

Safety evaluation of a thermolysin enzyme produced from *Geobacillus stearothermophilus*



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ABSTRACT

Thermolysin is a zinc metalloprotease that has potential uses in the food industry. The safety of thermolysin has not been demonstrated before, and therefore a series of standard toxicological tests to assess its potential toxicity was undertaken. The thermolysin used in this study was derived from the thermophilic bacterium *Geobacillus stearothermophilus*, which had undergone chemical mutagenesis to generate strains with increased thermolysin production. Acute toxicity studies in rats and mice showed that thermolysin powder is not acutely toxic with an oral LD₅₀ of more than 18,000 mg/kg (2520 mg/kg thermolysin protein) in rats and more than 24,000 mg/kg (3360 mg/kg protein) in mice. Subchronic feeding studies in rats for 91 days at doses up to 1000 mg/kg (390 mg/kg protein) revealed no significant differences between treated and non-treated groups and a No Observed Effect Level (NOEL) of 1000 mg/kg (390 mg/kg protein) per day was established. Results from genotoxicity tests such as *in vitro* chromosomal aberration assay and *in vivo* mouse micronucleus were negative. Allergenicity sequence analysis revealed no evidence suggesting that thermolysin is an allergen. The data presented in this study support the conclusion that thermolysin is safe for use in food production.

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1. Introduction

Thermolysin (EC 3.4.24.27; CAS 9073-78-3) derived from *Geobacillus stearothermophilus* is a 34.4 kDa thermostable neutral protease that has been described as one of the best studied zinc metalloproteases (O'Donoghue et al., 1994; Blumberger et al., 2007). The production organism, *G. stearothermophilus*, is a thermophilic, rod-shaped, Gram-positive bacterium (Nazina et al., 2001). *G. stearothermophilus* (National Center for Biotechnology Information taxonomy database 1422) was previously classified as *Bacillus stearothermophilus* and recently transferred to a new thermophilic genus *Geobacillus* due to a phenotypic and 16S rRNA gene sequence analysis (Nazina et al., 2001). The strain was confirmed to be *G. stearothermophilus* by a third party laboratory based on nutrient usage, morphology, ability to grow at various temperatures, and ability to produce certain compounds (data not shown; Gordon et al., 1973).

The thermolysin produced by *G. stearothermophilus* is bacterial extracellular zinc endoprotease involved in catalyzing the hydrolysis of the peptide bond at the N-terminus region of large hydrophilic residues (Blumberger et al., 2007). Thermolysin is bound to a single zinc ion, which has been shown to be essential for its

catalytic function. The mature 316 amino acid and gene sequence from this specific production strain has been described (Kubo and Imanaka, 1988) and is available at <http://www.ncbi.nlm.nih.gov/nuccore/M21663>. In addition, the three dimensional structure of thermolysin was determined through X-ray crystallography (Matthews et al., 1972). Thermolysin has potential for commercial applications in fields such as the food industry as it has demonstrated stability at high temperatures and tolerance to organic solvents (Blumberger et al., 2007). The heat resistance of thermolysin has been attributed to the presence of the four calcium cations present in the interior of the enzyme, which prevents large conformational changes (Blumberger et al., 2007).

Microbially derived enzymes have had a long and established history of safe use for commercial applications in a variety of fields, such as the food industry. Enzymes are ubiquitous in living organisms and are consumed on a regular basis. Enzymes consumed by humans as part of a normal diet are not considered to be toxic and “are considered intrinsically safe” (Olempska-Beer et al., 2006). Enzymes used in the food industry are made of proteins, and are digested and metabolized like other types of dietary proteins (Olempska-Beer et al., 2006).

The production strain *G. stearothermophilus* is a non-pathogenic, Gram positive bacterium with a history of safe use for the production of amylase enzymes used in food processing primarily for enzymes such as α -amylases (Olempska-Beer et al., 2006). For

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example, *G. stearothermophilus* is the production organism for a 1,4- α -glucan branching enzyme preparation, which is used as a processing aid for the production of modified food starch ingredients, and is the subject of a Generally Recognized as Safe (GRAS) Notice (GRN 405) submitted to the Food and Drug Administration (FDA). FDA subsequently issued a letter indicating that the Agency had no questions about the submitter's safety conclusion. Given that *G. stearothermophilus* has a history of safe use in the food industry as described in the review articles on food-processing enzymes from recombinant microorganisms (Olempska-Beer et al., 2006) and the 1,4- α -glucan branching enzyme GRAS Notice above, it is not anticipated to have any unintended pleiotropic effects that would lead to the production of toxins or other unwanted metabolites, which is a point of consideration commonly used to assess the safety of enzymes (Pariza and Johnson, 2001).

