A Thermolysin Enzyme
from *Anoxybacillus caldiproteolyticus* Rokko

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: IFF AUSTRALIA PTY LTD (Trading as Danisco Australia Pty Ltd)

8th November 2023

CONTENTS

List of Figures

APPENDICES :

APPENDICES - CONFIDENTIAL :

1 General information

1.1 Applicant details

(a) Applicant:
This application is made by Danisco Australia (IFF)

(b) Company:
Danisco Australia Pty Ltd

(c) Address:
IFF Australia Ltd
[redacted]

(d) Contact Details:
[redacted]

(e) Email address:
See above.

(f) Nature of Applicants Business:
Danisco Australia Pty Ltd – A subsidiary of International Flavors and Fragrances Inc (IFF), manufacturer/marketer of specialty food ingredients, food additives and food processing aids. Danisco Australia is also an affiliate of Genencor International Ltd, the manufacturer of the product and another subsidiary of International Flavors and Fragrances Inc (IFF). Entity Relationship letter, Section 1.1.1.

(g) Details of Other Individuals.:
No other individuals, companies or organisations are associated with this application.

1.1.1 Entity relationship statement

September 11, 2022

Where science
& creativity meet

Re: Statement Regarding Subsidiary Entities

To Whom It May Concern,

We are pleased to inform you that on February 1, 2021, International Flavors & Fragrances Inc. ("IFF") completed its previously announced combination of IFF and the Nutrition & Biosciences business (the "N&B Business") of DuPont de Nemours, Inc. ("DuPont") (the "Merger").

As part of the Merger, various legal entities associated with the N&B business, were transferred by DuPont to IFF. The N&B legal entities listed on the following website are among the legal entities associated with the N&B business that were transferred by DuPont to IFF.

https://www.iff.com/where-we-operate/subsidiaries

For your purposes, please consider the following N&B legal entities as operating under IFF ownership as IFF Health & Biosciences business.

Danisco Australia Pte. Ltd.
97 Waterloo Rd
Macquarie Park NSW 2113
Australia

Health & Biosciences
97 Waterloo Rd,
Macquarie Park NSW
2113, Australia

iff.com

Thank you for your attention.

Sincerely,

[redacted]

IFF Australia Pty Ltd

1.2 Purpose of the application

This application seeks to modify Schedule 18 to Standard 1.3.3 Processing Aids to permit the use of a new Processing Aid, Thermolysin from the Rokko strain of *Anoxybacillus caldiproteolyticus* (formerly classified as *Geobacillus caldoproteolyticus*) that has not been genetically modified. The intended use of the processing aid is protein processing to improve the physical properties of foods. Thermolysin may be used for processing dairy foods, egg, meat and fish, protein concentrates and isolates, yeast processing, and in beer brewing.

This application is made solely on behalf of IFF Health & Biosciences (IFF), the manufacturer/marketer of the Processing Aid. When approved, the Processing Aid would be available for use by any food manufacturer in Australia and New Zealand.

1.3 Justification for the application

1.3.1 Regulatory impact information

A. Costs and Benefits of the application

Thermolysin is an enzyme produced by submerged fermentation of the *Anoxybacillus caldiproteolyticus* Rokko strain. The enzyme is characterised as a thermostable extracellular metalloendopeptidase (EC 3.4.24.27). A collection of information detailed in Section 3 supports the safety of the production organism and the enzyme for use in the applications outlined in Section 4.

The enzyme is intended to be used for protein hydrolysis in the processing of various protein containing foods. Thermolysin may be used for processing dairy foods, egg, meat and fish, protein concentrates and isolates, yeast processing, and in beer brewing.

More information on the benefits of this enzyme can be found in Section 2.4.

Enzyme preparations are widely used as processing aids in the manufacture of food products. In 2018 FSANZ approved the use of thermolysin from *Anoxybacillus caldiproteolyticus* strain TP-7 as a processing aid for the manufacture or processing of foods under Application A1146. Approval of this application would provide food processors with an alternate source of the same enzyme, also from a non-genetically modified strain, offering the benefits and advantages as discussed in Section 2.4.

It is not anticipated that the introduction of this alternate source of Thermolysin will have a significant impact to costs for the consumer, as outlined by the applicant in A1146, the availability of a range of food products is the same, irrespective of the method employed to achieve the results. However, the availability of an alternate source of thermolysin is likely to have a positive impact in the marketplace by introducing some competition.

B. Impact on international trade

The inclusion of this alternative source of Thermolysin from *Anoxybacillus caldiproteolyticus* Rokko in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products produced with this enzyme product and reduce technical barriers to trade.

1.4. Support for the application

No marketing or promotional activities have been undertaken for Thermolysin derived from *Anoxybacillus caldiproteolyticus* Rokko in the Australia/New Zealand market. Hence at this stage, no requests from food manufacturers are provided in support of this application.

However, the need and justification for use of the processing aid are discussed in Section 1.3, and it is anticipated that support from the food processing industry will be submitted during the period for public comment on the application Draft Regulatory Measure/Assessment Report.

1.5. Assessment procedure

This application seeks to modify Schedule 18 to Standard 1.3.3 Processing Aids to permit the use of a Processing aid that is currently not permitted. Based on guidance in the Application Handbook, IFF considers General Procedure Level 1 (up to 350 hours) to be the appropriate procedure for assessment of the application.

1.6. Confidential commercial information (CCI)

Certain (identified) technical and manufacturing information included in Appendices L-U and other information including amino acid sequences labelled with Confidential Commercial information is regarded by the applicant as **Confidential Commercial Information** and is provided in the application strictly on this basis. This information is the result of a significant research and development effort and investment by the applicant; it is not in the public domain and is considered as either proprietary or commercially sensitive. It would be disadvantageous to the applicant if this information were released into the public domain.

1.7. Exclusive commercial capturable benefit (ECCB)

According to Section 8 of the FSANZ Act, this application is not expected to confer Exclusive Capturable Commercial Benefit (ECCB).

1.8. International and other national standards

Thermolysin from *Anoxybacillus caldiproteolyticus* Rokko meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex. Thermolysin has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application. Protease from *Geobacillus caldoproteolyticus* (*Anoxybacillus caldiproteolyticus*) is listed in CODEX IPA Database for processing aids .

Thermolysin from *Anoxybacillus caldiproteolyticus* Rokko has been determined to be Generally Recognized as Safe (GRAS) as a processing aid for food protein processing by a panel of scientific experts in the USA (Appendix N, **Confidential Commercial Information**). It is also approved for various purposes in both France (Appendix L, **Confidential Commercial Information**), Denmark (Appendix M, **Confidential Commercial Information**), Mexico,(Appendix K) and China (Appendix O, **Confidential Commercial Information**).

1.9. Statutory declaration

I, [redacted]

of [redacted]

make the following declaration under the Oaths and Declaration Act 1959:

the information provided in this application fully sets out the matters required; and
the information is true to the best of my knowledge and belief; and
no information has been withheld which might prejudice this application to the best of my knowledge and belief.

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the Statutory Declarations Act 1959, and I believe that the statements in this declaration are true in every particular.

