Hemoglobin Unit Tyrosine base (HUT) units for proteases. The conversion factor for PPI to FCC HUT units is 0.00377.

[2] Based on consumption of 1-2 capsules/tablets with three (3) daily meals.

[3] Based on 1 PPU = 16,667 PPI (PPU in micromoles/minute converted to PPI picomoles/second: 1,000,000/60 = 16,667)

6.4.2 Published Human Studies

The acid prolyl endopeptidase produced by *Aspergillus niger* strain GEP formulated for use in dietary supplements (AN-PEP) was the subject of multiple human studies, summarized in Table 6-2.

The studies each involved between 12 and 16 subjects that received the enzyme once daily at up to 1,600,000 PPI (equivalent to 96 PPU or 1.5 g TOS) on 3 or 4 separate occasions, or at 2,950,059 PPl/day (equivalent to 177 PPU or 2.77 g TOS/day) for up to 4 weeks (n = 7 subjects). Gluten degradation was the primary study endpoint, but adverse events were monitored during each of the studies. No serious adverse events were observed in any of the studies. Complaints were generally limited to mild and transient gastrointestinal discomfort.

Table 6-2 Summary of human studies of DSM's acid prolyl endopeptidase formulated for use in dietary supplements

Reference	Number of subjects completing the study	Study details	Adverse events and other measures possibly related to safety
Tack *et al.* (2013)	14 Sixteen (16) adult outpatients with initial diagnosis of celiac disease were enrolled; 2 were discontinued after first 2-week period due to histological deterioration (2 and 3 Marsh grades).	Test material Topping with AN-PEP enzyme or without (Control) applied to bread (7 g gluten) consumed at breakfast Amount of enzyme administered 2,950,059 PPI/day 177 PPU[1]/day [equivalent to 2.77 g TOS/day] Duration 2 weeks for 7 subjects; 4 weeks for 7 subjects (with 2-week washout period in between)	No serious adverse events. No significant differences between AN-PEP enzyme and Control in: • Gastrointestinal (GI) symptoms reported, most of which were mild and transient • Duodenum mucosa immunohistology • Serum antibodies associated with response to gluten in celiac disease
Salden *et al*. (2015)	12 Seventeen (17) healthy adult subjects were enrolled, 5 dropped out due to discomfort related to the nasoduodenal tube; 11 completed all 4 test days, and 1 completed 3 out of 4 test days.	Test Material AN-PEP enzyme or Control solution administered by nasogastric tube with high- or low-calorie meals containing 4 g gluten Amount of enzyme administered 1,600,000 PPI/day 96 PPU[1]/day [equivalent to 1.50 g TOS/day] [Polyethylene glycol (PEG-3350) used as dilution marker; acetaminophen used as marker of gastric emptying.] Duration Single administration on 4 separate days (with at least 1 week in between)	No serious adverse events. No significant differences between groups in GI symptoms reported.

Reference	Number of subjects completing the study	Study details	Adverse events and other measures possibly related to safety
Konig *et al*. (2017)	16 Eighteen (18) subjects with self-reported gluten sensitivity were enrolled. Two subjects dropped out after the first test day, one due to discomfort associated with nasogastric tube, the other for health reasons unrelated to the study.	<u>Test Material</u> AN-PEP enzyme or placebo tablet administered orally with oatmeal porridge (following placement of nasogastric tube) <u>Amount of enzyme administered</u> 83,300 or 166,700 PPI/day 5 or 10 PPU[1]/day [equivalent to 78 or 156 mg TOS/day] <u>Duration</u> Single administration on 3 separate days (with at least 1 week in between)	No serious adverse events. Three study participants reported n = 4 mild adverse events, of which n = 3 were rated as 'not related to study product', and n = 1 was rated as 'suspected relation to study product'.
[1]Based on 1 PPU = 16,667 PPI (PPU in micromoles/minute converted to PPI picomoles/second: 1,000,000/60 = 16,667). TOS calculated based on an average of 64.0 PPU/g TOS for Tolerase™ G from analyses of 5 ccUF batches.			

6.5 Toxicity Studies

Acid prolyl endopeptidase produced by *Aspergillus niger* strain GEP was subjected to a series of toxicity studies (genotoxicity and 90-day oral toxicity in rats), performed according to internationally accepted guidelines (OECD/EU) and in compliance with the principles of Good Laboratory Practice (GLP). The findings of these studies have not been published. Summaries of the studies are provided below.

The batch (of the enzyme used for the toxicity studies was produced on a pilot-plant scale. Compared to the commercial batches listed in Table 2-4, the batch used in these studies had slightly lower activity versus TOS ratio. This means it was slightly less pure than the commercial batches and may therefore represent a 'worst-case' situation with respect to potential toxicity. All other data are comparable to the commercial batches as presented. Consequently, the test material may be regarded as representative of the commercial enzyme. The downstream processing (purification process) produced the final, non-standardized ultra-filtrate concentrate (ccUF). The composition and specifications of the test material are summarized below.

Batch no:	
Ash (%)	0.7
Water (%)	74.1
TOS (%)	25.2
Activity (PPU/g)	11.0
PPU/mg TOS	0.044
Protein (%)	13.9
Lead (mg/kg)	<0.08
Salmonella sp. (per 25 g)	absent
Total coliforms (per g)	< 1
Escherichia coli (per 25 g)	absent

6.5.1 Bacterial reverse mutation (Ames) test (TNO Nutrition and Food Research report no. V5005/10, 2004).

The study was conducted in compliance with the following guidelines:

- OECD 471 Genetic Toxicology: Bacterial Reverse Mutation Test (adopted July, 1997)

The potential of mutagenicity of the enzyme (non-standardized ultra-filtrate concentrate) was tested in four histidine-dependent *Salmonella typhimurium* mutant strains (TA 98, TA 100, TA 1535, and TA 1537) and one tryptophan-requiring *Escherichia coli* mutant strain WP2uvrA, in two independent experiments. Tester bacteria were exposed to five concentrations ranging from

62 to 5,000 µg dry matter/plate (corresponding with 60 to 4865 µg TOS/plate) in the absence and presence of a rat liver-derived metabolic activation system (S-9 mix). Negative (water) and positive controls were run simultaneously with the test.

An increase in the number of reverse mutation colonies, together with a (slightly) more dense bacterial background lawn, was observed in the first assay, indicating the presence of histidine or proteins. Therefore, a second assay was performed according to the treat-and-plate method to exclude false positive results. In the second assay, no increase in the number of revertants was observed. Precipitation and toxicity were not observed. The positive control substances, sodium azide, daunomycine, methylmethanesulphonate, 4-nitroquinolone, 2-aminoanthracene, and 9-aminoacridine, gave the expected increase in the number of revertants.

Based on the results of this study, the enzyme was not considered mutagenic in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay.

6.5.2 *In vitro* Chromosomal Aberration Test in Human Lymphocytes (TNO Nutrition and Food Research report no. V5002/08, 2003)

The study was conducted in compliance with the following guideline:

- OECD 473 Genetic Toxicology: *In vitro* Mammalian Chromosome Aberration Test (adopted July, 1997)

The enzyme (non-standardized ultra-filtrate concentrate) was tested for its potential to induce chromosomal aberrations in cultured human peripheral lymphocytes in the presence and absence of a metabolic activation system (Aroclor-1254 induced rat liver S9-mix). Negative (culture medium) and positive controls were run simultaneously.

