

Supporting document 1

Risk and technical assessment report (at Approval) –
Application A1098

Serine Protease (Chymotrypsin) as a Processing Aid (Enzyme)

Executive summary

Application A1098 seeks approval for the use of a new enzyme, a serine protease (chymotrypsin), sourced from a genetically modified strain of *Bacillus licheniformis* containing genes for serine protease from *Nocardiopsis prasina*, as a processing aid. The stated purpose of this enzyme, namely production of peptides and small proteins with different functionalities via the hydrolysis of animal or vegetable proteins, is clearly articulated in the Application. The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified to be effective in achieving its stated purpose. The enzyme preparation meets international purity specifications for enzymes used in the production of food.

There are no public health and safety issues associated with the use of the enzyme preparation, containing a serine protease (chymotrypsin) produced by genetically modified *B. licheniformis*, as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic, pathogenic or sporogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Further, *B. licheniformis* has a history of safe use as the production organism for a number of enzyme processing aids that are already permitted in the Code.
- Residual enzyme may be present in the final food but would be inactive.
- Bioinformatic analysis indicated that the enzyme has no biologically relevant homology to known protein allergens or toxins.
- The enzyme caused no observable effects at the highest tested doses in a 90-day toxicity study in rats. The NOAEL was 500 mg Total Organic Solids (TOS)/kg body weight per day, the highest dose tested.
- The enzyme preparation was not genotoxic *in vitro*.

Based on the reviewed toxicological data, it is concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) 'not specified' is appropriate. A dietary exposure assessment is therefore not required.

Table of Contents

EXECUTIVE SUMMARY	1
1 INTRODUCTION	2
1.1 OBJECTIVES OF THE ASSESSMENT	2
2 FOOD TECHNOLOGY ASSESSMENT	2
2.1 CHARACTERISATION OF SERINE PROTEASE (CHYMOTRYPSIN).....	2
2.1.1 <i>Identity of the enzyme</i>	2
2.1.2 <i>Enzymatic properties</i>	3
2.1.3 <i>Physical properties</i>	3
2.2 PRODUCTION OF THE ENZYME	3
2.3 SPECIFICATIONS.....	4
2.4 TECHNOLOGICAL FUNCTION OF THE ENZYME	4
2.5 FOOD TECHNOLOGY CONCLUSION	4
3 HAZARD ASSESSMENT	5
3.1 BACKGROUND.....	5
3.1.1 <i>Chemistry</i>	5
3.1.2 <i>Description of the genetic modification</i>	5
3.1.3 <i>Scope of the hazard assessment</i>	6
3.2 HAZARD OF THE PRODUCTION ORGANISM – <i>B. LICHENIFORMIS</i>	6
3.3 HAZARD OF THE ENCODED PROTEIN – SP-C.....	6
3.3.1 <i>Bioinformatic analyses for potential allergenicity</i>	7
3.3.2 <i>Bioinformatic analyses for potential toxicity</i>	8
3.4 EVALUATION OF UNPUBLISHED TOXICITY STUDIES	8
3.4.1 <i>Genotoxicity</i>	8
3.4.2 <i>Subchronic toxicity study</i>	9
3.5 HAZARD ASSESSMENT CONCLUSIONS.....	10
4 CONCLUSION	10
5 REFERENCES	10

1 Introduction

FSANZ received an application from Novozymes Australia Pty Ltd seeking approval for the enzyme serine protease (chymotrypsin specificity, EC 3.4.21.1) as a processing aid to be used in the production of food. The enzyme is produced from a genetically modified (GM) strain of *Bacillus licheniformis* expressing two serine protease (chymotrypsin) genes from *Nocardiopsis prasina*.

The Applicant proposes to use serine protease (chymotrypsin) to produce smaller proteins and peptides from various animal and vegetable proteins. The Applicant claims that the smaller proteins and peptides can be used as ingredients in a variety of food and beverage products for different functionalities (e.g. protein fortification, reduced allergenicity, improved emulsifying capacity).

1.1 Objectives of the Assessment

As there are no permissions for the enzyme serine protease (chymotrypsin) currently in the *Australia New Zealand Food Standards Code* (the Code), any application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market assessment.