Signature _____

Declared at _____ on _____ of _____ 202___

Before me, _____

_____
Signature

1.10. Checklist

	Mandatory Requirements	Check	Page Number	Remarks
General requirements for applications	A. Form of the application	✓	N.A.	
	Table of contents	✓	N.A.	
	Executive summary	✓	N.A.	Supplied separately
	B. Applicant details	✓	2	Section 1.1
	C. Purpose of application	✓	4	Section 1.2
	D. Justification for the application	✓	4	Section 1.3
	D.1 Regulatory impact information	✓	4	Section 1.3.1
	D.1.1 Costs and benefits of the application	✓	4	Section 1.3.1
	D.1.2 Impact on international trade	✓	4	Section 1.3.1
	E Information to support the application	✓	4	Section 1.4
	E.1 Data requirements	✓	N.A.	
	F. Assessment procedure	✓	5	Section 1.5
	G. Confidential commercial information (CCI)	✓	5	Section 1.6
	H. Other confidential information	✓	5	
	I. Exclusive capturable commercial benefit (ECCB)	✓	5	Section 1.7
	J. International and other national standards	✓	5	Section 1.8
	K. Statutory declaration	✓	6	Section 1.9
	L. Checklist	✓	7	Section 1.10
3.3.2. Processing aids	A. Technical information on the processing aid	✓	9	Section 2
	A.1 Information on the type of processing aid	✓	9	Section 2.1
	A.2 Information on the identity of the processing aid	✓	9	Section 2.2
	A.3 Information on the chemical and physical properties of the processing aid	✓	9	Section 2.3
	A.4 Manufacturing process	✓	15	Section 2.4
	A.5 Specification for identity and purity	✓	18	Section 2.5
	A.6 Analytical method for detection	×		Not applicable for enzymes used as processing aids
	C. Information related to the safety of an enzyme processing aid	✓	19	Section 3
	C.1 General information on the use of the enzyme as a food processing aid in other countries	✓	19	Section 3.1
	C.2 Information on the potential toxicity of the enzyme processing aid	✓	20	Section 3.2
	C.3 Information on the potential allergenicity of the enzyme processing aid	✓	20	Section 3.3

	C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available	✓	21	Section 3.4
	D. Additional information related to the safety of an enzyme processing aid derived from a microorganism			Section 3
	D.1 Information on the source microorganism	✓	22	Section 3.5
	D.2 Information on the pathogenicity and toxicity of the source microorganism	✓	23	Section 3.6
	D.3 Information on the genetic stability of the source organism	✓	23	Section 3.7
	E. Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism			Section 3
	E.1 Information on the methods used in the genetic modification of the source organism	✓	23	Section 3.8
	F Information related to the dietary exposure to the processing aid		24	Section 4
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	✓	24	Section 4.1
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	✓	24	Section 4.2
	F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption	✓	27	Section 4.3
	F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid	✓	27	Section 4.4
	F.5 Information relating to the levels of residues in foods in other countries	✓	27	Section 4.5
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	✓	28	Section 4.6

2. Technical information

2.1. Type of processing aid

The Thermolysin enzyme is an enzyme produced by submerged fermentation of Thermolysin from a non-genetically modified strain of *Anoxybacillus caldiproteolyticus*.

This Processing Aid falls into the category "Enzymes of microbial origin" from the Food Standard Code section 1.3.3-6 Enzymes.

2.2. Identity

2.2.1. Chemical/Common Name:

The systematic name of the principal enzyme activity is Thermolysin. Other names used are Neutral proteinase; thermoase; and thermoase.

The enzyme Thermolysin is derived from *Anoxybacillus caldiproteolyticus* sp. (Rokko) which is not genetically modified.

- EC number: 3.4.24.27 (Appendix A)
- CAS number: 9073-78-3 (Appendix B)

2.2.2. Marketing Name of the Processing Aid:

The marketing name of this enzyme preparation will depend on the application. An example marketing name of Thermolysin is ALPHALASE® XXX.

2.2.3. Molecular and Structural Formula:

Thermolysin is a protein. The amino acid sequence is known. Full details of the amino acid sequence are provided in Appendix P (**Confidential Commercial Information**).

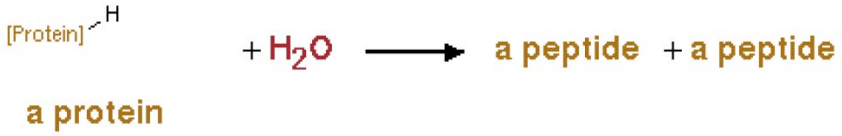
2.3. Chemical and physical properties

Thermolysin is a thermostable bacterial neutral metallo-endopeptidase. It is effective in raw material processing applications where a high level of thermostability is desirable. Thermolysin has broad substrate specificity, and it will efficiently hydrolyse casein, whey proteins, gelatin, soy proteins, wheat gluten, fish proteins and other proteins.

2.3.1. Substrate specificity

The food enzyme catalyses the hydrolyses of proteins, with preference for cleavage of bonds in which amino-acids Leucine (Leu) and Phenylalanine (Phe) are involved with the main reaction products being protein fragments of various lengths, peptides and free amino acids. Such enzyme activity is widely present in nature and in particular in food ingredients. The substrates and its reaction products are themselves present in food ingredients.

Simple schematic reaction diagram:



2.3.2. Activity

The activity of the enzyme is defined in Thermolysin activity units (U/g) based on the enzyme's ability to cleave p-nitroanilide from a synthetic peptide.

A Thermolysin preparations' enzyme activity will depend on the final product. A detailed assay method is present in Appendix C.

2.3.3. Temperature optimum

Temperature optimum of Thermolysin was determined using the analytical method provided. The activity was measured in 50mM potassium HEPES-buffer pH 7.5 at the indicated temperature. Temperature optimum is ranging from 60-70°C. The exact temperature optimum will depend on many process variables, such as pH, time, substrate composition and concentration.

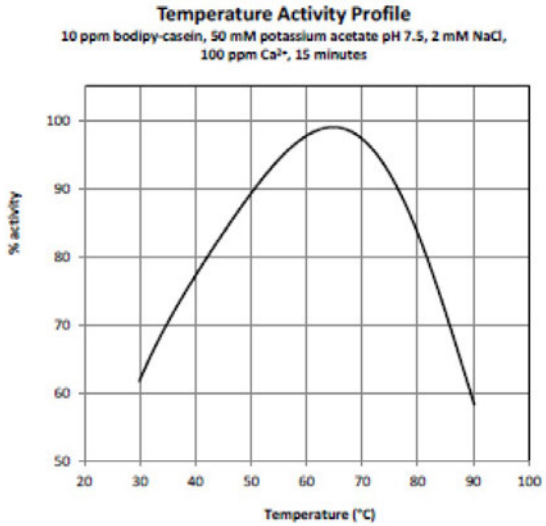


Figure 1: Temperature profile of Thermolysin (pH7.5)

2.3.4. Thermal stability

The temperature stability was measured a 50-fold dilution in pH 7.5 buffer as described in the assay. The samples were incubated for 10 minutes at the given temperatures. No enzyme activity is left at temperatures above 90°C.

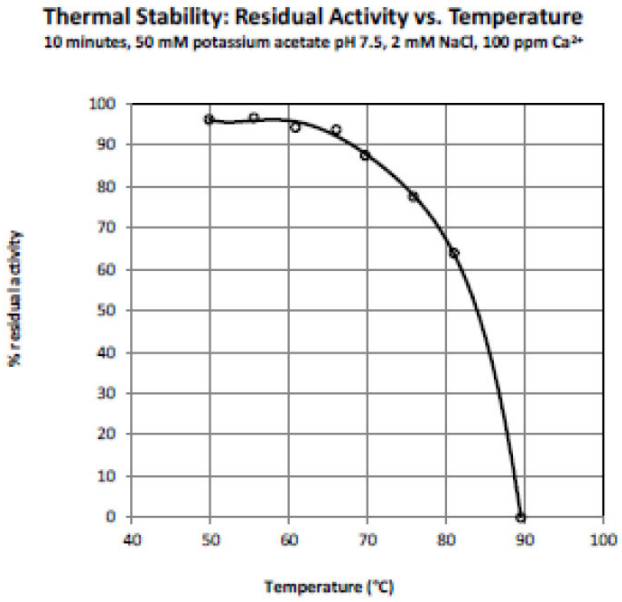


Figure 2: Activity was determined after 10 minutes of incubation at pH 7.5

2.3.5. pH optimum

The pH optimum of Thermolysin was determined using the analytical method provided. The activity was measured at 65°C for 15 minutes at the indicated pH-range. The pH optimum is ranging from pH 5-6. Results are shown in Figure 3. The exact pH optimum will depend on process variables, including temperature, time, substrate composition and concentration.