In the absence of S9-mix: in the first experiment, the enzyme was tested up to 5,000 µg dry matter/ml (corresponding with 4865 µg TOS/ml) for a 4 h and 24 h treatment time, with a 24 h fixation time. In the second experiment, it was tested up to 5,000 µg dry matter/ml for a 24 h treatment time with a 24 h fixation time as well as for a 48 h treatment time with a 48 h fixation time.

In the presence of 1.8% (v/v) S9-mix: the enzyme was tested up to 5,000 µg dry matter/ml (corresponding with 4865 µg TOS/ml) for a 4 h treatment time with a 24 h fixation time in the first experiment, and for a 4 h treatment time with a 48 h fixation time in the second experiment.

The enzyme did not induce a statistically significant increase in the number of cells with chromosome aberrations at any of the dose levels at any time point analyzed, in the absence or presence of S9-mix. Positive control chemicals, mitomycin C and cyclophosphamide, each produced a statistically significant increase in the incidence of cells with chromosome

aberrations, indicating that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

Based on the results of this study, the enzyme was not considered clastogenic to human lymphocytes *in vitro*.

6.5.3 Sub-chronic (90-day), Repeated-Dose Oral (gavage) Toxicity Study in Wistar Rats (Rallis Research Centre, report no. 3716/03)

The study was conducted in compliance with the following guidelines:

- Commission Directive 87/302/EEC, Annex, Part B: Subchronic oral toxicity test: 90-day Repeated oral dose using Rodent species (EU, 1988)
- OECD guideline 408. Repeated Dose 90-day Oral Toxicity Study in Rodents (OECD, 1998)
- EPA Health Effects Test Guideline No. OPPTS 870.3100: 90-Day Oral Toxicity in Rodents (USA, 1996).

The sub-chronic oral toxicity of the enzyme (non-standardized ultra-filtrate concentrate) was examined in a 90-day study with groups of 10 male and 10 female Wistar rats. The rats received the test article by gavage at 2000, 7000 and 20,000 mg/kg bw/day, equivalent to 504, 1764 and 5040 mg TOS/kg bw/day, respectively (25.2% TOS in batch [redacted], as noted above). A similarly constituted vehicle control group received double distilled water.

The parameters evaluated were clinical signs, physical examinations, functional observations, body weight, food consumption and ophthalmologic examinations. At termination of the study, hematology and clinical chemistry, organ weights, macroscopy, histology, and clinical pathology were performed.

No treatment-related findings were observed on general health, neurological findings, hematological parameters, biochemical parameters, fasting body weights, organ weights and their ratios, gross pathology, or histopathology. Compared to the corresponding vehicle controls, animals exposed to 20,000 mg/kg/day had higher body weights (females) and reduced food consumption (males). However, based on the absence of any corroborative functional disturbances or morphological changes in the high-dose animals, the alterations in body weights and food consumption were considered attributable to the extra energy intake from the enzyme preparation and of no toxicological significance.

Based on the results of this study, the highest level tested, 20,000 mg/kg bw/day, equivalent to 5040 mg TOS/kg bw/day, was considered the no-observable-adverse-effect level (NOAEL).

6.6 Allergenicity assessment

As proteins, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens.

Enzymes have a long history of safe use in food. Since new enzymes are generally (based on) existing enzymes, it is very unlikely that a new enzyme would be a food allergen. Moreover, exposure to an enzyme associated with ingestion is typically very low and residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system (Grimble, 1994). For the most part, ingestion of food enzymes is not considered to be a concern with regards to food allergy (Bindslev-Jensen *et al*., 2006).

The potential allergenicity of DSM's acid prolyl endopeptidase protein produced by *Aspergillus niger* GEP was evaluated by comparing the amino acid sequence of the enzyme with known food allergens. This comparison did not reveal any relevant matches with known food allergens.

For the comparison, the database AllergenOnline™ (available at http://www.allergenonline.org/, last updated March 23, 2018) was used. The comparison was done in March 2014. AllergenOnline™ allows the search in NCBI, SwissProt, PIR, PRF, PDB and the WHO-IUIS databases using a FASTA algorithm. The WHO-IUIS list is set up by the IUIS Allergen Nomenclature Sub-committee operating under the auspices of the International Union of Immunological Societies and the World Health Organization. The objectives of the IUIS Allergen Nomenclature Sub-committee are to maintain a unique and unambiguous nomenclature for allergen molecules and maintain the 'official list of allergens'.

The search was performed following the guidelines developed by EFSA in the safety evaluation document of the newly expressed proteins in genetically modified plants (EFSA, 2006) and the guidelines from the FAO/WHO consultation (FAO/WHO, 2001, 2009). According to the guidelines, cross-reactivity between the expressed protein and a known allergen has to be considered when there is:

- more than 35% identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty or
- identity of short contiguous amino acid segments (*i.e*., at least 8 contiguous amino acids).

Although the 2001 WHO/FAO consultation suggested searching for matches of 6 identical amino acid segments or longer, it is recognized that a search for such small sequences would lead to the identification of too many false positives (EFSA, 2006; FAO/WHO, 2009). It has been

reported that an immunologically significant sequence similarity requires a match of at least 8 contiguous identical residues (Metcalfe *et al*., 1996; Fuchs and Astwood, 1996).

The amino-acid sequence comparison of DSM's acid prolyl endopeptidase did not show 35% or more overlap with known allergens, using a window of 80 amino acids. Matches of 8 amino acids or more were not observed.

In the absence of any significant matches, the acid prolyl endopeptidase protein was not considered likely to produce an allergenic or sensitization response upon ingestion.

6.7 Other information possibly related to safety

Various authors (Panda *et al*., 2015; Colgrave *et al*., 2017; Krishnareddy *et al*., 2017) have raised concerns about the use of this and similar enzymes in products that make statements related to gluten reduction and/or gluten content, alleging that these products have minimal published evidence of efficacy and may therefore be hazardous to individuals with Celiac disease.

As noted previously, Tolerase™ G (AN-PEP) is a version of DSM's acid prolyl endopeptidase formulated specifically for use in dietary supplements. Tolerase™ G has been in use in multiple dietary supplement products marketed in the U.S. and elsewhere since April 2015. DSM is not aware of any adverse events linked to Tolerase™ G.

DSM does not expect the use of acid prolyl endopeptidase enzyme in the production of beer and other fermented beverages, as proposed in this notice, to be associated with the concerns raised by these authors. First, DSM does not promote any version of acid prolyl endopeptidase for products intended for individuals with Celiac disease, and finished product manufacturers interested in using the enzyme are advised accordingly. In addition, any statements related to gluten content that DSM customers may wish to make for a finished food product would be subject to the rule established by U.S. FDA for (see 78 FR 47154, 2013) for voluntary gluten-free labeling of foods.

6.8 Summary of the basis for a GRAS conclusion

Combined, the elements described in this dossier support DSM's conclusion that: (1) there is sufficient information available to support the safety of DSM's acid prolyl endopeptidase produced by a genetically engineered strain of *Aspergillus niger* (GEP) when used as an enzyme in the production of beer and other fermented beverages; (2) there is a basis to conclude that this technical evidence of safety would be generally known and accepted by qualified experts.

Part 7: Supporting Data and Information

References

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APPENDIX 1: Method of analysis for acid prolyl endopeptidase

Title:

Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

Product:	Validated method	Date of issue:
Fermentation broth Fermentation filtrates Ultra filtrate Finished product	YES	28 MAY 2009

AUTHORIZATION

Compiled by	A. Vriend	Date: 20090518
Approved by expert	M. Stor	Date: 20090518
Approved by QA/QC Analysis	M.M. Immerzeel	Date: 26-05-2009
Approved by Team manager Service Lab Delft	F.C. van der Heeft	
Approved by external QA/QC (if applicable)	n.a.	Date:
Approved by external QA/QC (if applicable)	n.a.	Date:

Title:
Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

1 SAFETY AND ENVIRONMENT

Restrictions for working with chemicals and ML-I samples are mentioned in the work instructions concerning management, storage and use of chemicals, the handling of dangerous substances and standard rules for ML-I laboratories. These restrictions are also applicable for material that has been in contact with ML-I samples.