The final production strain of *G. stearothermophilus* has undergone seven rounds of classical chemical mutagenesis with the commonly used mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and has been screened for strains that have increased production of thermolysin. After chemical mutagenesis treatment with NTG, the nucleotide sequence of the thermolysin gene in the final mutagenized *G. stearothermophilus* production strain was confirmed through DNA sequence analysis (data not shown) to be unaffected by the NTG treatment. Therefore, the thermolysin gene and subsequent thermolysin enzyme produced from the mutagenized *G. stearothermophilus* production strain is identical to the thermolysin produced from the wild type parental strain.

Proteases in general have been used in the food industry for over 70 years (Garcia-Carreño, 1991). Thermolysin (EC 3.4.24.27) from a closely related bacterial species *Bacillus thermoproteolyticus* Rokko has already been used to synthesize N-carbobenzoxyl-L-Asp-L-Phe methyl ester (ZDFM), which is a precursor for the artificial sweetener aspartame, on a commercial scale (Inouye et al., 2007). However, studies that specifically evaluate the toxicological profile for thermolysin have not been published. Therefore, we have conducted a series of standard toxicological studies including acute toxicity feeding studies in rodents, a 91-day oral gavage toxicity study in rats, chromosomal aberration in cultured Chinese hamster cells, and a micronucleus test in mice.

8. Materials and methods

2.1. Test material preparation and characterization of enzyme preparation

G. stearothermophilus is cultivated utilizing standard fermentation processes under controlled pH and temperature conditions. The fermentation broth was sterilized prior to seeding with the *G. stearothermophilus*, pH adjusted, and fermented at the optimal time and temperature. Although all the ingredients used in the fermentation broth are appropriate for food applications, the fermentation broth is removed from the final product in a series of filtration steps, as described in this section. As the proteases are secreted into the fermentation broth, the *G. stearothermophilus* cells are removed with a filtration press step. Purification and concentration of thermolysin protein includes an adjustment to achieve a pH of 7.2–7.5 and crystallization of the protein. A series of filtration steps, including another filter press, dissolution of the crystal, and a 0.2 μ m microfiltration step which removes any intact bacteria, are performed. There is a small amount of carbohydrate left over from the fermentation media. Minerals such as NaCl, CaCl₂, Na₂HPO₄, and Ca(OH)₂ are added to the protein solution and spray dried. An extra purification step involving minerals such as Ca(CH₃COO)₂ and CH₃COONa may be executed and the protein is freeze dried. Five individual lots of the thermolysin powder containing an average of 39% (w/w) protein, which was used for the 91-day study, had an average enzymatic activity of 4,390,000 units/g. This value serves as a reference point for the protein activity levels of other thermolysin preparations that contain different percentages of the thermolysin protein.

2.2. Acute toxicity study in rats

Thirty grams of thermolysin powder containing 14% thermolysin protein was dissolved in 100 ml of distilled water to form the test article (30% suspension). At the time of administration, the animals were about 5 weeks of age with body

weights of 120–145 g for males and 105–125 g for females. Groups of 10 Wistar rats per sex were given the test article (thermolysin powder) at doses of 10,400 mg/kg, 12,500 mg/kg, 15,000 mg/kg, and 18,000 mg/kg once by oral gavage, which, due to the 14% thermolysin protein content of the powder, reflect thermolysin protein doses of 1456 mg/kg, 1750 mg/kg, 2100 mg/kg, and 2520 mg/kg, respectively. The animals were observed for general condition and mortality at 5 h, 15 h, 24 h, 48 h, 72 h, and 168 h after administration of the test article. Body weight was measured on the day before the test article administration and at the end of a 7-day observation period. At the end of the observation period, necropsy was performed on all animals.

2.3. Acute toxicity study in mice

Forty grams of thermolysin powder containing 14% thermolysin protein was dissolved in 100 ml of distilled water to form the test article (40% suspension). At the time of administration, the animals were about 5 weeks of age with body weights of 22–24 g for both males and females. Groups of 10 ddY-N stain mice (Japan SLC Inc.) per sex were given the test article (thermolysin powder) at doses of 13,900 mg/kg, 16,700 mg/kg, 20,000 mg/kg, and 24,000 mg/kg once by oral gavage, which, due to the 14% thermolysin protein content of the powder, reflect thermolysin protein doses of 1946 mg/kg, 2338 mg/kg, 2800 mg/kg, and 3360 mg/kg, respectively. The animals were observed for general condition and mortality at 5 h, 15 h, 24 h, 48 h, 72 h, and 168 h after administration of the test article. Body weight was measured on the day before the test article administration and at the end of a 7-day observation period. At the end of the observation period, necropsy was performed on all animals.