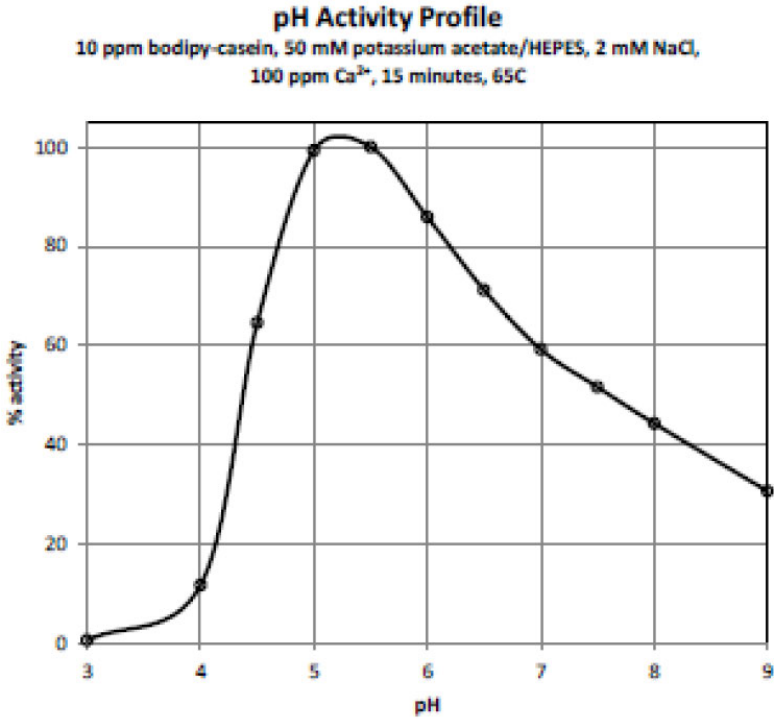


Figure 3: pH profile of Thermolysin

2.3.6. Storage stability

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

According to Standard 1.2.5 of Food Standards Code, the date of minimum durability or use-by-date is indicated on the label of the food enzyme preparation. If necessary, special conditions of storage and/or use will also be mentioned on the label.

The figure below shows storage stability of an example commercial product of Thermolysin. As seen in the figure, at refrigeration temperature (4°C), the enzyme is stable for 2 years with close to 95% remaining activity.

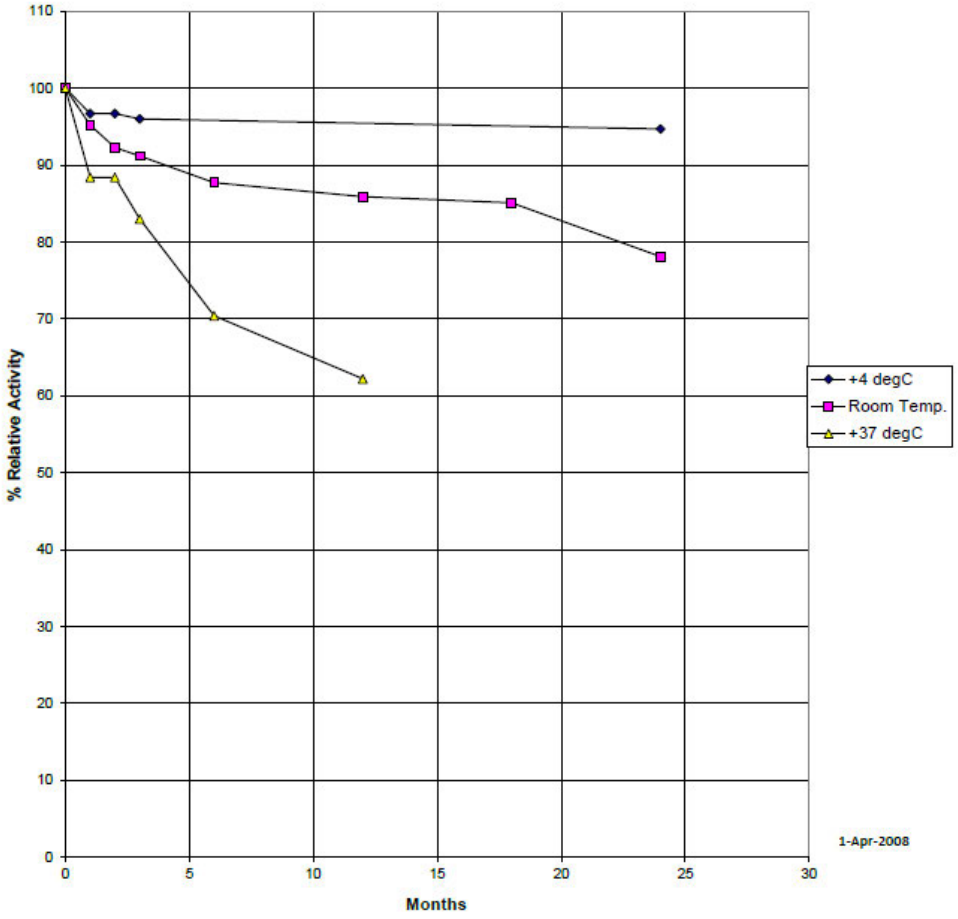


Figure 4: Storage Stability of Thermolysin

2.3.7. Interaction and fate in foods

The Thermolysin enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food, or present in negligible amounts with no technical function in the final food.

The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing, lack of water activity, wrong pH, etc. In some cases (e.g., after alcohol distillation, products resulting from starch processing), the enzyme may no longer be present in the final food.

Based on the conditions of use described in Section 2.3.9 and the activity of the enzyme under such conditions, it can be concluded that the food enzyme does not exert any (unintentional) enzymatic activity in the final food.

2.3.8. Nutritional implications

Thermolysin is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Thermolysin are very low, and as with other enzymes that are currently approved and used as Processing Aids, use of this preparation would not have any nutritional significance.

2.3.9. Efficacy and benefits of the Thermolysin enzyme preparation

Like any other enzyme, Thermolysin acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs any technological function.

Depending on the specific food product, the same effect might be achieved by other means as well. However, these methods often involve more expensive or less environmentally friendly productions processes, the use of chemicals or recipe changes.

The substrate(s) for all proteases (including Thermolysin) are the proteins and peptides which can be found in all living organisms including wheat, and other grains, vegetables, as well as in meat, fish and milk (Poutanen, 1997; Hamada *et al*., 2013; Tester *et al*., 2007; Jones, 2005; Malacarne *et al*., 2002). Consequently, the substrate for Thermolysin occurs naturally in food.

Protein processing

Protein hydrolysates are produced by hydrolysis of proteins or protein-containing raw materials from different origins, e.g.:

- Vegetable (derived) raw materials, such as soy, wheat, barley (in brewing), maize, etc
- Animal (derived) raw materials, such as milk and milk derived products (whey proteins, caseins), meat, fish, collagen, gelatine.