The objectives of this risk assessment are to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid
- evaluate any potential public health and safety concerns that may arise from the use of serine protease (chymotrypsin) as a processing aid.

2 Food Technology Assessment

2.1 Characterisation of serine protease (chymotrypsin)

2.1.1 Identity of the enzyme

Information regarding the identity of the enzyme that was taken from the Application has been verified using enzyme nomenclature references (JECFA 2012; IUBMB 2014). Additional information has also been included from these references.

Generic common name	Serine protease
Accepted IUBMB name:	Chymotrypsin
IUBMB enzyme nomenclature:	EC 3.4.21.1
C.A.S. number:	9004-07-3
Other names:	Chymotrypsins A and B; α -chymar ophth; avazyme; chymar; chymotest; enzeon; quimar; quimotrase; α -chymar; α -chymotrypsin A; α -chymotrypsin

Reaction – enzyme preparation: Protease with preferential cleavage at the C-terminus (carboxyl end) adjacent to tyrosine, phenylalanine, leucine residues and methionine

Commercial name: CTL3 conc BG

JECFA have also assessed serine protease (chymotrypsin) from *N. prasina* expressed in *B. licheniformis* (EC number 3.4.21.1) (JECFA 2012).

2.1.2 Enzymatic properties

Chymotrypsin belongs to the superfamily of serine proteases that comprise around one third of all proteases (Hedstrom 2002). The serine protease enzyme that is the subject of this application has chymotrypsin specificity and catalyses the hydrolysis of peptide bonds in proteins to produce smaller proteins and peptides of variable lengths. Its catalytic characteristic is that it cleaves on the carboxyl end (C-terminal) of phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr) on peptide chains; in addition, it also catalyses the hydrolysis of peptide bonds at the carboxyl end of other amino acids, primarily methionine (Met) and leucine (Leu), albeit at a slower rate (Choudhuri et al. 2012).

The enzyme is used for partial or extensive hydrolysis of both animal and vegetable proteins (e.g. whey, casein, gluten and proteins from soy, corn, rice, peas, lentils, meat and fish). The hydrolysed proteins are then used as ingredients in a variety of food and beverage products for different functionalities (e.g. protein fortification, reduced allergenicity, improved emulsifying capacity).

The enzyme preparation (granulated powder) is active during the processing of the protein-containing food/food ingredient, with deactivation of the enzyme occurring when it is denatured by the high temperatures used in the manufacturing process. The enzyme has no action or function in the final food product.

2.1.3 Physical properties

The commercial enzyme preparation is supplied as a light brown granulate in which the enzyme is stabilised with sucrose.

2.2 Production of the enzyme

The enzyme is produced by a submerged fed-batch pure culture fermentation process which is common for the production of many food enzymes.

The production steps can be summarised as a fermentation process, a purification process, formulation of the final commercial enzyme preparation, and a quality control process. The raw materials used are food grade. More detail of the individual steps is provided in the Application.

The fermentation process involves two steps, the initial inoculum fermentation to produce enough of the microorganism for the production fermentation and then the main fermentation. The production organism secretes the enzyme into the fermentation broth.

The downstream processing steps taken after the main fermentation to produce the enzyme consist of: removal of the production strain and other solids, ultrafiltration to concentrate and further purify the enzyme, filtration, stabilisation using sucrose, and finally further concentration and granulation.

2.3 Specifications

There are international specifications for enzyme preparations used in the production of food that have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2001) and the Food Chemicals Codex (U.S. Pharmacopeial Convention 2014). The Applicant states that the serine protease (chymotrypsin) preparation meets these specifications. See Table 1 for a comparison of the product specifications and the JECFA specifications. The specification monographs listed above are primary references for specifications in Standard 1.3.4 – Identity and Purity.