When working with strong acids, bases, carcinogenic matters and toxic matters etc. take all necessary precautions.
Dioxane is a risk class 2 chemical. The use of this chemical is restricted to authorized technicians only. The use of this chemical needs to be registered.

When working with highly concentrated enzyme preparations take all necessary precautions. Avoid inhalation of dust and/or prolonged contact with unprotected skin.

2 PRINCIPLE

2.1 Application

This method is applicable for the determination of proline specific endoprotease from *Aspergillus niger*. The method is validated for fermentation broth, fermentation filtrate, Ultra Filtrate and finished products.

2.2 Description of the method

EndoPro catalyses the hydrolysis of N-carbobenzoxy-glycine-proline-*p*-nitroanilide (Z-Gly-Pro-pNA). The amount of liberated *p*-nitroaniline (pNA) formed in time is a measure for the EndoPro activity and is determined spectrophotometrically by measurement of the absorption at 405 nm.

The enzyme is incubated in the presence of Z-Gly-Pro-pNA in a citric acid-phosphate buffer pH 4.6 at and 37 °C. The liberated pNA in time is measured spectrophotometrically at 405 nm. The increase in absorbance at 405 nm in time is a measure for the EndoPro activity.
This is an absolute method. The results are related to the molar extinction coefficient of p-nitroaniline at 405 nm and pH 4.6.

2.3 Unit definition

The activity is expressed in **P**roline **P**rotease **U**nits (PPU).
One PPU is defined as the amount of enzyme required to release one micromole of pNA from Z-Gly-Pro-pNA in one minute under the defined assay conditions (pH 4.6, T=37°C and at a substrate concentration of 0.37 mM Z-Gly-Pro-pNA).

Title:
Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

2.4 Measuring range

The measuring-range of this method is 0.02 – 0.12 PPU/ml.

2.5 Summary of the validation report

validation item	sample type	criterium	determined	acceptation
system precision	control	record	0,3%	yes
repeatabilty	UF	record	0,6%	yes
	Ferm. Broth	record	0,6%	yes
	Ferm Filtr.	record	0,7%	yes
intermediate precision	UF	<5%	5,5%	no
	Ferm. Broth	<5%	1,6%	yes
	Ferm Filtr.	<5%	1,9%	yes
accuracy	UF	>90% and<110%	103,9%-99,5%-97.5%	yes
	Ferm. Broth	>90% and<110%	100,8%-96,7%-91.0%	yes
	Ferm Filtr.	>90% and<110%	101,1%-99.9%-93.3%	yes
linearity	control	linear in the 0.02-0.12 PPU/ml range, p>0.1	linear in the 0.02-0.12 PPU/ml range, p=0.122	yes
robustness	control	record	99,1%-105,9%	yes

Table 1: Results from validation of method 61914
UF = UF concentrate; Ferm. Broth = Fermentation broth sample; Ferm. Filtr. = Fermentation filtrate sample.

3 APPARATUS AND CONDITIONS

3.1 Apparatus

Clinical analyser	: COBAS Mira Plus
Balance, accurately to 0.001 g	: Mettler AE200 or AJ100
Balance, accurately to 0.1 mg	: Mettler AT201
Diluter	: Hamilton Microlab 500, provided with 0.5 and 5.0 mL cylinders
pH meter	: Radiometer PHM 82
Magnetic stirrer	: Variomag
Centrifuge, g = 14.000 rpm	: Eppendorf, 5417R P

Or equivalent equipment.

3.2 Conditions

Not applicable.

4 MATERIALS

4.1 Chemicals

Citric acid monohydrate, p.a.	: Merck 1.00244
Disodium hydrogenphosphate dihydrate ($Na_2HPO_4.2H_2O$), p.a.	: Merck 1.06580
N-carbobenzoxy-glycyl-L-proline-4-nitroanilide, ≥99.0% (Z-Gly-Pro-pNA)	: Fluka 96286
1,4-dioxane	: Merck 1.09671

Or equivalent quality.

4.2 References, standards and controls

Standard:
p-Nitroaniline standard preparation from Sigma, cat no N-2128, with an officially assigned content.

Control:

Use an EndoPro control preparation with an officially assigned activity. The activity is expressed in PPU. Store the control preparation and amounts for daily use in the freezer.

4.3 Reagents

- Water:
Ultra High Quality (UHQ) water, conductivity ≤ 0.10 µS.cm

- Citric Acid solution 1 mol/L:
Weigh 210 g citric acid monohydrate in a 1000 mL volumetric flask. Dissolve in water, make up to volume with water and mix. This solution is stable for 1 year at room temperature.

- Citric acid - phosphate buffer pH 4.6:
Dissolve 10.0 g citric acid monohydrate and 15.1 g disodium hydrogenphosphate dihydrate in approximately 800 mL water. Adjust the pH to 4.60 +/- 0.03 at approximately 20°C with citric acid solution 1 mol/L. Quantitatively transfer the solution to a 1 L volumetric flask with water. Make up to volume with water and mix. This solution is stable for 1 month in the refrigerator.

- Dioxane / Citric acid-phosphate buffer pH 4.6 mixture (40 / 60):
Mix 100 mL dioxane with 150 mL of citric acid-phosphate buffer pH 4.6.

- Substrate solution, 2.0 mM:
Dissolve 21.33 mg +/- 0.05 mg Z-Gly-Pro-pNA in 10.0 mL 1.4-dioxane in a 25 mL volumetric flask. While stirring vigorously, make up to volume by slowly adding citric acid - phosphate buffer pH 4.6. Always use a freshly prepared solution.

Title:
Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

Remark:
Due to the low solubility of the substrate in aqueous solution, proline specific endoprotease activity in this assay is not determined under V_{max} conditions.
Slight variations in substrate concentration will influence the measured activity.
It is therefore essential to weigh the amount of substrate within the range prescribed.

5 PROCEDURE

5.1 Preparation

Not applicable.

5.2 Pretreatment reference

Not applicable.

5.3 Pretreatment standard

Weigh approximately 138 mg pNA standard accurately to within 0.1 mg in a 100 mL volumetric flask. Dissolve in approximately 80 mL of dioxane / citrate-phosphate buffer pH 4.6, make up to volume with same and mix (= stock solution). Dilute the stock solution 20 times with citric acid-phosphate buffer pH 4.6 (= working solution). Introduce in duplicate approximately 0.5 mL of this working solution into sample cups for analysis. Only use a freshly prepared pNA solution.

Remark: The molar extinction coefficient of pNA is determined in triplicate during 5 days using freshly prepared reagents daily. Based on the results obtained, the molar extinction coefficient is calculated and used as a fixed value (ε_{Fixed}) in the calculation program.

For each different analyser the molar extinction coefficient has to be determined independently!! **For each sample series the molar extinction coefficient (ε_{daily}) of pNA is determined and checked against the requirements (section 7.1). Daily calculated molar extinction coefficients are monitored in time as check for equipment performance.**

5.4 Pretreatment control

Before use, allow the control preparation to attain room temperature. Weigh accurately to within 1 mg and in duplicate amounts of control sample corresponding to approximately 7 PPU in 100 mL volumetric flasks.
Add approximately 80 mL citric acid-phosphate buffer pH 4.6 and dissolve by stirring on a magnetic stirrer. Make up to volume with citric acid/phosphate buffer and mix. Store these diluted control solutions on ice until starting the incubation.