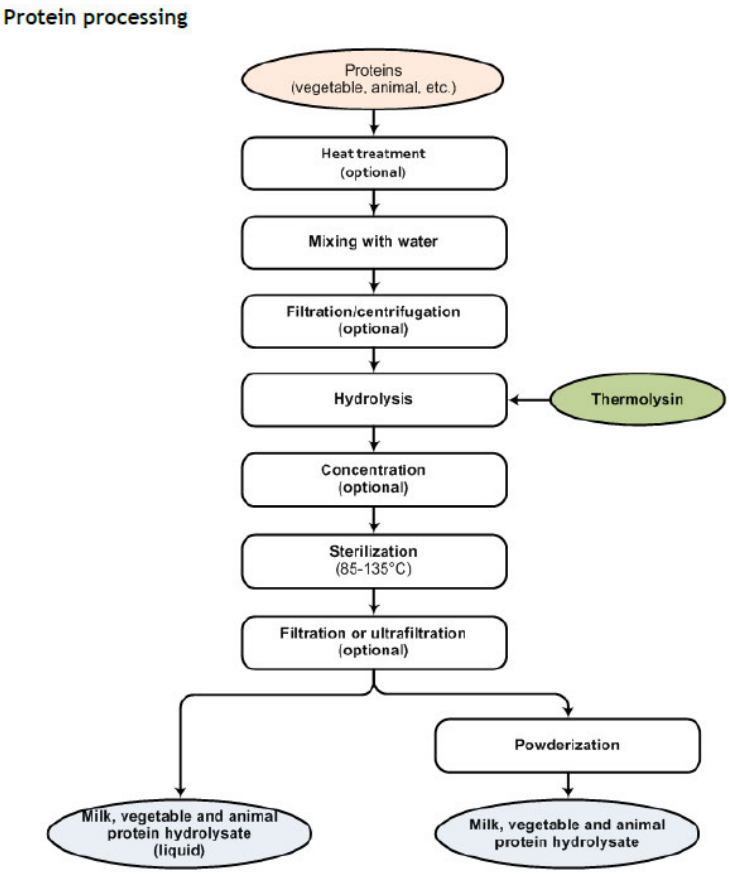
The main intention of the use of Thermolysin in protein processing is to facilitate protein hydrolysis. The benefits of protein are countless including:

- Flavour improvement
- Increased yields (extracts for example)
- Improved physical properties.
- Improved nutritional quality.
- Processing efficiencies
- Utilisation of food waste streams

Traditionally, protein hydrolysis was carried out using e.g., hydrochloric acid or by boiling meat and fish pieces. From the 1960s, proteases came into use because of their effective hydrolysis activity, leading to increased yield and enhanced flavours (Whitehurst and Law, 2010). In the beginning, a limited number of proteases were used (Criswell *et al*., 1964, Sripathy *et al*., 1962), but currently a bigger range of proteases (animal, plant, fungal, bacterial origin and alkaline, acid, neutral, heat resistant etc.) including Thermolysin are used (Hale, 1969, Koury and Spinelli, 1974, Shimono and Sugiyama, 2010). Enzymatic hydrolysis has been utilised for over four decades (Feldman *et al*., 1974).

Sometimes other enzymes (aminopeptidase, nuclease etc.) are used at the same time for greater efficiency.

The typical protein process in which Thermolysin may be applied is illustrated in the process flow scheme given below:



Thermolysin protein is inactivated by heat in a specific inactivation step or in a sterilisation/pasteurisation process.

2.3.9.1. Efficacy Examples

This section describes just some examples of the efficacy of Thermolysin in food processing.

2.3.9.1.1. Thermolysin in brewing

In brewing proteases such as thermolysin are used to improve malting, fermentation, clarification and chilling and storage quality (Gomaa, 2018). Thermolysin is used in the brewing process during mashing to cleave proteins and release free amino acids and nitrogen for yeast to utilise as a nutrient source. The use of thermolysin in the mashing process increases soluble protein thereby improving viscosity to optimise processing conditions.

In a brewing protocol with 100% barley and a standard mashing protocol (no liquefaction at high temperature) the thermostable protease Thermolysin (A THP) was more efficient than another thermostable protease (O DCO+) and a neutral protease (A NP) to provide free amino nitrogen (FAN), soluble protein and slightly higher extract.

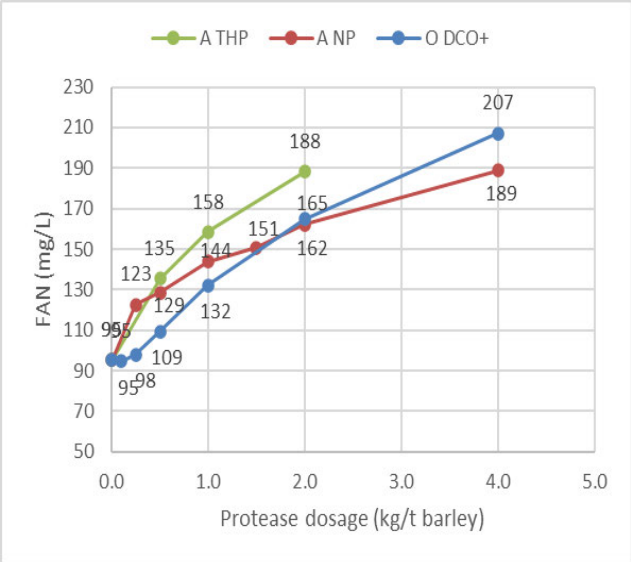


Figure 5: Free amino nitrogen

Figure 5 demonstrates that A THP provided a free amino nitrogen level sufficient for a proper fermentation of 150 mg/L with a dosage less than 1.0 kg/t barley whereas 1.5 kg/t was required of A NP and O DCO+.

2.3.9.1.2. Thermolysin in food processing

As highlighted above, in principle, the enzymatic conversion of proteins with the help of Thermolysin may be used in the processing of all food raw materials which naturally contain proteins.

A specific example where Thermolysin can be advantageous, is the fish processing industry. Thermolysin can be added to the press-liquor of fish meal and fish oil co-product streams to increase the product yields and quality. Advantages to Thermolysin use in fish processing are increased productivity through increased annual outputs, and environmental advantages by better utilisation of waste streams.

	Standard process	Enzymatic process	Benefit of Thermolysin
Volume Index	100	113	**+ 13%**
Free FA	11.45%	2.38%	**- 80%**
Colour	92 units	60.2 units	**-35%**

Figure 6: Thermolysin impact on yield and quality of fish oil

Figure 6., shows that the addition of Thermolysin to press liquor provides a 13 % increase in oil yield. Additionally, crude fish oil quality is increased with an 80 % reduction in free fatty acids and 35 % reduction in colour index.

2.4. Manufacturing process

The manufacturing process for Thermolysin production will be conducted in a manner similar to other food and feed enzyme production processes. It is conducted in accordance with food good manufacturing practice (GMP) and the resultant product meets the general requirements for

enzyme preparations of the Food Chemicals Codex, Thirteenth Edition (FCC, 2015) and the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Join FAO/WHO Expert Committee on Food Additives (JEFCA, 2006).

The manufacturing process is a three-part process consisting of fermentation (growth of organism and production of enzyme), recovery (separation of cell mass from enzyme and concentration/purification of enzyme) and formulation/drying (preparation of a stable enzyme formulation). The production process follows standard industry practices (Aunstrup *et al*, 1979; and Aunstrup, 1979).

2.4.1. Raw materials

The raw materials used in the fermentation and recovery process for the Thermolysin enzyme concentrate are standard ingredients used in the enzyme industry. All the raw materials conform to the specifications of the Food Chemical Codex, Thirteenth Edition, except for those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. IFF uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix Q (**Confidential Commercial Information**).

2.4.2. Fermentation

Thermolysin is manufactured by submerged fed-batch pure culture fermentation of the non-genetically modified strain *Anoxybacillus caldiproteolyticus* Rokko. The fermentation is an aerobic process and requires continuous addition of air to the fermenter. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken, and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

The fermentation process consists of three operations: laboratory propagation of the culture, seed fermentation and primary fermentation. These processes, except for the laboratory propagation are carried out in sealed vessels carefully designed to prevent both the release of the production organism and/or the entry of other microorganisms.