Table 1: Product specifications for commercial serine protease (chymotrypsin) preparation compared to JECFA specifications for enzymes

Analysis	Product spec	JECFA spec
Lead (mg/kg)	≤ 5	≤ 5
Arsenic (mg/kg)	≤ 3	
Mercury (mg/kg)	≤ 0.5	
Cadmium (mg/kg)	≤ 0.5	
Standard plate count (cfu/g)	≤ 10,000	
Coliforms (cfu/g)	≤ 30	≤30
Salmonella (in 25 g)	Not detected	Absent
E. coli (in 25 g)	Not detected	Absent

The Application states that the serine protease (chymotrypsin) preparation contains no detectable antimicrobial activity. This is also a requirement of the JECFA specifications for enzymes used in food processing. The Applicant also stated that the product complies with the JECFA purity recommendations for mycotoxins.

The final enzyme preparation meets international specifications for enzyme preparations used in the production of food.

The enzyme preparation does not contain any allergenic substances that would require mandatory labelling declarations.

2.4 Technological function of the enzyme

The enzyme is used as a processing aid for partial or extensive hydrolysis of proteins from both animal and vegetable sources (e.g. casein, whey, gluten, and proteins from meat, fish, corn, soy, rice, peas and lentils). Based on the information provided by the company, the serine protease (chymotrypsin) has benefits that include higher yields of soluble proteins and peptides, milder process conditions, reduced amounts of salts (compared with acid hydrolysed protein) and protein hydrolysates with controlled peptide profile due the specificity of the enzyme.

2.5 Food technology conclusion

The stated purpose for this enzyme, namely for use as a processing aid in the production of protein hydrolysates, is clearly articulated in the Application. The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme preparation meets international purity specifications.

3 Hazard Assessment

3.1 Background

3.1.1 Chemistry

Details of the chemistry of the serine protease (chymotrypsin) (henceforth referred to as SP-C¹) produced by *B. licheniformis* including relevant physicochemical and enzymatic properties, and product specifications, are provided in the Food Technology Assessment (Section 2).

3.1.2 Description of the genetic modification

SP-C is produced by a GM production strain of *B. licheniformis*, derived from host strain Ca63 (DSM 9552) and an intermediate strain SJ6370, which expresses two serine protease (chymotrypsin) genes (henceforth referred to as *sp-c*) encoding the same protein.

The *sp-c* expression cassette comprises:

- a triple tandem promoter comprising three promoter fragments originating from three different donor organisms; this promoter was inserted into two loci, *amyL* and *xylA*, of strain SJ6370 prior to the transformation event that added the serine protease coding sequences and terminator
- the two *sp-c* coding sequences (referred to as the tandem gene) derived from the actinomycete *Nocardiopsis prasina*. While members of the genus *Nocardiopsis* are regarded as human pathogens (Yassin et al. 1997), the *sp-c* coding sequences are not pathogenic in themselves and do not cause pathogenic symptoms in humans.
- a *B. licheniformis* terminator.

A double homologous recombination strategy, using two different plasmids, was employed to insert into SJ6370 the tandem gene/terminator construct at the promoter sites present at the *amyL* and *xylA* loci. Thus the production strain contains two copies of the *sp-c* cassette, and hence four copies of genes that encode the same SP-C protein. Southern blot and sequence analysis indicate that the intended modifications are indeed present at the *amyL* and *xylA* loci and have the expected sequence. Southern blot analyses showed there are no other coding sequences from the plasmids present in the final production strain; this means, in particular, there are no antibiotic resistance genes present.

To test the stability of the insert in the production strain, Southern blot analysis, using a probe derived from part of the tandem gene, was done on DNA from cells from three separate end-of-production batches and was compared to reference genomic DNA of the production strain. The band patterns (number and size) obtained for all the production batches were identical to the band pattern of the reference production strain, thus indicating stability of the insert.

¹ The Applicant has requested the identity of the serine protease gene and protein be considered confidential commercial information. FSANZ has agreed with this request and therefore, chosen an abbreviation for use in this document that is entirely artificial and does not reflect any official nomenclature.

3.1.3 Scope of the hazard assessment

The hazard of SP-C derived from the *B. licheniformis* production strain was evaluated by considering the:

- hazard of the production organism, including any history of safe use in food production processes
- hazard of the encoded protein, including potential allergenicity
- toxicity studies on the enzyme preparation intended for commercial use.

