Application to Amend Standard 1.3.3 of the Australia New Zealand Food Standards Code to Include Thermolysin from *Thermaerobacillus caldiproteolyticus* AE-TP as a Processing Aid

Executive Summary

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Amano Enzyme, Inc. wishes to request an amendment to Schedule 18 of Standard 1.3.3, "Processing Aids," of the Food Standards Code. The amendment would include thermolysin derived from *Thermaerobacillus caldiproteolyticus* (basonym: *Anoxybacillus caldiproteolyticus*) strain AE-TP as a processing aid.

Thermolysin catalyzes the hydrolysis of peptide bonds. It can use proteins from various origins as a substrate.

Thermolysin from *Anoxybacillus caldiproteolyticus* strain TP-7 is listed in Schedule 18, section S18-9(3), "Permitted Processing Aids—Various Purposes" (section 1.3.3-11).

It has been approved for use in processing dairy products, eggs, meat, fish, proteins, yeast, and flavors.

Strain AE-TP and the currently permitted strain TP-7 belong to the same Safe Strain Lineage (SSL). Strain TP-7 was derived from strain AE-TP through natural selection and non-genetically modified strain improvement. While they share a common biological lineage and safety history, AE-TP and TP-7 are separately identified production strains.

Accordingly, this application seeks recognition of thermolysin produced by strain AE-TP as a permitted source of thermolysin and an extension of the permitted uses of thermolysin to include breads and bakery, fruits and vegetables, and cereals processing.

The thermolysin is an enzyme derived from non-genetically modified strain of *Thermaerobacillus caldiproteolyticus*. The production process of the thermolysin enzyme comprises a cultivation step with *Thermaerobacillus caldiproteolyticus*, followed by several filtration and purification steps to result in thermolysin concentrate.

All of the raw materials used in the manufacture of the thermolysin are safe and suitable for use. The enzyme is produced according to Good Manufacturing Practices (GMP) and the FSSC22000 quality control system. Production controls are in place to monitor the strain during the fermentation and ensure the avoidance of genetic drift. Furthermore, the product specifications along with extensive batch analysis of thermolysin demonstrate the purity of the enzyme preparation, including the absence of microbiological and heavy metal contaminants, as well as the lack of antibiotic activity.

Thermolysin is stable at least 18 months from the manufacturing date under the sealed condition. The optimum pH range of thermolysin is from 7.5 to 8.0 and the optimum temperature is 65℃. Thermolysin is inactivated when exposed to temperature above 85°C in 5 minutes. Also, as far as Amano Enzyme is

aware, Amano Enzyme's thermolysin described in this dossier does not have any enzymatic side activities which might cause adverse effects.
The safety of thermolysin derived from *Thermaerobacillus caldiproteolyticus* is supported by its history of use and toxicity studies. EFSA evaluated the enzyme and concluded that its use in food processing would not significantly affect the human body.

As for the toxicity studies, the food enzyme has been subjected to a standard package of toxicological tests, with the following results:

- Bacterial reverse mutation (Ames) test: No mutagenic activity under the given test conditions
- Micronucleus test: No chromosome damage under the given test conditions
- Systemic toxicity: The No Observed Adverse Effect Level (NOAEL) is 1400 mgTOS/kg bw/day

Thermolysin derived from *Thermaerobacillus caldiproteolyticus* also does not pose any allergenicity concerns, given the long history of use of the enzyme. Additionally, the amino acid sequence of thermolysin does not indicate that thermolysin has a potency of allergenicity concerns.

A bioinformatic analysis was conducted using whole-genome sequence-based screening of the strain. According to the results, the strain contains one AMR gene above the EFSA thresholds. According to the bioinformatic analysis, the gene is not associated with mobile genetic elements. It was present in all the *Anoxybacillus caldiproteolyticus* genomes available in databases and can be considered as intrinsic, typical finding for this species. Also, one potential virulence factor was identified. It has a role in normal microbial protein metabolism, and in the absence of other virulence factors, its presence does not raise safety concerns.

Theoretical Maximum Daily Intake was calculated using the Budget Method. Based on this method, the Total TMDI of thermolysin was calculated as 0.92 mg TOS/kg body weight/day. As described above, NOAEL of the enzyme is 1400 mgTOS/kg/day. Consequently, the safety margin of thermolysin is 1522 (1400/0.92).

As such, no safety concerns are anticipated with the proposed use of thermolysin as a processing aid in Australia/New Zealand.