

Supporting document 1

Risk and technical assessment report – Application A1107

Asparaginase from *Bacillus subtilis* as a Processing Aid (Enzyme)

Executive summary

Application A1107 seeks approval for the use of asparaginase isolated from a genetically modified strain of *Bacillus subtilis* as a processing aid. The stated purpose of this enzyme, namely the hydrolysis of the amide in asparagine to aspartic acid and the subsequent reduction in acrylamide formation during food manufacture, 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 asparaginase preparation containing asparaginase produced by genetically modified *B. subtilis* strain MOL2940, as a food processing aid on the basis of the following considerations:

- The production organism *B. subtilis*, 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. subtilis* 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 is expected to be present in the final food but would be inactive and susceptible to digestion like any other dietary protein.
- Bioinformatic analysis indicated that the enzyme has no biologically relevant homology to known protein allergens or toxins.
- The enzyme preparation caused no observable effects at the highest tested doses in a 90-day toxicity study in rats. The No Observable Adverse Effect Level (NOAEL) was 1.207 g TOS (Total Organic Solids)/kg bw/d, 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 for asparaginase from *B. subtilis*. A dietary exposure assessment is therefore not required.

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1 Introduction

FSANZ received an application from Novozymes Australia Pty Ltd seeking approval for the enzyme asparaginase (EC 3.5.1.1) as a processing aid to be used in the production of food. The enzyme is produced from a genetically modified (GM) strain of *Bacillus subtilis* expressing a thermo-tolerant asparaginase gene from *Pyrococcus furiosus*.

The Applicant proposes to use the asparaginase to reduce the concentration of asparagine in certain foods (potato chips, breakfast cereals and coffee beans) to reduce the risk of acrylamide formation in these foods during high temperature processing.

1.1 Objectives of the Assessment

As there are no permissions for the enzyme asparaginase from *B. subtilis* currently in the *Australia New Zealand Food Standards Code* (the Code), any application to amend the Code to permit the use of this enzyme from this source 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 asparaginase as a processing aid.

2 Food Technology Assessment

2.1 Acrylamide

Acrylamide is formed by the heat-induced reaction between reducing sugars and asparagine, which is one of the reaction pathways of the Maillard reaction. The Maillard reaction is the process that gives the brown colour and tasty flavour of baked, fried and toasted foods.

2.2 Characterisation of asparaginase

2.2.1 Identity of the enzyme

Information regarding the identity of the enzyme that was taken from the Application has been verified using enzyme nomenclature references (IUBMB 2015). Additional information has also been included from the IUBMB reference.

Systematic name: L-asparagine amidohydrolase

Accepted IUBMB¹ name: Asparaginase

IUBMB enzyme nomenclature: EC 3.5.1.1

C.A.S. number: 9015-68-3

¹ International Union of Biochemistry and Molecular Biology

Other names: asparaginase II; L-asparaginase; colaspase; elspar; leunase; crasnitin; α -asparaginase

Reaction: L-asparagine + H₂O = L-aspartate + NH₃

Commercial name: Acrylaway HighT

2.2.2 Enzymic properties

The enzyme asparaginase catalyses the hydrolysis of the amide of the amino acid L-asparagine to the corresponding acid, L-aspartate, and ammonia (IUBMB 2015).