3.2 Hazard of the production organism – *B. licheniformis*

The production strain (S10-34zEK4), containing four *sp-c* genes, is genetically engineered from strain SJ6370 and has undergone classical mutagenesis to increase productivity. In turn, SJ6370 has been derived from the host strain Ca63, a natural isolate of *B. licheniformis*, through a series of targeted steps designed to inactivate the gene essential for sporulation and genes encoding two proteases.

Morphological and biochemical characteristics of the host strain Ca63 (DSM 9552) are consistent with *B. licheniformis* (Priest et al. 1998; De Vos et al., 2009). The Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) confirmed the species designation in 1995.

Virulence is not generally associated with *B. licheniformis*, asporogenic forms of which are designated as Risk Group 1 (RG1) – agents that are not associated with disease in healthy adult humans (NIH 2013). There are, however, strains of *B. licheniformis* that have been implicated as an opportunistic pathogen in human infection of immunocompromised individuals and neonates (EPA 1997; De Vos et al., 2009). Toxin-producing isolates of *B. licheniformis* have been isolated from raw milk, commercially-produced baby food and other foods involved in food poisoning incidents.

B. licheniformis is widely used to produce food-grade enzymes and other food products (Schallmey et al. 2004). FSANZ has previously assessed the safety of *B. licheniformis* as the source organism for a number of food processing aids and the following enzymes derived from *B. licheniformis* (both GM and non-GM) are listed as permitted in Standard 1.3.3 of the Code: α -amylase, maltotetrahydrolase, pullulanase, glycerophospholipid cholesterol acyltransferase, and serine proteinase.

Data submitted with the application indicate that the *B. licheniformis* production strain is not detectable in the final enzyme preparation to be used as a food processing aid. The organism is removed during a multi-step recovery of the purified enzyme following submerged fed-batch pure culture fermentation. A pre and germ filtration stage towards the end of the manufacturing process involves filtrations at defined pH and temperature intervals that result in an enzyme concentrate solution free of the production strain.

3.3 Hazard of the encoded protein – SP-C

Many organisms across eukaryotes, prokaryotes, archae and viruses naturally produce serine proteases with chymotrypsin-like activity and, indeed, chymotrypsin-like proteases are the most abundant in nature with over 240 being recognised (Hedstrom 2002).

A BLASTP² sequence comparison of the SP-C protein with human, bovine and porcine chymotrypsin protein sequences (National Center for Biotechnology Information - <http://www.ncbi.nlm.nih.gov/>) indicates that there is less than 50% identity.

The SP-C produced by *B. licheniformis* is a 188 amino acid protein and has preferential cleavage at Tyr, Phe, Leu and Met. The safety of this enzyme has been assessed by JECFA (Choudhuri et al. 2012) and allocated an Acceptable Daily Intake (ADI) of 'not specified'.

The intention is that the enzyme preparation (designated CTL3 conc BG and comprising sucrose, water and SP-C, formulated to achieve the desired enzyme activity – see Table 2) is used as a processing aid for the production of partly or extensively hydrolysed proteins of vegetable and animal origin. The enzyme is expected to be inactivated by high temperature once the desired degree of hydrolysis is obtained, and therefore any residual enzyme in the final food would be present as denatured protein and undergo normal proteolytic digestion in the gastrointestinal tract.

Bioinformatic analyses were performed to assess the similarity to known allergens and toxins of the chymotrypsin.

3.3.1 Bioinformatic analyses for potential allergenicity

The *in silico* analyses compared the SP-C sequence with known allergens in two datasets:

- the FARRP (Food Allergy Research and Resource Program) dataset within AllergenOnline (University of Nebraska; <http://www.allergenonline.org/>).
- the dataset within the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. (<http://www.allergen.org/>).