2.4. 91-Day oral gavage toxicity study in rats

The study was conducted in accordance with the GLP standards of OECD Principles of Good Laboratory Practice, EPA Pesticide Programs and Toxic Substances Control, and FDA Non-clinical Laboratory Studies.

The test article was prepared weekly by making a stock solution using a thermolysin powder containing 39% thermolysin protein in a container with the vehicle, 1% methyl cellulose, to make a brown suspension. The test material was stored at 4 °C and dispensed as daily aliquots. Thermolysin in the dose suspension was shown by enzyme assay to be stable for at least 7 days at all concentrations used.

Sprague–Dawley CD rats were obtained from Charles River GmbH Wig, West Germany, and were acclimatized to the laboratory conditions for 14 days (7 days pre and 7 days post randomization) prior to administration of the test article. Randomization was performed using a computer-generated random algorithm according to body weight. At the start of the study, the animals were approximately 6 weeks of age with body weights of 188–231 g for males and 128–161 g for females.

Rats were fed the standard pelleted laboratory animal diet (RMH-B from Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. Rats were housed under controlled conditions: air-conditioned room 7.5–15 air changes per hour, 18–22 °C, relative humidity of 40–85%, and 12 h light/dark cycles. Five animals (same sex) were housed in one stainless steel suspended cage with wire mesh floors.

Groups of 20 rats per sex were given the test article (thermolysin powder) at doses of 0 mg/kg (control), 40 mg/kg, 200 mg/kg, and 1000 mg/kg, which, due to the 39% thermolysin protein content of the powder, reflect thermolysin protein doses of 15.6 mg/kg, 78 mg/kg, and 390 mg/kg, respectively, by oral gavage, 7 days per week for 91 days. The volume administered was 10 ml/kg body weight. The animals were observed and recorded twice daily for mortality/viability, and weekly for clinical signs, food consumption, and body weight. Ophthalmological observations were performed on all animals by indirect ophthalmoscope (DORC) at the start of the administration and by direct ophthalmoscope (Beta-200 Heine-ophthalmoscope) at week 13 following instillation of a pupil dilator.

Blood samples were collected under light ether anesthesia. The animals were fasted overnight with water provided before blood sampling. Blood samples were drawn from the retro-orbital sinus of 10 rats per sex per group and collected into tubes prepared with EDTA for haematological parameters (0.6 ml) and untreated tubes for clinical biochemistry parameters (2.0 ml). The haematological and clinical biochemistry parameters were analyzed.

All animals that survived to the end of the observation period were anaesthetized with pentobarbitone, euthanized by exsanguination, and subjected to necropsy. Descriptions of all macroscopic abnormalities were recorded. Samples of tissues and organs were collected from all animals at necropsy and fixed in neutral phosphate buffered 4% formaldehyde solution. During necropsy, adrenal glands, kidneys, liver, and testes were weighed and recorded. Slides of tissues collected during necropsy from the control group and the top dose group [1000 mg/kg (390 mg/kg thermolysin protein)], slides of sciatic nerve, brain, and spinal cord from intermediate groups [40 mg/kg (15.6 mg/kg thermolysin protein) and 200 mg/kg (78 mg/kg thermolysin protein)], and all gross lesions of all animals were examined by a pathologist.

The following statistical methods were used to analyze the body weight, food consumption, organ weights, and clinical laboratory data. Univariate one-way analysis of variance was used to assess the significance of intergroup differences. If the variables could be assumed to follow a normal distribution, the Dunnett-test (many

to one *t*-test) based on a pooled variance estimate was applied for the comparison of the treated groups and the control group. The Steel-test (many-one rank test) was applied when the data could not be assumed to follow a normal distribution. All tests were two-sided and in all cases $p < 0.05$ was accepted as the lowest level of significance. Group means were calculated for continuous data and medians were calculated for discrete data. Individual values, means, standard deviations and statistics were rounded off before printing.

2.5. Chromosomal aberration test in cultured Chinese hamster cells

The *in vitro* chromosomal aberration assay was conducted in accordance with the OECD Guideline for Test No. 473, and GLP standards.