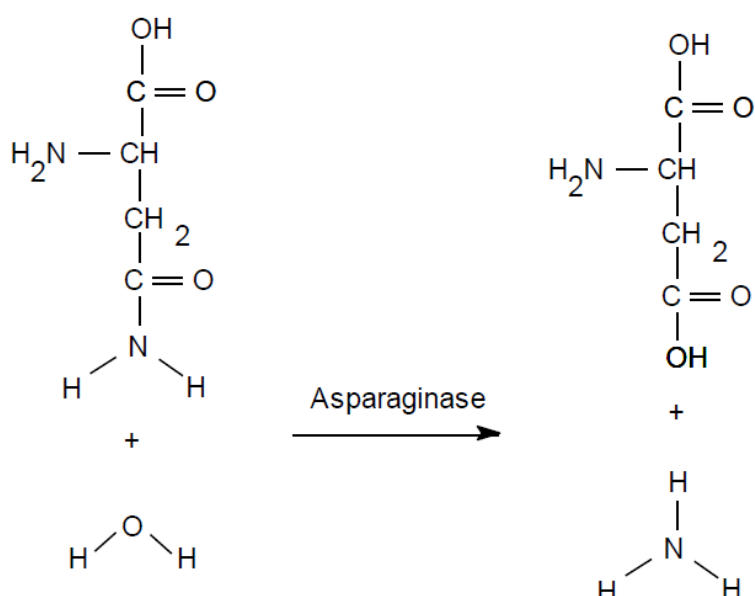
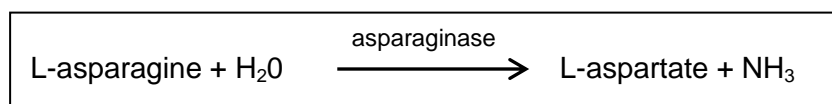


Figure 1: Reaction catalysed by asparaginase (Source: Application)

Asparagine is one of the precursors of the Maillard browning reaction, reacting with reducing sugars to form acrylamide during high temperature manufacturing processes. The combination of time and temperature during processing is critical in the formation of acrylamide (FoodDrinkEurope 2014). Therefore, treating food to reduce the concentration of asparagine prior to heat processing would reduce the amount of acrylamide formed in foods such as potato chips, breakfast cereals and coffee beans.

The enzyme is largely inactivated by excessive heat treatment during later processing steps – i.e. the toasting of breakfast cereals, roasting of coffee beans, frying potato-based snacks and chips.

2.2.3 Physical properties

The commercial enzyme preparation is supplied in two forms:

- Acrylaway HighT L – a light brown liquid preparation with approximately 0.8% Total Organic Solids (TOS), 5% sodium chloride, 46.7% sorbitol, 47% water, 0.3% sodium benzoate and 0.2% potassium benzoate.

- Acrylaway HighT BG – a light yellow granulate with approximately 0.8% TOS, 3% sodium chloride, 4% water, 91.9% corn flour and 0.3% dextrin.

The enzyme preparations are standardised to an activity of 6,000 Thermostable Asparaginase Units (TASU)/gram. The maximum recommended dosage is 15,000 TASU/kg dry matter.

2.2.4 Temperature

Minimum time-temperature conditions to ensure that the enzyme is inactivated in the final product are recommended by the Applicant for breakfast cereal applications (Table 1). Food companies interested in using the enzyme preparation to reduce the formation of acrylamide in other food matrices should contact the Applicant seeking similar time and temperature inactivation data.

Table 1: Time-Temperature combinations to inactivate the asparaginase in breakfast cereals

| Temperature (°C) | Time (min) |
|------------------|------------|
| 140 | 1 |
| 120 | 5 |
| 110 | 15 |
| 105 | 20-25 |

2.2.5 Flavour

The current Acrylamide Toolbox (2013) prepared by FoodDrinkEurope to help food companies reduce the levels of acrylamide in their products notes there can be off flavours produced when asparaginase is used (FoodDrinkEurope 2014). Effects on flavour for the different food categories are noted below. It will be important for food manufacturers to perform their own evaluations of using asparaginase to check for any flavour taints, as well as establishing treatment conditions that minimise the formation of acrylamide.

2.2.5.1 Potato-based products

In fabricated potato-based products, the use of asparaginase with potatoes containing a high level of asparagine may result in the production of excessive amounts of aspartic acid and ammonia, imparting an off-flavour to the end product (FoodDrinkEurope 2014).

2.2.5.2 Green coffee beans

FoodDrinkEurope (2014) reported that trials with the asparaginase treatment of green coffee beans can result in negative flavour impacts depending on the coffee variety and the treatment conditions used. This was the case for Arabica coffee beans where there were off-flavours.

2.2.5.3 Breakfast cereals

The Application reported that there are not adverse effects of using the enzyme preparation on the quality of the final cereal.

2.3 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 and the enzyme preparations are reported to be made according to Good Manufacturing Processes for food.