5.5 Pretreatment samples

5.5.1 Fermentation broth

Before use allow the sample to attain room temperature. Weigh from the sample approximately 2.0 g sample, accurately to within 1 mg, in a 50 mL volumetric flask. Add approximately 40 mL of citric acid/phosphate buffer pH 4.6 and mix by stirring for 15 minutes on a magnetic stirrer. Make up to volume with citric acid/phosphate buffer and mix. Centrifuge part of the samples suspensions for 10 minutes at a speed of 3000 x g . Dilute each supernatant with citric acid/phosphate buffer to a final activity of 0.07 PPU/mL. Store the diluted sample solution on ice until starting the incubation.

5.5.2 Fermentation filtrate

Before use, allow the sample to attain room temperature. Dilute the sample with citric acid - phosphate buffer pH 4.6 to a final activity of 0.07 PPU/mL. Store the diluted sample solution on ice until starting the incubation.

5.5.3 Ultra Filtrate

Before use allow the sample to attain room temperature. Weigh from the sample approximately 2.0 g, accurately to within 1 mg, in a 50 mL volumetric flask. Add approximately 40 mL of citric acid/phosphate buffer pH 4.6 and mix. Make up to volume with citric acid/phosphate buffer and mix. Dilute this solution with citric acid/phosphate buffer pH 4.6 to a final activity of 0.07 PPU/mL. Store the diluted sample solution on ice until starting the incubation.

5.6 Preparation measurement

Start up, and if necessary check the analyser according to the appropriate work instruction Introduce 0.5 mL of the sample and control solution into sample cups.

5.6.1 Priming

- Press the "INFO" button.
- Enter "6" (system check).
- Enter "1" (prime).
- Enter "2" ("up-samp" appears on screen next to Z-position).
- Press "F1" (start) and flush 10 times. The sample needle will be positioned above the central wash position in order to allow checking of diluents stream continuity at daily start up.
- Check the syringe for the absence of air bubbles.
- Press "F1" (stop).
- Enter "3" ("up-reagent" appears on screen next to Z-position).
- Press "F1" (start) and flush 10 times. The reagent needle will be positioned above the central wash position in order to allow checking of diluents stream continuity at daily start up.
- Check the syringe for the absence of air bubbles.
- Press "F1" (stop).

5.6.2 Racks

On the appropriate position on the Cobas place one "reagent rack 5s, number 2" provided with:
at position 1, one 35 mL container filled with citric acid/phosphate buffer pH 4.6
at position 2-1, one 4 mL container filled with substrate solution (= used for endoprotease activity determination).
at position 2-B, one 10 mL container filled with citric acid/phosphate buffer pH 4.6 (= used for endoprotease activity determination).
at position 4-B, one 10 mL container filled with citric acid/phosphate buffer pH 4.6 (= used for pNA standard determination).
- Place one to three "sample 30 racks", dependent on the number of runs, coded 1, 2 and 3 on the Cobas.
- Place the 0.7 mL sample cups filled with control, sample and standard solutions in the sample racks, starting at rack 1 position 1.

5.6.3 Entering work list

- Press "ROUTINE".
- Enter "1" (sample position).
- Press "F2" (to).
- Enter the number of runs to be determined and press "ENTER".
- Press "F4" (next set).
- Press "1" (lev 1).

Press "X" (Endo Protease program) or "J" (pNA standard program) and press "ENTER". *For the content of the programs see annex 1.*
The work list has been completed now.
Note: The work list should be empty before starting a new test. If the work list is not empty, it should be cleared as follows:

When previous series were completed:

- Press "INFO".
- Enter "2" (patient file).
- Press "F2" (interim report).
- Press "F4" (delete) followed by "ENTER".
- Press "SPACE".

When previous series were not completed:

- Press "ROUTINE".
- Press "F1" (display).
- Press "F3" (delete) followed by "ENTER".
- Repeat this until all tests have been deleted.
- Press "START" to begin the analysis.

5.6.4 Switching off the Cobas Mira analyser

- Remove the cuvette segments used.
- Press "INFO" button.
- Enter "6" (system check)

- Enter "1" (prime)
- Enter "2" ("up-samp" appears on screen next to Z-position)
- Enter "F1" (start) and flush 10 times.
- Press "INFO".
- Enter "2" (patient file).
- Press "F2" (interim report).
- Press "F4" (delete) followed by "ENTER".
- Press "SPACE".
- Empty external waste collecting vessel and wash container.
- Remove sample cups from sample racks.
- Remove reagent containers from rack 5s.
- Switch off the Cobas Mira.

5.7 Measurement

Analyze the standard solutions, controls and samples as follows:
Start with the standard solutions followed by the samples and controls in a random order.

6 CALCULATION

Carry out the calculation with the aid of the computer program available for this analysis
If this program is not available carry out the calculation as follows:

6.1 Molar extinction coefficient (ε_M) of p-nitroaniline

$\varepsilon_M = (A_{405nm} \times 100 \times 20 \times 1.6667 \times 138.1 \times 13.5 \times 100 / (W \times P))$ $[M^{-1}. cm^{-1}]$

where:
A_{405nm} = absorbance at a wavelength of 405 nm
100 = volume of stock solution of pNA standard [ml]
20 = dilution factor stock solution of pNA standard
1.6667= correction factor for cuvette length of 6 mm (10 mm / 6 mm)
138.1 = molecular weight of pNA [g/mol]
13.5 = sample dilution factor Cobas Mira (0.27 mL / 0.02 mL)
W = weight of pNA [mg]
P = purity of pNA [%]

6.2 Activity

Activity is calculated as follows:

$\{(A / \varepsilon_{Fixed}) \times 1.6667 \times 13.5 \times 1000 \times Df \times 100\} / (RCF \times W) = PPU/g$

Or

$\{(A / \varepsilon_{Fixed}) \times 1.6667 \times 13.5 \times 1000 \times Df \times 100\} / RCF = PPU/mL$

where:

A	= (A_{405nm} / min) increase of absorbance per minute measured at a wavelength of 405 nm.
ε_{Fixed}	= Fixed molar extinction coefficient of pNA at 405 nm and pH 4.6 [M^{-1}. cm^{-1}] (see 5.3 and 6.1).
RCF	= Response Correction Factor (correction for non-linear reaction)
RCF	= 320.46 x $(Activity)^2$ – 132.46 x (Activity) + 102.53
Activity	= $(A / \varepsilon_{Fixed}) \times 1.6667 \times 13.5 \times 1000$)
1.6667	= correction factor for cuvette length of 6 mm (10 mm / 6mm)
13.5	= dilution factor Cobas Mira (0.27 mL / 0.02 mL)
1000	= correction factor: conversion *mol/L* to *µmol/mL*
Df	= total dilution factor of sample
W	= sample weight [g]

In Delft applied fixed molar extinction coefficient (ε_{Fixed}) of Cobas Mira plus Nr. 928866 RA 07 is determined at 10500 M^{-1}. cm^{-1} [3].
Due to the non-saturating substrate concentration in the assay mixture (see validation report), the reaction response over the measuring range is not completely linear [5]. Introduction of a response correction factor corrects for this phenomena within set measuring range.