The Chinese hamster lung fibroblast (CHL/IU) cells were obtained from the Health Science Research Resources Bank. The cells were subjected to characteristic testing on a regular basis. The cells that were verified to have appropriate characteristics (i.e., the cells grew as a monolayer, the doubling time was within 15–20 h, the average number of chromosomes was 25, and there was no mycoplasma contamination). The passage number of the cells at the time of use was 7 for the cell-growth inhibition test, 9 for the short-term treatment, 13 for the continuous treatment, and 15 for the continuous treatment (retest). The test article, thermolysin powder, containing 30.9% thermolysin protein, was dissolved in a vehicle (water) to prepare the test solution.

A cell-growth inhibition test was conducted as a preliminary test to select doses for the chromosome aberration test. Two separate experiments were performed: short-term treatment and continuous treatment. Each group was conducted in duplicate. In the short-term treatment, for each plate, 2×10^4 cells in 5.0 mL of culture were seeded and incubated at 37 °C in humidified air containing 5% CO₂. After 3 days of cultivation, cells were observed under an inverted phase-contrast microscope and confirmed to have no abnormality. The medium was replaced with freshly added culture medium, and the cells were treated with 0.500 mL of the test solution at each concentration or vehicle in the absence or presence of 0.833 mL of S9-mix to a total of 5.0 mL. The concentrations of thermolysin powder in the test solution in the medium were 39.1, 78.1, 156, 313, 625, 1250, 2500, and 5000 µg/mL, which, due to the 30.9% thermolysin protein content of the powder, reflect thermolysin protein doses of 12.1, 24.1, 48.2, 96.7, 193.1, 386.3, 772.5, 1545.0 µg/mL, respectively. After 6 h, the cells were rinsed with isotonic sodium chloride solution containing bovine serum, 5.0 mL fresh culture medium was added, and the culture was cultivated for another 18 h. After the cultivation, the cells were rinsed with isotonic sodium chloride solution and methyl alcohol (purity: $\geq 99\%$), fixed and stained with 0.1% crystal violet to prepare specimens for measurement of cell density. Cell density was measured with a monolayer culture densitometer (Monocellater, Olympus Optical Co., Ltd.), and the 50% cell growth inhibition concentration of the test article treated groups was calculated, where the value of the negative control group was taken as 100%. In the continuous treatment, a negative control group and test article treatment groups were provided for both 24 and 48 h treatments.

The chromosome aberration test consisted of two separate experiments: short-term treatment and continuous treatment (retest). Each group was conducted in quadruplicate. In the short-term treatment, for each plate, 2×10^4 cells in 5.0 mL of culture were seeded and incubated at 37 °C in humidified air containing 5% CO₂. After 3 days of cultivation, cells were observed under an inverted phase-contrast microscope and confirmed to have no abnormality. The medium was replaced with freshly added culture medium, and the cells were treated with 0.500 mL of the test solution at each concentration (32.2, 38.6, 46.3, 55.6, 66.7, and 80.0 µg/mL of thermolysin powder, which, due to the 30.9% thermolysin protein content of the powder, reflect thermolysin protein doses of 9.9, 11.9, 14.3, 17.2, 20.6, 24.7 µg/mL, respectively) or vehicle in the absence or presence of 0.833 mL of S9-mix to a total of 5.0 mL. In the positive control group, 0.833 mL of S9-mix and 0.100 mL of cyclophosphamide (CP, 0.70 mg/mL solution, Wako Pure Chemical Industries, Ltd.) were added. In the treatment without metabolic activation, 0.150 mL mitomycin C (MMC, 0.0025 mg/mL solution, Kyowa Hakko Kirin Co., Ltd.) was added. After 6 h, the cells were rinsed with isotonic sodium chloride solution, 5.0 mL fresh culture medium was added, and the culture was cultivated for another 18 h. For 2 plates for each 4-plate set, 0.1 mL of colcemid (demecolcine solution, 10 µg/mL, Wako Pure Chemical Industries, Ltd.) was added 2 h before the end of the culture period to prepare specimens for chromosome observation. At the end of the culture period, 0.25% trypsin solution (Invitrogen Co.) was added to harvest the cells, and the cells were collected by centrifugation, treated with hypotonic 0.075 M potassium chloride solution for 15 min and fixed in methyl alcohol/acetic acid solution (3:1). A small amount of the fixed cell suspension was dropped onto 2 places per glass slide. Two chromosome preparations per plate were prepared. The slides were air-dried for 1 day and stained with 2% Giemsa solution for 15 min. After 18 h of cultivation, the remaining 2 plates were treated in the same manner as the foregoing.