A new glycerol stock culture vial of the *Anoxybacillus caldiproteolyticus* Rokko production organism is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

The fermentation media is sterilised at 121°C for at least 20 minutes. The medium is sampled for microbiological testing prior to inoculation. The fermentation takes place at controlled temperatures.

All stages of the production process are controlled to ensure that the final product conforms to specifications. The culture liquid is sampled at intervals during fermentation for microbiological and enzyme activity tests. Operational parameters such as temperature, pH, air flow, agitation and oxygen content are monitored and controlled to desired values/ranges throughout the fermentation. In addition, at all stages, microbial growth is checked for correct morphological development of the microorganism and for the lack of contamination. Once the fermentation is completed, the fermentation broth is transferred to processing tanks.

2.4.3. Recovery

The purpose of the recovery process is to separate the biomass, purify, concentrate, and stabilise the desired enzyme, i.e., Thermolysin.

Separation of the cell debris from the liquid from the fermentation broth is achieved by either filtration or centrifugation, or a combination of both. Exactly which cell separation technique is used is dependent upon the manufacturing site. The broth may be treated with flocculating agents to maximise separation and is then fed into the filter or the centrifuge. The relatively solids-free stream then passes a polishing filter to further clarify the liquid and achieve clear, cell-free filtrate.

The liquid containing the enzyme is concentrated via ultrafiltration, which removes low molecular weight compounds. Diafiltration may follow ultrafiltration to help reach the activity target, remove colour and smaller particles, and carbon treatment may additionally be used to reduce colour. The final recovery step is a polish filtration using either microfiltration membranes, fine filtration aids such as diatomaceous earth or sterile filtration pads.

The ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier, or stabilised by e.g., glycerol to produce a liquid product.

A manufacturing flow chart is provided in Appendix D.

2.4.4. Formulation

The ultra-filtrated concentrate is then formulated and analysed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the FCC.

Depending on the final commercial product, the formulation may be different. Below is an example of product formulation:

Water	51 -67 %
Sorbitol	23 -30 %
Sodium chloride	8 -12 %
Thermolysin (enzyme)	1 - 5 %
Potassium sorbate	0.2-0.3%
Calcium acetate	1.2-1.6%
Total	100 %

2.5. Specification for identity and purity

Impurity profile:
Appropriate GMP controls and processes are used in the manufacture of Thermolysin to ensure that the finished preparation does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits is as follows:

Metals:	
Lead	less than 5 mg/kg
Microbiological:	
Total viable count	less than 10,000 CFU/g
Total coliforms	less than 30 CFU/g
E. coli	absent in 25 g
Salmonella	absent in 25 g
Antibiotic activity	Negative by test
Production strain	Negative by test
Physical properties:	
Appearance	Off white powder

The methods by which these specifications are measured are standardised/and or validated methods and given in Appendix E. Certificates of Analysis for three lots of product are given in Appendix F.

Standard for identity:
Thermolysin meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

Allergenicity of the Thermolysin:
An allergen statement is given in Appendix G. Wheat or soy could be used as fermentation nutrients in the manufacture of Thermolysin. IFF have carried out a risk assessment to determine that the presence of wheat or soy in the enzyme preparation is not expected to introduce any additional food allergen risk to consumers, these are also provided in Appendix G.

Refer to Section 3.3 for additional information on the safety of the enzyme as to its allergenicity potential.

3. Safety

Anoxybacillus caldiproteolyticus Rokko is the source organism of thermolysin, an enzyme that has been used as processing aid in the food industry since the 1960's. Thermolysin has been used for several applications, including food (e.g., hydrolysis of proteins that cannot be hydrolysed by other proteases, e.g., that of barley protein which contains a specific inhibitor toward serine protease (Aunstrup, 1980), and aspartame manufacturing (Ooshima *et al*, 1985).

Thermolysin is a metallo-protease (neutral protease, IUB E.C.3.4.24.27, CAS 9073-78-3) that has been extensively characterised. After the initial description by Endo (1962) various properties were described by Ohta *et al* (1966). Thermolysin specifically hydrolyses peptide bonds on the imino side of bulky hydrophobic residues such as Leu, Ile, Val, and Phe (Matsubara, 1966). The specificity of thermolysin was further analysed by Morihara *et al* (1968) and Morihara and Tsuzuki (1971). Thermolysin has one zinc ion necessary for activity (Latt *et al,* 1969) and four calcium ions for stability (Feder *et al*, 1971). The amino acid sequence (Titani *et al*, 1972) and three-dimensional structure (Colman *et al*, 1972) of this enzyme have been determined. The enzyme has been cloned and expressed in *Bacillus subtilis* (O'Donohue *et al*, 1994), and the catalytic mechanism enzyme has been characterised by Matthews (1988), Beaumont *et al* (1995) and others.

No toxic effects of Thermolysin or other neutral (metallo) proteases are described in literature.

3.1. Use of the enzyme as a food processing aid in other countries

Enzyme products are developed for a specific function, i.e., to catalyse a specific chemical reaction. That reaction determines the IUBMB classification. Enzyme variants may be selected to have a better performance of that function under the specific conditions of the application (e.g., temperature or pH). Enzymes of a certain IUBMB classification share conserved structural elements, called domains, which are needed for their specific function. As such the enzymes of our approval procedures do resemble those already permitted by FSANZ both in function and in structure.

Figure 7 below, shows an example of natural variation of alpha-amylases. The same holds for any other enzyme type. While significant differences in sequence amongst the various species exist, they all catalyse the same reaction and therefore fit under the same IUBMB entry. There will also be natural variation within one species. All this also applies to the enzymes under the current approval procedures by FSANZ:

% amino acid sequence identity	*B. amyloliquefaciens*	*B. licheniformis*	*G. stearothermophilus*	*A. niger*	*A. oryzae*	*Z. mays*	*O. sativa*	*H. vulgare*	*P. vulgaris*	*H. sapiens*
Bacillus amyloliquefaciens	100									
Bacillus licheniformis	80	100								
Geobacillus stearothermophilus	65	65	100							
Aspergillus niger	21	21	22	100						
Aspergillus oryzae	23	24	24	66	100					
Zea mays (corn)	24	26	25	28	27	100				
Oryza sativa (rice)	25	27	25	27	26	89	100			
Hordeum vulgare (barley)	25	23	24	25	28	70	69	100		
Phaseolus vulgaris (bean)	26	27	25	24	27	67	65	64	100	
Homo sapiens (human)	25	33	29	22	28	23	22	23	24	100

α-amylases in nature have divergent

amino acid sequences but have the same catalytic activity and IUBMB number

Figure 7. Variation of enzymes in nature

Thermolysin derived from *Anoxybacillus caldiproteolyticus*, has been determined to be GRAS in the United States, and been used for protein hydrolysis in foods in multiple countries including France (since 2007), Denmark (since 2009) and China (since 2021). These approvals are detailed in Section 2.4. There have not been any adverse events reported since thermolysin has been in commercial use in these countries.

Please refer to section 1.9 for details on the jurisdictions where Thermolysin is approved.

3.2. Toxicity of the enzyme

Toxin homology study

The mature sequence for Thermolysin from *Anoxybacillus caldiproteolyticus* Rokko is given in Appendix P (**Confidential Commercial Information**).

A BLAST search for homology of the protease sequence with known toxins and antinutrients was performed using the UniProtKB annotated Protein Knowledge database (Magrane *et al*., 2011; http://www.uniprot.org/), UniProt release 2023_08. This database contains 569,793 reviewed proteins (https://www.uniprot.org/uniprotkb?facets=reviewed%3Atrue&query=%2A), of which 6,798 are manually annotated as toxins and 7,216 as venom proteins (http://www.uniprot.org/program/Toxins).