7 ASSESSMENT

7.1 Requirements

Calculated molar extinction coefficient (ε_{daily}) of pNA for each series must fit within window: $\varepsilon_{Fixed} + 3 \times SD_{overall} > \varepsilon_{daily} > \varepsilon_{Fixed} - 3 \times SD_{overall}$
($SD_{overall}$ = overall standard deviation of the average ε_{daily} calculated from past series)
A (diluted) sample solution must have an activity fitted within the measuring-range.
The level of each control value must fit in the range : $C_{assigned} \pm 3 \times SD_{overall}$
($C_{assigned}$ = Assigned control value; $SD_{overall}$ = overall standard deviation of the average control value calculated from past series).
The *relative (absolute) difference* in level between (duplicate) *control* values within a daily series is not allowed to exceed a value of 2.8 x $RSD_{within\ day}$.

(Relative absolute difference in control values = $(|\text{control value 1} - \text{control value 2}| / \text{Average control value}) \times 100\%$; $RSD_{within\ day}$ = relative overall standard deviation "within a day" calculated from past series using control values e.g. as determined in validation of the method).

- The *relative (absolute) difference* in level between (duplicate) *sample* values is not allowed to exceed a value of 2.8 x $RSD_{within\ day}$.

(Relative absolute difference in sample values = $(|\text{sample value 1} - \text{sample value 2}| / \text{Average sample value}) \times 100\%$; $RSD_{within\ day}$ = relative overall standard deviation "within a day" calculated from past sample series with a comparable type of matrix e.g. as determined in validation of the method).

The results of the control sample must be expressed as percentage of the assigned value.
The results of the control samples must be imported into the control charts available for this method of analysis. All results have to be evaluated.

7.2 Actions

Repeat analysis when the examined molar extinction coefficient of pNA (ε_{daily}) does not comply with the requirements.
Repeat a sample analysis with an adjusted dilution when results (of the diluted sample) are out of the measuring range.
Repeat a sample series analysis (completely) when control values do not comply with the requirements.
Repeat analysis of a sample exceeding the "difference of duplicate" requirement.

7.3 Authorisation

After a training period by a for this method authorized laboratory technician, a technician will be authorized for this method when she/he succeeds on performing the test single-handed, whereby the control and selected samples meet all criteria mentioned above.

8 REFERENCES

[1] Method of analysis 62186 "Proline specific endoprotease activity determination, ELAN method"
[2] Validation Report no. 61914 (*version 1*): "Validation of the proline specific endoprotease determination, COBAS method", E.S. Edink, June 23rd, 2003; RD-LS127626.
[3] Memo *ANA-2003-0103*: "Optimization of endoprotease activity determination, ELAN method (62186) for application on COBAS", E.S. Edink, May 16th 2003; RD-LS127432.
[4] Memo *ANA-2003-0158*: "Stability of proline specific EndoPro substrate solution", E.S. Edink, July 15th, 2003; RD-LS127595.
[5] Memo *ANA-2006-0218*: "Response correction factor for proline specific endoprotease activity measurement" (R. Busink).
[6] ANA memo 2006-0218: "Response factor proline specific endoprotease activity measurement", by R. Busink, dated 20061009.

9 REMARKS

Not applicable.

10 ANNEXES

Annex 1: Cobas Mira program.
Annex 2: Response correction factor curve.

Title:
Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

Annex 1: Cobas Mira program

Programming diluents name

- Press "PROG". The program screen lights up.
- Enter "6" (system parameters). The system parameters screen light up.
- Enter "2" (diluents name). The diluents name screen lights up.
- Press "F1" (modify).
- Select position 11.
- Enter "CITR" and press "ENTER".

The diluents name has now been programmed.

Programming racks

- Press "PROG". The program screen lights up.
- Enter "5" (racks). The racks screen light up.
- Enter "1" (reagent 5s). The rack reagent 5s screen lights up.
- Enter "2" and press "ENTER". The rack reagent 5s nr 2 lights up.
- Press "F1" (modify).
- Go to rack position 1.
- Enter "1" (SR on) and press "ENTER".
- Enter "CITR" and press "ENTER".
- Go to rack position "2" for endoprotease activity determination and "4" for pNA standard determination, "ENTER". .
- Enter "1" (SR on).
- Enter "EPRO" for endoprotease activity determination and "PNA" for pNA standard determination, "ENTER".

The rack has now been programmed.

Programming the test

- Press "PROG". The program screen lights up.
- Enter "2". The test screen lights up.
- Press "F4"(test level)
- Press "1" (level 1)
- Press "X" for endoprotease activity determination or "J" for pNA standard determination, "ENTER". The test routine screen lights up.
- Enter "EPRO" for endoprotease activity determination or "PNA" for pNA standard determination, "ENTER".
- Press "ENTER". The test routine "EPRO" or "PNA" screen lights up.
- This new screen must be filled out as follows:

Title:
Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

GENERAL

- Measurement mode: "ABSORB".
- Reaction mode: enter "3" (R-S-SR1) for endoprotease activity determination and "1" (R-S) for pNA standard determination, "ENTER".
- Calibration mode: enter "1"(FACTOR), "ENTER".
- Reagent Blank: enter "1"(NOBLANK), "ENTER".
- Cleaner: enter "1" (NO), "ENTER".
- Wavelength: enter "2" (405), "ENTER".
- Decimal position: enter "4", "ENTER".
- Unit: enter "32" (ΔA/min) for endoprotease activity determination and "31" (delta A) for pNA standard determination, "ENTER".

ANALYSIS

- Post dil. factor: press "SPACE" (NO).
- Conc. factor: press "SPACE" (NO).
- Sample cycle: enter "1" for endoprotease activity determination and enter "3" for pNA standard determination, "ENTER".
- Volume: enter "20", "ENTER".
- Diluents name: enter "11" (citr), "ENTER".
- Volume: enter "10", "ENTER".
- Reagent cycle: enter "1", "ENTER".
- Volume: enter "180" for endoprotease activity determination and enter "240" for pNA standard determination, "ENTER".
- Start R1 cycle: enter "2" for endoprotease activity determination, "ENTER".
- Volume: enter "50", for endoprotease activity determination "ENTER".
- Diluents name: enter "11" (citr) for endoprotease activity determination "ENTER".
- Volume: enter "10" for endoprotease activity determination, "ENTER".

CALCULATION

- Sample limit: enter "3.500" (NO), "ENTER".
- Point: enter "CB".
- Reac. direction: enter "1" (increase), "ENTER".
- Check: enter "1"(on), "ENTER".
- Convers. factor: enter "1.0000, "ENTER".
- Offset: enter "0.0000", "ENTER".
- Test range low: press "SPACE" (NO).
- Test range high: press "SPACE" (NO).
- Norm. range low: press "SPACE" (NO).
- Norm. range high: press "SPACE" (NO).
- Number of steps

Title:
Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

Calc. step A: enter "3" (kinsearch) for endoprotease activity determination and "1" (endpoint) for pNA standard determination

- Readings first: enter "6", for endoprotease activity determination and "2" for pNA standard determination "ENTER".
- Readings last: enter "20", for endoprotease activity determination and "6" for pNA standard determination "ENTER".
- Reaction limit: press "SPACE" (NO)