The fermentation process involves two steps, the initial inoculum fermentations to produce enough of the microorganism for the production fermentation and then the main fermentation. The downstream processing steps taken after the main fermentation to produce the enzyme preparation consist of: removal of the production strain and other solids, ultrafiltration to concentrate and further purify the enzyme, filtration, preservation and stabilisation and, if required, further concentration. More detail of the individual steps is provided in the Application.

2.3.1 Potential presence of allergens

Soybean meal is one of the potential vegetable protein raw material sources for the fermentation. Starch hydrolysates, which can be produced from wheat starch, may also be used as a fermentation raw material. Soybean products and wheat products are identified as substances requiring declaration in clause 4 of Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations of the existing Code (subsection 1.2.3 – 4 in the revised Code).

2.4 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 asparaginase preparation meets the Food Chemicals Codex and JECFA purity criteria for food grade enzymes. Table 2 compares a batch of the product with various specifications. The specification monographs listed above are primary references for specifications in Standard 1.3.4 – Identity and Purity.

Table 2: Product specifications for a batch of commercial asparaginase preparation compared to specifications for enzymes

| Analysis | Specification (as listed in Application) | Analytical data for enzyme preparation |
|---|---|---|
| Heavy metals (mg/kg) [♦] | ≤ 30 | 4.6 |
| Lead (mg/kg) | ≤ 5 ^{*Δ} | ≤ 0.5 |
| Arsenic (mg/kg) | ≤ 3 | ≤ 0.1 |
| Mercury (mg/kg) | ≤ 0.5 | ≤ 0.03 |
| Cadmium (mg/kg) | ≤ 0.5 | ≤ 0.05 |
| Total viable count (cfu/g) | ≤ 50,000 | ≤ 100 |
| Coliforms (cfu/g) | ≤ 30 ^{*Δ} | ≤ 10 |
| <i>Salmonella</i> (in 25 g) | Not detected ^{*Δ} | Not detected |
| Enteropathogenic <i>E. coli</i> (in 25 g) | Not detected [*] | Not detected |
| Antibiotic activity | Not detected [*] | Not detected |
| Production strain (cfu/g) | Not detected | Not detected |

[♦] Heavy Metals = Sum of silver, arsenic, bismuth, cadmium, copper, mercury, molybdenum, nickel, lead, antimony, tin

^{*} JECFA specification (JECFA 2001)

^Δ Food Chemicals Codex Specification (U.S. Pharmacopeial Convention 2014)

The Application states that the asparaginase preparation contains no detectable antibiotic activity. Absence of antimicrobial activity is a requirement of the JECFA specifications for enzymes used in food processing.

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

2.5 Technological function of the enzyme

The enzyme preparation is intended to be used in the manufacture of foods such as potato chips, potato-based snacks and breakfast cereals and for the pre-treatment of green coffee beans to reduce the formation of acrylamide during manufacture. It is added to the food before the high temperature processing step (i.e. baking, frying or roasting) in the manufacture of the food.

The Acrylamide Toolbox 2013 (FoodDrinkEurope 2014) aims to assist manufacturers, national authorities and other bodies to prevent and reduce the formation of acrylamide in specific foods and manufacturing processes and is the result of collaboration between European Union national authorities and food industry. The Toolbox identifies potatoes, cereals and coffee as the three main product groups that are at higher risk of acrylamide formation. The enzyme preparation in this Application is proposed to be used in the manufacture of foods from these three main product groups.

2.5.1 Penetration of the enzyme into the food

FoodDrinkEurope (2014) report that the use of asparaginase in the treatment of raw potato slices for potato crisp manufacture is ineffective due to the enzyme being ineffectively in penetrating the slice / cell walls to act on the asparagine substrate.

The low moisture processing conditions of breakfast cereals makes it difficult for asparaginase to penetrate into the food matrix to react with asparagine (FoodDrinkEurope 2014). The size of the coarse flours and chopped grains also makes enzyme penetration into the food matrix difficult.

2.5.2 Reduction of acrylamide in foods

2.5.2.1 Breakfast cereals

A high water content in the wet mix hydrolysis step of infant cereal manufacture enables the use of asparaginase (FoodDrinkEurope 2014).