The conclusion is that the protease from *Anoxybacillus caldiproteolyticus* doesn't have any biologically relevant similarity with known toxic protein sequence. Please refer to Appendix U Toxin Homology Database Search for full report (**Confidential Commercial Information**).

Toxicological testing

To assess the safety of this Thermoylsin, different endpoints of toxicity were investigated and are evaluated and assessed in this document with the following results:

- Ames test: no mutagenic activity under the given test conditions
- Chromosomal aberrations: no clastogenic activity under the given test condition
- 90-day oral toxicity on rats: The No Observed Adverse Effect Level (NOAEL) is 337.5 mg TOS/kg bw/day, which is the highest dose in the study.

Toxicity test outcomes are summarised in Appendices H&I, and full study details are provided for reference.

3.3. Allergenicity of the enzyme

The ingestion of food enzymes has been shown to be an unlikely concern regarding food allergy (Bindslev-Jensen *et al*., 2006). Bioinformatic analyses based on sequence homology determined that the *Anoxybacillus caldiproteolyticus* Rokko Thermolysin is unlikely to pose a risk of food allergenicity.

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al*. (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al*. (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores (<10-7) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant

matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:
"A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

The *Anoxybacillus caldiproteolyticus* Rokko Thermolysin (mature) sequence is given in Appendix P (**Confidential Commercial Information**).

The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database (http://www.allergenonline.org/index.shtml, latest version releases on May 25, 2023) containing 2290 peer-reviewed allergen sequences (listed in http://www.allergenonline.org/databasebrowse.shtml) revealed no match to known allergens.

As for all enzyme products, an MSDS for the Thermolysin product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

Refer to Appendix R (**Commercial in Confidence**) for additional information on the safety of the enzyme as to its allergenicity potential.

3.4. Safety Assessment reports prepared by international agencies or other national government agencies.

Food enzymes are biological isolates of variable composition. Apart from the enzyme protein in question, microbial food enzymes will also contain some substances derived from the producing micro-organism and the fermentation medium. From a safety point of view, the similarity of the producing micro-organism is of higher importance than that of the enzyme protein in question. Therefore, sections below summarise not only authorised food enzymes with the same enzyme activity, but also authorised food enzymes from the same producing organism.

As documented below, Thermolysin from *Anoxybacillus caldiproteolyticus* (formerly *Geobacillus stearothermophilus*) has been approved in various jurisdictions.

3.4.1. CODEX standards

As discussed in section 1.8, Thermolysin from *Anoxybacillus caldiproteolyticus* Rokko has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

3.4.2. International legislation

3.4.2.1. United States

3.4.2.1.1. The enzyme

Thermolysin from *Anoxybacillus caldiproteolyticus* Rokko has been determined to be GRAS by expert opinion in the United States. Refer to Appendix N (**Commercial in Confidence**), for the GRAS expert Panel letter.

A similar Thermolysin from *Anoxybacillus caldiproteolyticus* was issued a "no questions" letter for GRAS by the US FDA for use as a processing aid in the production of yeast extract; cooked fish; egg white hydrolysates; enzyme-modified dairy ingredients; and protein hydrolysates (soy, wheat gluten, milk protein, fish) to improve the protein solubility, taste and digestibility of these products in 2015 (GRN 598).

3.4.2.1.2. Supporting approvals

A 1,4-α-glucan branching enzyme preparation from *Geobacillus stearothermophilus* strain TRBE14 was issued a 'no questions' letter by U.S. FDA (GRN 405).

3.4.2.2. Europe

Assessment and approval of Thermolysin from *Anoxybacillus caldiproteolyticus* Rokko have been carried out in both France and Denmark. Refer Appendix L & M (**Commercial in Confidence**) for safety reports/approval letter.

3.4.2.3. Other countries

Protease from *Geobacillus stearothermophilus* (*Anoxybacillus caldiproteolyticus*) is approved in the following countries.

- Japan.
- China, refer to Appendix O (**Commercial in Confidence**)
- Mexico

3.5. Information on the source organism

The host organism is a non-genetically modified strain of *Anoxybacillus caldiproteolyticus*. It is deposited in the IFF Culture Collection.

A. caldiproteolyticus Rokko, a spore forming thermophilic bacteria, is a classical industrial strain used for enzyme production by Genencor International (now IFF) or its parent companies and other enzyme manufacturers for decades. It was originally isolated from the Rokko Hot Spring in Japan (Endo, 1962).

Genencor International obtained the strain as part of acquisition of Rhodia Ltd in 2004. The strain originally came from Daiwa Kasei KK (Japan) who had defined the organism as *Bacillus stearothermophilus var. thermoproteolyticus*. Scientific literature invariably refers to the thermolysin producing bacterium as "*Bacillus thermoproteolyticus*". However, the name "*Bacillus thermoproteolyticus*" is not a formally recognised taxon, and the name has "no standing" in bacterial nomenclature. In 2001 Nazina *et al*. proposed that *Bacillus stearothermophilus* (and relatives) be transferred to a new genus *Geobacillus* as new combinations. The organism was subsequently reclassified to *Anoxybacillus caldiproteolyticus* (Coorevits , 2012).

Prior to the 2015 assessment of Thermolysin from *Anoxybacillus caldiproteolyticus* strain TP-7, neither *A. caldiproteolyticus* nor *G. caldoproteolyticus* had been assessed by FSANZ.

The taxonomy of *Anoxybacillus caldiproteolyticus* is searchable on the NCBI taxonomy database under Taxonomy ID: 247480. More details on the taxonomy of strain Rokko are provided in the Strain Identification report, Appendix T (**Confidential Commercial Information**).

The taxonomic identity of the species is as follows:

Name: *Anoxybacillus caldiproteolyticus*
Phyllum: Firmicutes
Class: Bacilli
Order: Bacillales
Genus: *Anoxybacillus*
Species: *caldiproteolyticus*

3.6. Pathogenicity and toxicity of the source microorganism

As the genus *Anoxybacillus* consists of obligatory thermophilic strains which grow optimally at high temperature (55-60 °C), as is also the case with this strain, no pathogens are expected.

Since this genus was previously known as *Bacillus*, and most of the strains (including this one) were previously assigned to the species *Bacillus stearothermophilus*, a search was performed for both names on pathogenicity in the PubMed literature database. No articles were found describing these organisms as pathogenic or even involved in opportunistic pathogenicity. In fact, one paper was found (Sattar *et al*, 1972) describing the use of *Bacillus stearothermophilus* as a safer replacement of *B. subtilis* as microbial tracer for aerobiological experiments. Also, *Bacillus stearothermophilus* is considered ideal for validating moist heat (saturated steam) sterilisation because this organism lacks pathogenicity, pyrogenicity and toxicity (e.g., WHO/SEA, 2003).

Bacillus stearothermophilus is not present on the pathogen lists of the USA (NIH Guidelines, CDC Office of Health and Safety (OHS)), EU, UK, Belgium and The Netherlands.

Bacillus stearothermophilus is listed in Germany as Risk Group 1, in The Netherlands as suitable for the construction of Risk Group I genetically modified organisms, and in several culture collections (DSMZ, ATCC) as Risk Group I / Biosafety Level 1.

It is concluded, from this review and tests made on preparations that the microorganism *A. caldiproteolyticus* Rokko provides no specific risks.

3.7. Genetic stability of the source organism

The strain *A. caldiproteolyticus* Rokko is a stable strain, which can easily be maintained as a homogeneous population under the usual laboratory and production conditions. The strain has been tested repeatedly for stability after growth for more than dozens of generations which occur in a large-scale industrial fermentation and no significant instability was detected. By plating the strain on agar media, a low frequency of phenotypic and genotypic variants may be found. In this aspect the strain does not differ from other, highly specialised industrial micro-organisms.