CALIBRATION

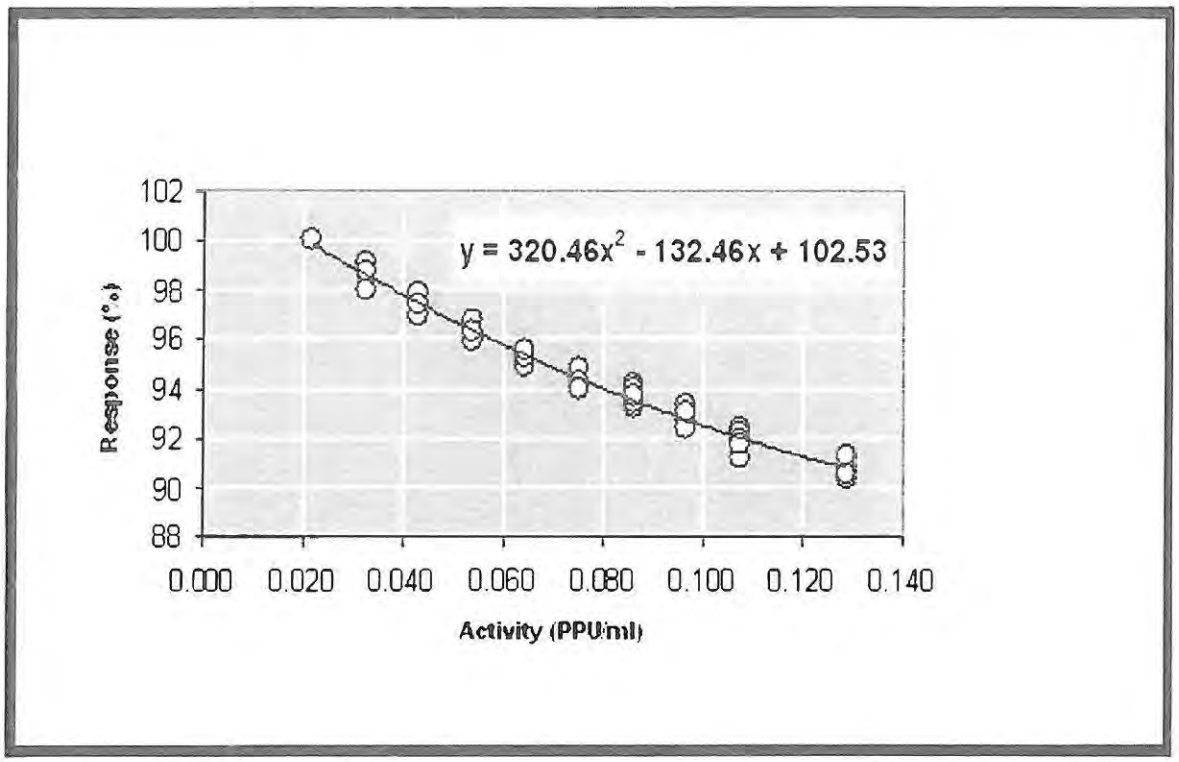
- Calib. factor: enter "1.0000", "ENTER".

CONTROL

- CS1 pos: press "SPACE" (NO).
- CS2 pos: press "SPACE" (NO).
- CS3 pos: press "SPACE" (NO).

Title:
Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

Annex 2: Response correction factor curve.



Title:
Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (Cobas Mira analyzer)

HISTORY

Version	Description of the modification
1	New Version
2	Validated method: Yes, validation results (table) added. Method in new format.
3	Cobas programming for pNA standard determination inserted. Introduction of a fixed and calculated (daily control) molar extinction coefficient for pNA standard. Wording of requirements clarified and adjusted. References updated.
4	Dilute with citric acid-phosphate buffer pH 4.6 under item 5.3. Introduce in duplicate 0.5 ml under item 5.3. Calculation formula samples adapted under item 6.2.
5	Introduction of Response Correction Factor (RCF) in calculation of activity under item 6.2. References updated under item 8. Method number from 61914 to B1944 for use in SampleManager. Centrifugation of fermentation sample suspensions added under item 5.5.1. Dilution of control sample adapted under item 5.4
6	Response correction factor calculated with 2nd degree polynomial approach instead of linear.
7	Reference 6 added. Response correction factor curve added.
8	VTW SLD-09-022 New lay out. Calculation formula corrected.

APPENDIX 2: Taxonomic identification of the *Aspergillus niger* strain GEP

Centraalbureau voor Schimmelcultures
Fungal Biodiversity Centre
Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

DSM Gist BV, DFS/SCU
t.a.v. Dhr. H. Spierenburg
Postpunt 624-0295
P.O. Box 1
2600 MA Delft

Utrecht, 14 oktober 2003

IDENTIFICATION SERVICE

Uw referentie: Dhr. H. Spierenburg

Onze referentie: det 247-2003

Hierbij sturen wij u de resultaten van onze identificatie van de door u ingezonden stammen.

DS 47447, GEP 712-9 = a	*Aspergillus niger* van Tieghem: Culture is morphologically degenerated and shows conidiophores with few biseriate heads.
PS-ETP 57738 = b	*Penicillium glabrum* (Wehmer) Westling

Uw stammen *kunnen* voor onze collectie van interesse zijn. Zonder tegenbericht nemen wij aan dat u geen bezwaren heeft dat wij deze stam(men) opnemen. Voor meer informatie kunt u telefonisch contact met ons opnemen, +31(0)302122600. Ons email adres is identification@cbs.knaw.nl.
Wanneer u contact met ons opneemt graag ons referentienummer gebruiken.

De factuur voor deze identificatie is bijgesloten.

Met vriendelijke groet,

[redacted]

Dr R.A.Samson
Hoofd Toegepast Onderzoek en Dienstverlening

Postal address: P.O. Box 85167, 3508 AD Utrecht, The Netherlands. Telephone: + 31 (0)30 2122600 E-mail: info@cbs.knaw.nl

Centraalbureau voor Scimmelcultures

Fungal Biodiversity Centre

Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

DSM Gist BV, DFS/SCU

Attn.. Mr. H. Spierenburg

Internal postal code 624-0295

P.O. Box 1

2600 MA Delft

Utrecht, 14 October 2003

IDENTIFICATION SERVICE

Your ref : Mr. H. Spierenburg Our ref : det 247-2003

Herewith we send you the results of our identification of the strains you have submitted.

DS 47447, GEP 712-9 = a	*Aspergillus niger* van Tieghem: culture is morphologically generated and shows conidiophores with few biseriate heads
PS-ETP 57738 = b	*Penicillium glabrum* (Wehmer) Westling

Your strains may be of interest for our collection. Without a message otherwise we assume that you have no objections that we include the strains (of course we objected). For more information you can contact us by telephone at +31(0)302122600. Our email address is identification@cbs.knaw.nl

Upon contact please use the reference number.

The invoice for the identifications is enclosed.

With kind regards,

Dr. R.A. Samsom

Head Applied Research and Services

APPENDIX 3: Certificates of Analysis of three different batches

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

CERTIFICATE OF ANALYSIS

Principal enzyme activity	Acid prolyl endopeptidase
IUBMB number	3.4.21.xx
Production organism	*Aspergillus niger*
Batch number	[redacted]

Parameter	Unit	Specification limits	Result
Activity	U/g		16
Lead	mg/kg	≤ 5	< 2
Coliforms	CFU/g	< 30	< 1
Salmonella	CFU/25 g	Negative by test	Negative by test
Escherichia coli	CFU/25 g	Negative by test	Negative by test
Antimicrobial activity	-	Negative by test	Negative by test
Mycotoxins	-	Negative by test	Negative by test*

Signature R&D QESH: [redacted]

Remarks (if any):

Determined mycotoxins are:
Zearalenone, Fumonisins, Trichothecenes, Aflatoxins, Ochratoxin A

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

CERTIFICATE OF ANALYSIS

Principal enzyme activity	Acid prolyl endopeptidase
IUBMB number	3.4.21.xx
Production organism	*Aspergillus niger*
Batch number	[redacted]