The enzyme preparation is added during the mixing of breakfast cereal ingredients and is reported to be most active during the cooking or pre-conditioning step of manufacture. The Application stated that the addition of the enzyme preparation can reduce the level of acrylamide in the final food by 50 – 75% compared to the control. The recommended dosage of the enzyme preparation is 250 – 5,000 TASU per kg flour, with dosage being dependent on moisture content, pH, processing temperatures and holding time of the product. Enzyme inactivation times and temperatures were provided in the Application and ranged from 1 minute at 140°C to 20-25 minutes at 105°C (see Table 1).

Data were provided to show reductions in acrylamide concentrations of:

- 54% for batch cooked wheat flakes (3,000 TASU/kg flour) in lab-scale testing
- 58% for batch cooked corn flakes (1,500 TASU/kg flour) in lab-scale testing
- 75% for whole wheat and bran extruded flakes (3,000 TASU/kg flour) in pilot-scale testing
- 56% for whole wheat and rice extruded flakes (6,000 TASU/kg flour) in pilot-scale testing

Further details about the reduction in acrylamide concentration and the testing conditions to achieve these reductions can be found in the Application.

2.5.2.2 Coffee

FoodDrinkEurope (2014) report that acrylamide could be reduced by 5-45%, depending on the processing conditions and the type of coffee.

No data were provided in the Application on the reduction of acrylamide concentration with the use of the enzyme preparation in coffee beans.

2.5.2.3 Potato-based products

Asparaginase significantly reduces the levels of acrylamide in fabricated potato-based products. On a commercial scale the enzyme's effectiveness is recipe and process dependent and requires a delicate balance of reaction conditions and contact time to be effective.

FoodDrinkEurope (2014) report that acrylamide can be significantly reduced by asparaginase treatment of fabricated potato based products. However, the efficacy is product recipe and process dependent. It requires a delicate balance between reaction conditions and contact time.

No data were provided in the Application on the reduction of acrylamide concentration with the use of the enzyme preparation in potato-based products.

2.6 Food technology conclusion

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. Food manufacturers will need to conduct evaluations on process conditions for using the asparaginase preparation with their food products to assess if acrylamide formation is reduced, to optimise the conditions and ensure negative product attributes do not form. The enzyme preparation meets international purity specifications for enzymes used in the production of food.

3 Hazard Assessment

3.1 Background

3.1.1 Chemistry

Details of the chemistry of the asparaginase produced by *B. subtilis* including relevant physicochemical and enzymatic properties, and product specifications, are provided in the Food Technology Assessment (Section 0).

3.1.2 Description of the genetic modification

Asparaginase is produced by a genetically modified (GM) production strain (MOL2940) of *B. subtilis*, which expresses an asparaginase (*asnPfu*) gene (Banke et al. 2014).

The expression cassette comprises:

- a fragment of a hybrid *Bacillus* promoter with promoter elements from *B. licheniformis*, *B. amyloliquefaciens* and *B. thuringiensis*
- a chemically synthesised coding sequence of the *asnPfu* gene derived from the extremophile *Pyrococcus furiosus* (ATCC 43587) from thermal marine sediments (Maeder et al. 1999)
- a *B. licheniformis* terminator from the *amyL* gene
- an integration fragment that enables site specific integration on the genome of the recipient strain.

The cassette was incorporated into expression plasmid pMOL2930 which was then mobilised into *B. subtilis* recipient strain PP2982 at three specific loci using a site-specific integration method. The resulting production strain therefore contained three copies of the expression cassette and was given the designation MOL2940. Southern blot analyses, polymerase chain reaction analysis and DNA sequencing of the inserts and flanking regions showed only elements between the promoter fragment and the integration element are present in the final production strain and there are no other coding sequences from the plasmid; this means, in particular, there are no antibiotic resistance genes present in the production strain.

To test the stability of the insert in the production strain, DNA from cells from four separate pilot plant fermentations was compared to DNA from cells in the master cell bank (MCB) by Southern blot analysis using part of the *asnPf* coding region as a probe. The band patterns obtained for all the pilot batches were identical to the band pattern of the MCB, thus indicating stability of the insert.