3.8. Method used in a genetic modification of the source organism.

The production organism of the Thermolysin preparation, the subject of this submission, is not genetically modified.

4. Dietary Exposure

4.1. List of food or food groups likely to contain the enzyme or its metabolites.

In the below applications Thermolysin will be used as a processing aid where the enzyme is either not present in the final food, or present in insignificant quantities having no function or technical effect in the final food.

Thermolysin is typically used in the following food manufacturing processes:

- Protein processing

This technological function lends itself to uses in a variety of foods including dairy foods, egg, meat and fish, protein concentrates and isolates, yeast processing, and in beer brewing.

4.2. Level of use and residue in foods

Thermolysin is intended for use in the hydrolysis of protein in a wide variety of foods. The Thermolysin enzyme preparation is used at the minimum level required to achieve the desired effect, in accordance with the principles of current Good Manufacturing Practice (GMP).

The recommended use level for proteins containing products ranges from 16.3 -163 mg TOS/ kg raw material. The maximum use level is 163 mg TOS/kg raw material. The product contains 10% Total Organic Solids (TOS).

4.2.1. Estimated food intake

Commercial food enzyme preparations are generally used following the Quantum Satis (QS) principle, i.e., at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer must be determined case by case, based on the desired effect and process conditions. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune this process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and Thermolysin is used according to the QS principle. A food producer who would add much higher doses than what is necessary would experience untenable costs as well as negative technological consequences.

The dosage of a food enzyme depends on the activity of the enzyme protein (in this case Thermolysin) present in the final food enzyme preparation (i.e., the formulated food enzyme). However, the activity Units, as such, do not give an indication of the amount of food enzyme added. Microbial food enzymes contain – apart from the enzyme protein in question – also some substances derived from the producing microorganism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS) (FAO/WHO, 2006). Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS.

The Table below shows the range of recommended use levels in the food process:

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximum exposure (mg TOS/kg RM)
Protein processing	Proteins of various origin	16.3-163	163

4.2.2. Estimated intake of Thermolysin

Thermolysin from *A. caldiproteolyticus* will be used as a processing aid in protein processing. While we expect the Thermolysin to be not present in the final food, or present as inactive residue in negligible amounts, the following conservative calculations assume that 100% of the enzyme remains in the processed food, as TOS.

As indicated above, Thermolysin from *A. caldiproteolyticus* may be used in the manufacture of a wide variety of protein containing foods and food ingredients. The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen, 1966; Douglass *et al*., 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.
The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake. This method was previously used by JECFA (FAO/WHO, 2001) and contains the following assumptions.

1. Level of consumption of foods and beverages:
   For solid foods, the daily intake is set at 25 g/kg bw based on a maximum lifetime energy intake of 50 Kcal/kg bw/day. For non-milk beverages, a daily consumption of 100 ml/kg bw is used corresponding to 6 litres per day for a 60-kg adult.

2. Concentration of enzymes in foods and beverages:
   The concentration of enzyme in foods and beverages is the maximum application rate.

3. Proportion of foods and beverages that contain the enzymes:
   a) A default of 50% of all solid foods is used to represent processed foods (i.e., 12.5 g/kg bw/day).
   b) A default of 25% is used to represent non-milk beverages that may contain the enzyme (i.e., 25 ml/kg bw/day).

4. Estimation of the theoretical maximum daily intake (TMDI).

To represent a worst-case scenario, TMDI for solid foods will be combined with the TMDI for beverages in the risk assessment.

The Budget Method is based on the following assumed consumption of targeted important foodstuffs and beverages (for less important foodstuffs, e.g., snacks, lower consumption levels are assumed). The regular assumption is for processed food (50% of total solid food) and for soft drinks (25% of total beverages).

Average consumption over the course of a lifetime/kg body weight/day	**Total solid food (kg)**	**Total non-milk beverages (l)**	**Processed food (50% of total solid food) (kg)**	**Soft drinks (25% of total beverages) (l)**
	0.025	0.1	0.0125	0.025

In Section 4.2.1 the recommended use levels of the enzyme Thermolysin are given, based on the raw materials used in the various food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation accounts for how much food or

beverage is obtained per kg raw material and it is assumed that all the TOS will end up in the final product.

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Example Final food (FF)	Ratio RM/FF	Maximal level in FF (mg TOS/kg food)
Beverages	Protein Processing	Proteins from Various Sources	163	Sport drinks	0.30	48.9
Solid food	Protein Processing	Proteins from various sources	163	Protein hydrolysates used in e.g., soups, bouillons, dressings.	0.17	27.7
				Protein bar	0.30	48.9

The Total TMDI can be calculated on basis of the **maximum** values found in food (in the above case, protein processing) multiplied by the average consumption of food kg body weight/day. The Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
48.9x0.0125=0.611	48.9x0.025=1.222	1.833

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that all producers of the above-mentioned foodstuffs use the specific enzyme Thermolysin from *A. caldiproteolyticus*.
- It is assumed that all producers apply the highest use level per application.
- For the calculation of the TMDI's in foodstuffs, only THOSE foodstuffs were selected containing the highest theoretical amount of TOS. Thus, foodstuffs containing lower theoretical amounts were not taken into account.
- It is assumed that the amount of TOS does not decrease as a result of the food production process.

It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime.

4.2.3. Safety assessment

Thermolysin is an enzyme produced from the non-GM strain *A. caldiproteolyticus* Rokko.

IFF has determined by scientific procedures that production organism *A. caldiproteolyticus* Rokko is safe as a production organism. For the determination of the safety of Thermolysin, different endpoints of toxicity were investigated. Summarising the results, the following conclusions can be drawn:

- No mutagenic activity under the given test conditions
- No clastogenic activity under the given test condition
- A sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 337.5 mg TOS/kg bw/day.

Determination of the margin of safety

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). As was shown in Section 4.2.2, the Total TMDI of the food enzyme is 1.833 mg TOS/kg body weight/day.

MoS = 337.2 mg TOS/kg body weight/day / 1.833 mg TOS/kg body weight/day
= 184

4.2.4. Conclusion

The safety of Thermolysin from *A. caldiproteolyticus* as a food processing aid in brewing and potable alcohol production is assessed in a battery of toxicology studies investigating its mutagenic and systemic toxicity potential. Under the condition of these assays, it was shown that Thermolysin is not a mutagen, a clastogen, or an aneugen. Daily administration of Thermolysin by gavage for 90 days did not result in overt signs of systemic toxicity. A NOAEL is established at 337.2 mg TOS/kg bw/day.

Based on a margin of safety of 184 the proposed uses of the Thermolysin are not a human health concern and are supported by existing toxicology data.

4.3. Likely level of consumption of foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs)

Not applicable. Thermolysin is not expected to be used in production of any foods or food groups that are currently not listed in NNSs. If such usage arises, an application would be made to inform FSANZ.

4.4. Percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

It is assumed that all raw materials containing protein are processed using the thermolysin object of this submission as a processing aid at the highest recommended dosage.

4.5. Levels of residues in food in other countries

Applications and levels of use of the Thermolysin preparation in other countries is the same as presented in section 4.2.

4.6. Likely current food consumption for foods where consumption has changed in recent years

Not applicable. Consumption of foods produced with Thermolysin is not expected to have a significant change.

5. References

Aunstrup, K. 1979. Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. and Goldsstein, L. pp. 28-68.

Aunstrup, K., Andersen, O., Falch, E. A., and Nielsen, T. K. 1979. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Aunstrup K., in A.H. Rose (Ed.), Microbial Enzymes and Bioconversions, Academic Press, New York, 1980, pp. 50–114.