Parameter	Unit	Specification limits	Result
Activity	U/g		18.5
Lead	mg/kg	≤ 5	< 0.8
Coliforms	CFU/g	< 30	< 1
Salmonella	CFU/25 g	Negative by test	Negative by test
Escherichia coli	CFU/25 g	Negative by test	Negative by test
Antimicrobial activity	-	Negative by test	Negative by test
Mycotoxins	-	Negative by test	Negative by test*

Signature R&D QESH:

[redacted]

Remarks (if any):

Determined mycotoxins are:
Zearalenone, Fumonisins, Trichothecenes, Aflatoxins, Ochratoxin A

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

CERTIFICATE OF ANALYSIS

Principal enzyme activity	Acid prolyl endopeptidase
IUBMB number	3.4.21.xx
Production organism	*Aspergillus niger*
Batch number	[redacted]

Parameter	Unit	Specification limits	Result
Activity	U/g		18.7
Lead	mg/kg	≤ 5	< 0.8
Coliforms	CFU/g	< 30	< 1
Salmonella	CFU/25 g	Negative by test	Negative by test
Escherichia coli	CFU/25 g	Negative by test	Negative by test
Antimicrobial activity	-	Negative by test	Negative by test
Mycotoxins	-	Negative by test	Negative by test*

Signature R&D QESH:

[redacted]

Remarks (if any):

Determined mycotoxins are:
Zearalenone, Fumonisins, Trichothecenes, Aflatoxins, Ochratoxin A

From: Vega, Katherine
To: Highbarger, Lane A
Subject: RE: EC number question re: GRN 832
Date: Friday, August 23, 2019 1:22:54 PM

For Internal Use Only

Dear Dr. Highbarger,

As you probably know, each enzyme receives a four-digit EC number from the International Union of Biochemistry and Molecular Biology (IUBMB), the first three digits of which define the reaction catalyzed (in this case, EC 3.4.21 for serine endopeptidases); the fourth digit is a unique identifier for the specific reaction of the particular enzyme subtype. Although there is an overlap in activity between DSM's acid prolyl endopeptidase and the prolyl oligopeptidase identified as EC 3.4.21.26, DSM's enzyme can catalyze the hydrolysis of proteins in addition to oligopeptides. DSM is exploring whether acid prolyl endopeptidase would fit best within an amended EC 3.4.21.26 entry (adding hydrolysis of protein as a reaction) or it would require its own unique identifier.

Please let me know if you have any questions. Thanks and have a great weekend.

Kind regards,

Katherine

Katherine Vega, PhD | Senior Manager | Regulatory Affairs | DSM Nutritional Products | 45 Waterview Blvd | Parsippany NJ 07054 | United States | T: 1-973-257-8136 | F: 1-973-257-8414 | M: 1-908-619-0303 | katherine.vega@dsm.com

---

From: Highbarger, Lane A <Lane.Highbarger@fda.hhs.gov>
Sent: Tuesday, August 20, 2019 8:56 AM
To: Vega, Katherine <Katherine.Vega@dsm.com>
Subject: EC number question re: GRN 832

Good morning Dr. Vega,

Quick question about GRN 832, acid prolyl endopeptidase enzyme preparation. On page 7 in the table describing the classification, the row ends with IUBMB Enzyme Commission (EC) number[2]: EC 3.4.21.**xx** Acid prolyl endopeptidase

Footnote 2 states:

> [2] Due to the fact that the name proline endopeptidase is also a synonym of prolyl oligopeptidase, EC (IUBMB) number 3.4.21.26, the enzyme protein described in this dossier has been classified as such in various documents, including some premarket submissions to other countries. However, in contrast to oligopeptidases, the enzyme also acts on proteins (Edens *et al*., 2005; Kubota *et al*., 2005; Takahashi, 2013).

I need to clarify that you are describing the EC number for this enzyme as 3.4.21.26, is this correct?

Thank you.

Lane A. Highbarger, Ph.D.
Microbiology and Regulatory Review
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Food Ingredients
(w) – 240-402-1204

Highbarger, Lane A

From: Vega, Katherine <Katherine.Vega@dsm.com>
Sent: Tuesday, September 10, 2019 12:09 PM
To: Highbarger, Lane A
Cc: Srinivasan, Jannavi
Subject: RE: EC number question re: GRN 832

For Internal Use Only

Hi Lane,

Thank you for clarifying. In response to the agency's question regarding the allergenic potential of the final enzyme preparation, DSM provides the following statement (bold text):

As noted in section 6.6 of the GRAS notice, DSM did not consider the acid prolyl endopeptidase protein likely to produce an allergenic or sensitization response upon ingestion because a comparison to the amino acid sequence of known food allergens did not reveal any significant matches.

With regard to allergenicity of the fermentation media, DSM has concluded that the data that it has and the public data and information allow to conclude that there is no published or unpublished data that suggest there is a potential allergen from the fermentation media in the finished enzyme product. To reach that conclusion, DSM relied on the following data:

1. **The Enzyme Technical Association in 2004 conducted a survey of its members, and collected information on the possible presence of protein from the fermentation media in the final enzyme product. ETA provided the supporting data and information to FDA in a letter in 2005, and sent an accompanying public statement which is posted on ETA's website (ETA, 2005). The statement concludes that no potentially allergenic protein from the fermentation medium has been found in the finished enzyme. Further, ETA points out that the typical manufacturing process of enzyme preparations includes a step to separate the biomass and fermentation media from the enzyme. This step ensures the enzyme product's purity and stability, and would likely remove most proteins present in the fermentation media.**

2. **In addition, the Food Allergy Research and Resource Program (FARRP) issued a paper in August of 2013 which concluded that, because of the nature of enzymes as catalysts, they are used in very small amounts, and that the fermentation media are consumed during the enzymatic process (FARRP, 2013). It is clear that any *de minimis* amount of protein present in the fermentation media that survived the fermentation process will not cause a significant public health risk to the consumer. FARRP also underscored the fact that the proteins would likely be removed during the filtration of the enzyme product, as discussed by ETA. Further, FARRP indicated that there is no reliable assay that could be used to detect the presence of most allergenic proteins in the final enzyme products, as the proteins would likely be degraded into fragments that would not reach levels of quantitation accessible with current commercial ELISA assays. The full August 2013 statement clearly concludes that any protein allergen present in the final enzyme product would not be present at a level that requires labeling or raises a public health concern.**

DSM therefore concludes that the acid prolyl endopeptidase preparation is not likely to produce any allergenic or sensitization reactions by oral consumption. This is supported by its safe use, with no reports of allergic reactions, in various other countries where it has been marketed for the last few years for use in food processing and as an ingredient in dietary supplements.

References

ETA (2005). ETA Position On Food Allergen Labeling of Microbially Derived Enzymes Under FALCPA as it Applies to Fermentation Media Raw Materials. Available online at https://www.enzymetechnicalassociation.org/wp-content/uploads/2018/10/Allergen-psn-paper-2.pdf.

FARRP, 2013. Testing of Microbially Derived Enzymes for Potential Allergens from Fermentation Media Raw Materials. Available online at https://farrp.unl.edu/microbially-derived-enzymes.

I would be happy to answer any other questions you may have.

Kind regards,

Katherine

Katherine Vega, PhD | Senior Manager | Regulatory Affairs | DSM Nutritional Products | 45 Waterview Blvd | Parsippany NJ 07054 | United States | T: 1-973-257-8136 | F: 1-973-257-8414 | M: 1-908-619-0303 | katherine.vega@dsm.com

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From: Highbarger, Lane A <Lane.Highbarger@fda.hhs.gov>
Sent: Monday, September 9, 2019 2:41 PM
To: Vega, Katherine <Katherine.Vega@dsm.com>
Cc: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: RE: EC number question re: GRN 832

Hi Katherine,

The request about a statement that there are no allergens in the final preparation is not a new one. We ask this question to avoid requiring notifiers to include explicit information about the formulation of the growth medium.