3.1.3 Scope of the hazard assessment

The hazard of asparaginase derived from the *B. subtilis* production strain was evaluated by considering:

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

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

The production strain (MOL2940), containing three *asnPfu* genes, is genetically engineered from recipient strain PP2982. In turn, PP2982 has been derived from strain A164, which is identical to strain ATCC6051a (the deposited type strain of *Bacillus subtilis*), through a series of targeted recombination events to inactivate genes encoding several proteases, as well as a gene essential for sporulation and a gene essential for the production of surfactin². The Applicant claims these modifications enhance the safety and stability of the asparaginase that is produced.

B. subtilis, is widely distributed in the environment by virtue of its natural occurrence in soil and is also detectable in water, air and decaying plant material (US EPA 1997). The bacterium is not pathogenic to humans or toxigenic (de Boer and Diderichsen 1991; US EPA 1997) and has been recommended for a qualified presumption of safety (QPS) by the Scientific Committee of the European Food Safety Authority (EFSA 2007).

FSANZ has previously assessed the safety of *B. subtilis* as the production organism for a number of enzymes used as food processing aids. Standard 1.3.3 of the Code permits the use of the following enzymes derived from *B. subtilis*: α -acetolactate decarboxylase, α - and β -amylase, β -glucanase, hemicellulase endo-1,4- β -xylanase, hemicellulase multicomponent enzyme, maltogenic α -amylase, metalloproteinase, pullulanase and serine proteinase. In the US, several enzyme preparations from *B. subtilis* have Generally-Recognised-As-Safe (GRAS) status (FDA 1999; FDA 2003; FDA 2006; FDA 2009).

The bacterium itself is commercially available in many countries as a dietary probiotic intended to improve human health (Duc et al. 2004; Henriksson et al. 2005). It is also used as an animal feed additive (Klose et al. 2009; Lee et al. 2010), although its efficacy has been questioned (Arthur et al. 2010; Danicke and Doll 2010), and growth promotant in aquaculture (Farzanfar 2006). Strains of *B. subtilis* are used to make fermented soybean products such as thua nao (Thailand) and natto (Japan) (Inatsu et al. 2006; Hosoi and Kiuchi 2008).

Data submitted with the application indicate that the *B. subtilis* 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 – asparaginase

Asparaginase hydrolyses the amide in asparagine to the corresponding acid (L-aspartate), and enzymes with asparaginase activity occur in bacteria, fungi, animals and plants (Olempska-Beer 2008).

² Surfactin is a lipopeptide with exceptional surface activity that is produced by *B. subtilis* and enhances the spreading of multicellular colonies on nutrient substrates.

The enzyme is used in food manufacture to reduce the level of asparagine which is a precursor of acrylamide formed when starchy foods are baked or fried at temperatures above 120° C. Asparaginase is also used as a chemotherapeutic enzyme in the treatment of certain leukaemias and lymphomas that have a diminished capacity to synthesise asparagine (Jennings and Beacham 1990).

The asparaginase produced by *B. subtilis* strain MOL2940 is a 326 amino acid protein (Banke et al. 2014). The intention is that the enzyme preparation (designated Acrylaway HighT and used in either a liquid (L) or granulated (BG) form) – see Table 0) is used as a processing aid during production of various food products such as breakfast cereals, potato based snacks, and sliced potato chips and in the pre-treatment of green coffee beans. In all of these applications, the enzyme treatment takes place upstream in the food production process and the enzyme is largely heat inactivated during subsequent steps where the heat treatment is excessive, i.e. extrusion and toasting of breakfast cereals, frying of potato based snacks or chips and roasting of coffee beans. Therefore any residual enzyme in the final food would be present as denatured protein and undergo normal proteolytic digestion in the gastrointestinal tract. It has also been noted (Olempska-Beer 2008) that as a result of the catalytic activity of asparaginase, low levels of L-aspartic acid and ammonia are expected to be formed in food but this is not of concern since both compounds are present in the human diet.