Three types of analyses were used to search the databases:

- a) the FASTA algorithm (Pearson and Lipman 1988) version 3.4, using the BLOSUM50 scoring matrix (Henikoff and Henikoff 1992). The E-score³ generated by this indicates whether there are any alignments that meet or exceed the Codex Alimentarius (Codex 2003) FASTA alignment threshold (35% identity over 80 amino acids) for potential allergenicity. This threshold aims to detect potential conformational IgE-epitopes.
- b) The same as a) but with scaling enabled in order to find any matches that may have high identity over windows shorter than 80 amino acids.
- c) The Needleman-Wunsch global alignment algorithm (Needleman and Wunsch 1970) in the program package EMBOSS (<http://www.ebi.ac.uk/Tools/emboss/>). This explores all possible alignments and chooses the best one (the optimal global alignment). Hence it can identify those hits with more than 35% identity over the full length of the sequences.

In none of the analyses was any significant homology with any known allergens found.

² the BLASTP (Basic Local Alignment Search Tool Protein) algorithm (Altschul *et al.*, 1997) is used to compare a protein sequence with a database of protein sequences.

³ Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. Commonly, for protein-based searches, hits with E-values of 10⁻³ or less and sequence identity of 25% or more are considered significant although any conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxeavanis 2005).

The conclusion from these bioinformatic analyses is that SP-C from *B. licheniformis* does not show biologically relevant homology to any known allergen and on this basis is unlikely to be allergenic.

3.3.2 Bioinformatic analyses for potential toxicity

The SP-C sequence was compared to sequences present in the UniProt database (<http://www.uniprot.org/>) containing entries from Swiss-Prot and TrEMBL and using the term 'toxin' to refine the search. The comparison method used a ClustalW 2.0.10 sequence alignment program (<http://www.clustal.org/clustal2/>) (Larkin et al. 2007) to align each sequence from the database with the chymotrypsin sequence.

The greatest homology found was 22.3%, which indicates that SP-C is unlikely to be toxic.

3.4 Evaluation of unpublished toxicity studies

Report submitted

Nielsen, L.A.; Pedersen, P.B.; Lund, T. Ø. (2013). Summary of toxicity data. Serine endopeptidase, batch PPA 26797 *Bacillus licheniformis*. File ID # 2013-03986-01, Novozymes A/S (unpublished).

Unpublished toxicity studies on a representative SP-C preparation were submitted by the Applicant and independently evaluated by FSANZ. These studies comprised two genotoxicity test – an Ames test conducted in accordance with OECD test Guideline 471 (OECD 1997a) and an *in vitro* chromosome aberration test conducted in accordance with OECD test Guideline 473 (OECD 1997b) – and a 13 week oral toxicity study in rats conducted in accordance with OECD Test Guideline 408 (OECD 1998). The test substance was chymotrypsin preparation (designated as batch PPA26797) prepared to the manufacturer's specifications. The test substance was supplied in liquid form (dissolved in water) and differed from the actual product, CTL3 conc BG, which would be supplied in granulate form comprising 85% (w/w) SP-C (TOS). A comparison of PPA26797 and CTL3 conc BG is given in Table 2.

Table 2: Comparison of PPA26797 and CTL3

Characteristic	PPA26797	CTL3 conc BG
Activity	54.6 KMCU/g ¹	650 KMCU/g
Water (% w/w)	88.1	5
Ash (% w/w)	2.4	-
Sucrose (% w/w)	-	10
Total Organic Solids (TOS) (% w/w)	9.5	85

¹ KMCU =Kilo Microbial Chymotrypsin Units (calculation method provided by Applicant)

3.4.1 Genotoxicity

Individual studies

Pedersen, P.B. (2009). Serine endopeptidases, PPA 26797: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*. Study ID # 20078045, Novozymes A/S (unpublished).

Whitwell, J. (2009). Serine endopeptidases (PPA26797): Induction of chromosome aberrations in cultured human peripheral blood lymphocytes. Ref # 20076030, Covance (unpublished).

The results of these *in vitro* studies are summarised in Table 3. Negative (water vehicle) and positive controls were tested in each study and gave expected results. The chromosomal aberration study comprised two experiments that involved two different exposure times and different dose concentrations. It is concluded that the SP-C preparation did not induce chromosome aberrations or gene mutations when tested up to 5,000 µg/ml (an acceptable maximum concentration for such studies).