Without an explicit formulation of the growth medium and no statement about allergens in the final formulation, our response will require the inclusion of a labeling paragraph pursuant to FALCPA.

I am including two example of how we approach the FALCPA labeling requirement:

1. In the situation where one of the big-eight food allergens is used in the final formulation, we have included a required allergen labeling paragraph. GRN 808 response letter
2. In a another situation, the notifier does not describe their growth medium formulation (permitting them to keep information proprietary), but makes a statement that the final formulation does no contain an allergen, and thus we do not include the FALCPA labeling paragraph. GRN 783 response letter

Your internal methodology to satisfy how you wish to conclude that there are no allergens in the final preparation is up to you. To reiterate, we will include a mandatory labeling requirement for the formulation unless we receive some form of statement that there are no allergens in the final formulation

If you have any additional questions, do not hesitate to contact me.

~Lane

From: Vega, Katherine <Katherine.Vega@dsm.com>
Sent: Friday, September 06, 2019 1:19 PM
To: Highbarger, Lane A <Lane.Highbarger@fda.hhs.gov>
Subject: RE: EC number question re: GRN 832

For Internal Use Only

Hi Lane,

Indeed, we did not explicitly state in the GRAS notice that there are no allergens in the final enzyme preparation; allergens would not be expected to be present, but it would be difficult to establish with absolute certainty. Can you indicate whether this is a new requirement? My understanding is that potential allergenicity is a key consideration in establishing the safety of the substance. However, a conclusion that the substance is GRAS in the context of its intended use would not necessarily require a statement to the absolute absence of allergens.

In the case of enzyme preparations, DSM and other members of the food enzyme industry have generally relied on a comparison of the enzyme protein amino acid sequence to that of known allergens, and a position paper by the Enzyme Technical Association (ETA) that provides a science-based approach to asserting that the use of microbially-derived enzyme preparations in food production would not present any public health concerns related to allergy, based on consideration of how the enzymes are produced and used (at very low levels) in food production, and the absence of any reported allergic reactions linked to fermentation media components, which in some cases may include soy- or wheat-derived ingredients. This has been the approach used in dozens of GRAS notices for enzyme preparations in the GRAS Notice Inventory.

ETA shared its position paper and associated report with FDA in September 12, 2005. To date, ETA has not received any comments from the agency. ETA members have been and continue operating under the presumption that FDA would have responded if they had found glaring deficiencies in ETA's argument and reason to be concerned about public health.

We would appreciate any further insight you can provide before we make any formal statements related to potential allergenicity. Please let me know if you have any questions.

Kind regards,

Katherine

Katherine Vega | Senior Manager | Regulatory Affairs | DSM Nutritional Products | 45 Waterview Blvd | Parsippany NJ 07054 | United States | T: 1-973-257-8136 | F: 1-973-257-8414 | M: 1-908-619-0303 | katherine.vega@dsm.com

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From: Highbarger, Lane A <Lane.Highbarger@fda.hhs.gov>
Sent: Friday, September 6, 2019 7:53 AM
To: Vega, Katherine <Katherine.Vega@dsm.com>
Subject: RE: EC number question re: GRN 832

Hi Katherine,

I have one more question. You do not state that there are no allergens in the final formulation. If this is a true, would please provide me with an email stating that there are no allergens present in the final enzyme formulation?

Thank you.

~Lane

From: Vega, Katherine <Katherine.Vega@dsm.com>
Sent: Friday, August 30, 2019 9:03 AM
To: Highbarger, Lane A <Lane.Highbarger@fda.hhs.gov>
Subject: RE: EC number question re: GRN 832

For Internal Use Only

Dear Dr. Highbarger,

Indeed, the enzyme preparation complies with FCC, 11th edition.

Please let me know if you have any other questions.

Kind regards,

Katherine

Katherine Vega, PhD | Senior Manager | Regulatory Affairs | DSM Nutritional Products | 45 Waterview Blvd | Parsippany NJ 07054 | United States | T: 1-973-257-8136 | F: 1-973-257-8414 | M: 1-908-619-0303 | katherine.vega@dsm.com

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From: Highbarger, Lane A <Lane.Highbarger@fda.hhs.gov>
Sent: Monday, August 26, 2019 10:38 AM
To: Vega, Katherine <Katherine.Vega@dsm.com>
Subject: RE: EC number question re: GRN 832

Dear Dr. Vega,

Thank you for your response to the EC Number question.

I have one additional question. Your dossier never explicitly states which FCC edition the enzyme preparation specification conform to, is the monograph the 11th edition, 2018?

Thank you for your attention to this matter.

Lane A. Highbarger, Ph.D.
Microbiology and Regulatory Review
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Food Ingredients
(w) – 240-402-1204

From: Vega, Katherine <Katherine.Vega@dsm.com>
Sent: Friday, August 23, 2019 1:22 PM
To: Highbarger, Lane A <Lane.Highbarger@fda.hhs.gov>
Subject: RE: EC number question re: GRN 832

Dear Dr. Highbarger,

As you probably know, each enzyme receives a four-digit EC number from the International Union of Biochemistry and Molecular Biology (IUBMB), the first three digits of which define the reaction catalyzed (in this case, EC 3.4.21 for serine endopeptidases); the fourth digit is a unique identifier for the specific reaction of the particular enzyme subtype. Although there is an overlap in activity between DSM's acid prolyl endopeptidase and the prolyl oligopeptidase identified as EC 3.4.21.26, DSM's enzyme can catalyze the hydrolysis of proteins in addition to oligopeptides. DSM is exploring whether acid prolyl endopeptidase would fit best within an amended EC 3.4.21.26 entry (adding hydrolysis of protein as a reaction) or it would require its own unique identifier.

Please let me know if you have any questions. Thanks and have a great weekend.

Kind regards,

Katherine

Katherine Vega, PhD | Senior Manager | Regulatory Affairs | DSM Nutritional Products | 45 Waterview Blvd | Parsippany NJ 07054 | United States | T: 1-973-257-8136 | F: 1-973-257-8414 | M: 1-908-619-0303 | katherine.vega@dsm.com

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From: Highbarger, Lane A <Lane.Highbarger@fda.hhs.gov>
Sent: Tuesday, August 20, 2019 8:56 AM
To: Vega, Katherine <Katherine.Vega@dsm.com>
Subject: EC number question re: GRN 832

Good morning Dr. Vega,

Quick question about GRN 832, acid prolyl endopeptidase enzyme preparation. On page 7 in the table describing the classification, the row ends with IUBMB Enzyme Commission (EC) number[2]: EC 3.4.21.**xx** Acid prolyl endopeptidase

Footnote 2 states:

[2] Due to the fact that the name proline endopeptidase is also a synonym of prolyl oligopeptidase, EC (IUBMB) number 3.4.21.26, the enzyme protein described in this dossier has been classified as such in various documents, including some premarket submissions to other countries. However, in contrast to oligopeptidases, the enzyme also acts on proteins (Edens *et al*., 2005; Kubota *et al*., 2005; Takahashi, 2013).

I need to clarify that you are describing the EC number for this enzyme as 3.4.21.26, is this correct?

Thank you.

Lane A. Highbarger, Ph.D.
Microbiology and Regulatory Review
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Food Ingredients
(w) – 240-402-1204

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