To confirm the digestibility of asparaginase, potential cleavage sites were investigated by FSANZ using the amino acid sequence of asparaginase and the PeptideCutter tool in the ExPASy Proteomics Site (http://web.expasy.org/peptide_cutter/). Asparaginase has multiple cleavage sites for pepsin (68 sites at pH 1.3 and 88 sites at pH >2), trypsin (35 sites), chymotrypsin (21 high-specificity sites, 67 low-specificity sites) and endopeptidases (63 sites). On this basis, asparaginase is considered likely to be as susceptible to digestion as the vast majority of dietary proteins.

Health Canada has a comprehensive Q & A section on the asparaginases from two GM sources on its website (http://www.hc-sc.gc.ca/fn-an/securit/addit/asparaginase_qs_as-eng.php)

Bioinformatic analyses were performed to assess the similarity to known allergens and toxins of the asparaginase produced by strain MOL2940.

Report submitted

Friis, E. (2013). Sequence homology of asparaginase from MOL2940 to known toxins, and Allergen analysis of asparaginase from MOL2940. Novozymes Report No. 2013-09763 (unpublished).

3.3.1 Bioinformatic analysis for potential allergenicity

The *in silico* analyses compared the asparaginase 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. The conclusion from these bioinformatic analyses is that asparaginase from *B. subtilis* strain MOL2940 does not show biologically relevant homology to any known allergen and on this basis is unlikely to be allergenic.

3.3.2 Bioinformatic analysis for potential toxicity

The asparaginase 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 asparaginase sequence.

The greatest homology found was 16.9%, which indicates that the asparaginase is unlikely to be toxic.

3.4 Evaluation of unpublished toxicity studies

Report submitted

Lichtenberg, J. (2013). Summary of toxicity data. Asparaginase PPV33595. File ID # 2013-02837-02, Novozymes A/S (unpublished).

Unpublished toxicity studies on a representative asparaginase 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 1997) and an *in vitro* micronucleus test conducted in accordance with OECD test Guideline 487 (OECD 2010)
- an *in vitro* cytotoxicity test
- a 90-day oral toxicity study in rats conducted in accordance with OECD Test Guideline 408 (OECD 1998).

The test substance was asparaginase preparation (designated as toxbatch PPV33595) prepared to the manufacturer's specifications by mixing three sub-batches.

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 (Baxevanis 2005).

The test substance was supplied in liquid form (dissolved in water) and differed from the actual product, Acrylaway HighT L and Acrylaway HighT BG. A comparison of PPV33595, Acrylaway HighT L and Acrylaway HighT BG is given in Table 0 .

Table 0: Comparison of typical Acrylaway HighT L, Acrylaway HighT BG and toxbatch PPV33595

| Characteristic | Acrylaway HighT L | Acrylaway HighT BG | PPV33595 |
|--|---------------------------|--------------------|---------------|
| Activity | 6,000 TASU/g ¹ | 6,000 TASU/g | 55,200 TASU/g |
| Total Organic Solids (TOS) (approx. % w/w) | 0.8 | 0.8 | 11.4 |
| Water (approx. % w/w) | 47 | 4 | 86.9 |
| Sodium chloride (approx. % w/w) | 5 | 3 | |
| Sorbitol (approx. % w/w) | 46.7 | - | |
| Sodium benzoate (approx. %w/v) | 0.3 | - | |
| Potassium sorbate (approx. w/v) | 0.2 | - | |
| Corn flour (approx. w/v) | - | 91.9 | |
| Dextrin (approx. w/v) | - | 0.3 | |
| Dry Matter (approx. w/v) | - | - | 13.1 |

¹ TASU = Thermostable Asparaginase Units (calculation method provided by Applicant)

3.4.1 Genotoxicity

Individual studies

Pedersen, P.B. (2013). Asparaginase, batch PPV33595: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*. Novozymes reference no. 20128071 (File 2013-01259-01), Novozymes A/S (unpublished).

May, K. (2012). Asparaginase, PPV33595: *In vitro* micronucleus test in cultured human lymphocytes Huntingdon Life Sciences study no. LKG0062, Novozymes reference no. 20126013 (File 2012-19300-01) (unpublished)

The outcomes of these *in vitro* studies are summarised in Table . Negative (water vehicle) and positive controls were tested in each study and gave expected results. The micronucleus study comprised two experiments that involved two different exposure times and S9 presence/absence. Both studies gave negative results.