Beaumont A, O'Donohue MJ, Paredes N, Rousselet N, Assicot M, Bohuon C, Fournie-Zaluski MC, Roques BP. "The role of histidine 231 in thermolysin-like enzymes. A site-directed mutagenesis study.", J Biol Chem. 270 (1995), 16803-16808.

Bindslev-Jensen C, Skov PS, Roggen EL, Hvass P and Sidelmann Brinch D (2006). Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food and Chemical Toxicology, 44, 1909–1915

Codex Alimentarius Commission. 2009. Foods Derived from Modern Biotechnology, Annex 1, Assessment of Possible Allergenicity, Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Rome, Italy, http://www.fao.org/docrep/011/a1554e/a1554e00.htm, pp. 21-23

Colman PM, Jansonius JN, Matthews BW. "The structure of thermolysin: an electron density map at 2-3 A resolution.", J Mol Biol. 70 (1972), 701-24.

Coorevits A, Dinsdale AE, Halket G, Lebbe L; De Vos P, Van Landschoot A, Logan NA (2012) Taxonomic revision of the genus *Geobacillu*s: emendation of *Geobacillus, G. stearothermophilus, G. jurassicus, G. toebii, G. thermodenitrificans* and *G. thermoglucosidans* (nom. corrig., formerly '*thermoglucosidasiu*s'); transfer of *Bacillus thermantarcticus* to the genus as *G. thermantarcticus* comb. nov.; proposal of *Caldibacillus debilis* gen. nov., comb. nov.; transfer of *G. tepidamans* to *Anoxybacillus* as *A. tepidamans* comb. nov.; and proposal of *Anoxybacillus caldiproteolyticus* sp. nov. International Journal of Systematic and Evolutionary Microbiology 62:1470-1485

Criswell LG, Litchfield JH, Vely VG and Sachsel GF (1964). Studies on improved recovery of protein from rendering plant raw materials and products: II. Acid and enzyme hydrolysis, Food Technol, 18, (9), 247-251

Douglass JS, Barraj LM, Tennant DR, Long WR, Chaisson CF (1997). Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contaminants, 14, 791-802

Endo S. "Study on protease by thermophilic bacteria.", J Ferment Technol. 40 (1962), 346 –353.

Feder J, Garrett LR, Wildi BS. "Studies on the role of calcium in thermolysin.", Biochemistry, 10 (1971), 4552-4556

Feldman J, Haas G, Lugay J and Wiener C (1974). Process for bland, soluble protein, Patent US3857966 A

Food Chemicals Codex (FCC) 13th Edition. 2015. US Pharmacopeia, Rockville, Maryland

Gomaa, A. (2018). Application of enzymes in brewing. J. Nutr. Food Sci. Forecast, 1(5).

Hale, MB (1969). Relative Activities of Commercially-Available Enzymes in the Hydrolysis of Fish Protein, Food Technol, 23, 107-110

Hansen, S.C. (1966). Acceptable daily intake of food additives and ceiling on levels of use. Food Cosmet. Toxicol., 4, 427-432.

Hamada S, Suzuki K, Aoki N and Suzuki Y (2012). Improvements in the qualities of gluten-free bread after using a protease obtained from Aspergillus oryzae. Journal of Cereal Science, 57, 91-97

JECFA (Joint FAO/WHO Expert Committee on Food Additives) 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing

Jones BL (2005). Endoproteases of barley and malt. Journal of Cereal Science 42 (2), 139-156

Koury B and Spinelli J (1974). Preparation of functional fish protein concentrates and isolates, Patent US3826848 A

Ladics GS, Cressman RF, Herouet-Guicheney C, Herman RA, Privalle L, Song P, McClain S (2011). Bioinformatics and the allergy assessment of agricultural biotechnology products: industry practices and recommendations. Regulatory Toxicology and Pharmacology, 60(1), 46-53

Latt SA, Holmquist B, Vallee BL. "Thermolysin: a zinc metalloenzyme.", Biochem. Biophys. Res. Commun. 37 (1969), 333-339.

Magrane, M and the UniProt consortium**.** 2011. **UniProt Knowledgebase: a hub of integrated protein data.** Database, 2011: bar009 (2011).

Malacarne M, Martozzi F, Summer A and Mariani P (2002). Protein and fat composition of mare´s milk: some nutritional remarks with reference to human and cow´s milk. International Dairy Journal, 12 (11), 869-877

Matthews BW, "Structural basis of the action of thermolysin and related zinc peptidases.", Acc. Chem. Res. 21 (1988), 333-340

Matsubara H. "Observations on the specificity of thermolysin with synthetic peptides.", Biochem. Biophys. Res. Commun., 24 (1966), 427-430

Morihara K, Tsuzuki H, Oka T. "Comparison of the specificities of various neutral proteinases from microorganisms.", Arch Biochem Biophys. 123 (1968) 572-588

Morihara K, Tsuzuki H. "Comparative study of various neutral proteinases from microorganisms: specificity with oligopeptides.", Arch Biochem Biophys. 146 (1971) 291-296

Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV. "Taxonomic study of aerobic thermophilic bacilli and descriptions of Geobacillus subterraneus gen. nov., sp. nov. and Geobacillus uzenensis sp. nov. from petroleum reservoirs and transfer of Bacillus

stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius and Bacillus thermodenitrificans to Geobacillus as the new combinations G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius and G. thermodenitrificans." Int. J. Syst. Evol. Microbiol. 51 (2001), 433-466.

O'Donohue MJ, Roques BP, Beaumont A, "Cloning and expression in Bacillus subtilis of the npr gene from Bacillus thermoproteolyticus Rokko coding for the thermostable metalloprotease thermolysin.", Biochem J 300 (1994), 599-603.

Ohta Y, Ogura Y, Wada A. "Thermostable protease from thermophilic bacteria. I. Thermostability, physicochemical properties, and amino acid composition.", J Biol Chem. 241 (1966) 5919-5925

Ooshima H, Mori H, HaranoY. "Synthesis of aspartame precursor by thermolysin solid in organic solvent.", Biotechnol Lett 7 (1985), 789-792.

Poutanen K (1997). Enzymes: An important tool in the improvement of the quality of cereal foods. Trends in Food Science & Technology, 8(9), 300-306

Sattar SA, Synek EJ, Westwood JC, Neals P. "Hazard Inherent in Microbial Tracers: Reduction of Risk by the Use of Bacillus stearothermophilus Spores in Aerobiology". Appl Microbiol. 23 (1972), 1053–1059.
Shimono M and Sugiyama K (2010). Salty taste enhancing agent and food or drink containing the same, Patent EP2263477 A1

Sripathy NV, Sen DP, Lahiry NL, Sreenivasan A and Subrahmanyan V (1962). Fish Hydrolysates II. Standardization of Digestion Conditions for .Preparation of Hydrolysates Rich in Peptones and Proteoses, Food Technol, 141-142

Tester R F, Yousuf R, Kettlitz B and Röper H (2007). Use of commercial protease preparations to reduce protein and lipid content of maize starch. Food chemistry, 105(3), 926-931

The UniProt Consortium. 2012. Reorganizing the protein space at the Universal Protein Resource (UniProt). Nucleic Acids Res. 40: D71-D75 (2012).

Titani K, Hermodson MA, Ericson LH, Walsh KA, Neurath H. "Amino acid sequence of thermolysin. Isolation and characterization of the fragments obtained by cleavage with cyanogen bromide.", Biochemistry 11 (1972), 2427-35.

Whitehurst RJ and Law B (2010). Enzymes in Food Technology, Sheffield academy press, 117-137

WHO/SEA (World Health Organization, South East Asia), "Quality Control in Sterilization", in: Quality Assurance in Bacteriology and Immunology, 2003