Table 3: Summary of genotoxicity test results

Test	Test system	Test article	Concentration or dose range	Result
Bacterial reverse mutation (Ames test)	<i>Salmonella typhimurium</i> strains TA 98, 100, 1535 & 1537 <i>Escherichia coli</i> strain WP2 <i>uvrApKM101</i>	SP-C obtained from <i>B. licheniformis</i> (Batch No.PPA26797) Water vehicle	156–5000 µg test substance (in solution) per millilitre ± S9 ¹	Negative
<i>In vitro</i> chromosome aberration assay	Human lymphocytes prepared from the pooled blood of 3 male donors	As above	1 st experiment: 1582, 2813 and 5000 µg/ml ± S9 2nd experiment: 1311, 2048 and 4000 µg/ml –S9 and 2048, 3200 and 5000 µg/ml +S9	Negative

¹ S-9 = metabolic activation system comprising liver preparation from rats induced with Aroclor 1254

3.4.2 Subchronic toxicity study

Individual study

Glerup, P. (2009). Serine endopeptidases (PPA 26797): A 13-week oral (gavage) toxicity study in rats. Study # 66063, LAB Research Scantox (unpublished).

[The study contained a statement of compliance with principles of GLP and a quality assurance statement. It was conducted in accordance with OECD Test Guideline 408 (OECD 1998).]

The SP-C preparation (Batch No. PPA26797) was administered by gavage to four groups of 10 SPF Sprague Dawley (strain Ntac:SD) rats/sex at doses of 0, 50, 165.1 or 500 mg TOS/kg bw/d for 90/91 days (water vehicle). Rats were sourced from Taconic Europe A/S (Ejby, Denmark), were approximately 5 weeks old and had body weights within a range of ± 20 g for each sex at the commencement of dosing. Rats were housed under standard conditions, with food and water available *ad libitum*. Standard gross toxicological endpoints were recorded during the treatment period (deaths, clinical signs, bodyweight and food consumption). Ophthalmoscopy was performed prior to treatment and on day 90. Pre-treatment and before termination, the animals were examined with respect to motor activity (open field test) and reactivity to different types of stimuli. Blood was collected on day 91 for the analysis of standard haematology and clinical chemistry parameters and a bone marrow smear was taken at necropsy. Following sacrifice, rats were necropsied, organ weights recorded and standard tissues prepared for histopathology.

There were no deaths, clinical signs, motor activity abnormalities or ophthalmic abnormalities attributable to administration of the test substance. Bodyweight gain and food consumption were comparable across all groups. There was no treatment-related effect on any haematology or clinical chemistry parameter. There were no treatment-related macroscopic abnormalities, differences in organ weights or histopathological findings. The NOAEL (No Observed Adverse Effect Level) for both males and females was 500 mg TOS/kg bw/d, the highest dose tested.

3.5 Hazard assessment conclusions

There are no public health and safety issues associated with the use of CTL3 conc BG, containing a serine protease (chymotrypsin) produced by GM *B. licheniformis*, as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic, pathogenic or sporogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Further, *B. licheniformis* has a history of safe use as the production organism for a number of enzyme processing aids that are already permitted in the Code.
- Residual enzyme may be present in the final food but would be inactive.
- Bioinformatic analysis indicated that the enzyme has no biologically relevant homology to known protein allergens or toxins.
- The enzyme caused no observable effects at the highest tested doses in a 90-day toxicity study in rats. The NOAEL was 500 mg TOS/kg bw per day, the highest dose tested.
- The enzyme preparation was not genotoxic *in vitro*.

Based on the reviewed toxicological data, it is concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) 'not specified' is appropriate. A dietary exposure assessment is therefore not required.

4 Conclusion

This risk assessment considered the technological suitability, potential hazard of the production microorganism and the potential hazard of serine protease (chymotrypsin).

It was concluded that the proposed use of the enzyme was technologically justified in the form and prescribed amounts, and was demonstrated to be effective. The evidence presented was sufficient to determine that no safety concerns with production microorganisms or the enzyme exist. Thus serine protease (chymotrypsin) derived from *B. licheniformis* is unlikely to pose any health risk when used as a food processing aid.

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