Table 4: 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> | asparaginase obtained from <i>B. subtilis</i> (Batch No.PV33595) Water vehicle | 156–5000 µg test substance (in solution) per millilitre ± S9 ¹ | Negative |
| <i>In vitro</i> micronucleus test | Human lymphocytes prepared from the pooled blood of 2 healthy, non-smoking male donors | As above | 1 st experiment (3 h exposure): 0.16, 0.31, 0.63, 1.25, 2.5 % (v/v) ± S9 2nd experiment (20 h exposure) : 0.16, 0.31, 0.63, 1.25, 2.5 % (v/v) –S9 | Negative |

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

3.4.2 Cytotoxicity

Individual study

Birkved, F.K. (2012). Asparaginase, batch PPV33595 *In vitro* cytotoxicity test: Neutral red uptake in BALB/c 3T3 cell culture. Novozymes reference no. 20128030. File No: 2012-07837-01 (unpublished).

The neutral red uptake assay is a widely used cytotoxicity test (Repetto et al. 2008).

BALB/c 3T3 fibroblasts were incubated for 48 h in asparaginase batch PPV33595 at concentrations ranging from 0 – 30 mg/ml. The cells were then incubated in a medium containing neutral red and the uptake of dye (as measured by the amount of dye subsequently extracted from the cells) was determined spectrophotometrically by reading the absorbance at 540 nm. The amount of dye provides a quantitative estimation of the number of viable cells remaining after exposure to the test article. The concentration of test article required to reduce cell viability to 50% of that of the untreated control is designated the NRU₅₀.

The NRU₅₀ for asparaginase, batch PPV33595 was determined to be 5.72 mg/ml. This figure serves to corroborate the negative results from the *in vitro* micronucleus test.

3.4.3 Subchronic toxicity study

Individual study

Ellemann-Laursen, S. (2012). Asparaginase PPV33595: 90-days oral gavage toxicity study in rats. Study # 74826, CiToxLAB Scantox, Novozymes reference no. 20126001 (File 2012-18820-01) (unpublished).

The PPV33595 asparaginase preparation was administered by gavage to groups of 10 male and 10 female SPF Sprague Dawley (strain Ntac:SD) rats/sex. Rats were sourced from Taconic Europe A/S (Ejby, Denmark), were approximately 5 weeks old and had body weights within a range of 123 – 165 g for males and 108 – 153 g for females at the commencement of dosing. The asparaginase preparation was tested at doses of 0, 1.0, 3.3 or 10.0 ml/kg/d for 90/91 days (water vehicle), meaning that the actual administered doses were different for male and female rats.

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 1.207 g 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 Acrylaway HighT, containing an asparaginase produced by GM *B. subtilis* strain MOL2940, as a food processing aid on the basis of the following considerations:

- The production organism *B. subtilis*, 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. subtilis* 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 is expected to be present in the final food but would be inactive and susceptible to digestion like any other dietary protein.
- Bioinformatic analysis indicated that the enzyme has no biologically relevant homology to known protein allergens or toxins.
- The enzyme preparation caused no observable effects at the highest tested doses in a 90-day toxicity study in rats. The NOAEL was 1.207 g TOS/kg bw/d, the highest dose tested.
- The enzyme preparation was not genotoxic *in vitro*.

It is noted that JECFA allocated an acceptable daily intake (ADI) “not specified” for asparaginase from both *Aspergillus oryzae* expressed in *A. oryzae* (JECFA 2007) and *A. niger* expressed in *A. niger* (Mueller et al. 2009). 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 for asparaginase from *B. subtilis*. A dietary exposure assessment was therefore not required.

4 Conclusion

This risk assessment considered the technological suitability, potential hazard of the production microorganism and the potential hazard of the asparaginase enzyme preparation isolated from a genetically modified *Bacillus subtilis* expressing an asparaginase gene from *Pyrococcus furiosus*.

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

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