In the continuous treatment (retest), a negative control group, test article treatment groups, and a positive control group, were provided for both 24 and 48 h treatments.

A test was considered negative if incidence of cells with structural and numerical chromosome aberrations is less than 5%.

2.6. Micronucleus test in mice

Male ICR mice [Crj: CD-1 (ICR)] were obtained from Charles River Japan Co., Ltd. and were 7 weeks of age with body weights of 24–36 g. The mice were acclimated a week prior to the dosing. Mice were fed a commercial laboratory diet (CRF-1, Oriental Yeast Co., Ltd.) and tap water *ad libitum*, and were housed under controlled conditions: 12 air changes per hour, 20–24 °C, relative humidity of 40–70%, and 12 h light/dark cycles. Animals were housed individually in stainless steel suspended cages with wire mesh floors. The body weight range of the animals at the beginning of dosing was between 28.2 and 36.0 g.

Thermolysin powder containing 65% protein was suspended in 0.1% CMC (Junsei Chemical Co., Ltd.) aqueous solution and was administered orally to mice by stomach catheter. Mitomycin C (Otsuka Pharmaceutical Factory, Inc.) was dissolved in distilled water and was injected to mice intraperitoneally with an injection syringe. The administration volume of both solutions was adjusted to 10 mL/kg.

Groups of animals, 5 per dose, received an oral treatment at dose levels representing a dose of the thermolysin powder at 0 (negative control, 0.1% CMC), 1250, 2500, and 5000 mg/kg body weight, which, due to the 65% thermolysin protein content of the powder, reflect thermolysin protein doses of 0, 812.5, 1625, 3250 mg/kg, respectively. The oral treatment was administered in a single dose, and the animals were sacrificed 30 h after treatment. The highest dose level of thermolysin powder at 5000 mg/kg (3250 mg/kg thermolysin protein) was based upon the results of the foregoing acute oral toxicity of thermolysin in mice. During the acute oral toxicity tests, no death was observed at the maximum thermolysin powder dose of 24,000 mg/kg (3360 mg/kg thermolysin protein) but the increase in body weight was slightly suppressed. The thermolysin powder used in the acute oral toxicity tests contained approximately 14% of thermolysin protein, while the thermolysin used here contained about 65% protein. Therefore, the dose of thermolysin powder that corresponded to that in the acute oral toxicity was about 5200 mg/kg. Mice injected with 2 mg/kg of Mitomycin C intraperitoneally and sacrificed 24 h later served as positive controls.

For each animal, the following items were observed and recorded: (1) Micronucleated polychromatic erythrocytes (MNPCE) in 1000 polychromatic erythrocytes; (2) Micronucleated normochromatic erythrocytes (MNNCE) in normochromatic erythrocytes; (3) Micronucleated erythrocytes (MNE) in whole erythrocytes; and (4) Polychromatic erythrocytes (PCE) in whole erythrocytes.

The data of MNPCE, MNNCE, and MNE, were evaluated by binominal test and the data of PCE, was evaluated by Student's *t*-test. The significant level was $p < 0.05$ for each test.

3. Results

3.1. Acute toxicity study in rats

All animals survived the course of treatment and observation. No significant differences in body weight were observed among the treated groups during the course of the study. All animals grew normally except that loose stools were observed in all treated animals 5 h after administration. Excretion returned to normal by 15–24 h. No abnormalities were observed in the main organs. The oral LD₅₀ of the test article was determined to be more than 18,000 mg/kg, which corresponds to 2520 mg/kg thermolysin protein in both male and female rats.

3.2. Acute toxicity study in mice

All animals survived the course of treatment and observation. No significant differences in body weight were observed among the treated groups during the course of the study. However, it was noted that the male mice in the high dosage groups [20,000 mg/kg (2800 mg/kg thermolysin protein) and 24,000 mg/kg (3360 mg/kg thermolysin protein)] showed a slight tendency of slower growth measured by body weight, compared with the low dosage groups [13,900 mg/kg (1946 mg/kg thermolysin protein) and 16,700 mg/kg (2338 mg/kg thermolysin protein)]. No abnormalities were observed in the main organs. The oral LD₅₀ of the test article was determined to be more than 24,000 mg/kg (3360 mg/kg thermolysin protein) in both male and female mice.

3.3. 91-Day oral gavage toxicity study in rats

All control and treated animals survived the course of the study. There were no statistically significant differences identified

in body weight (Fig. 1) between treated and control groups. Food intake of treated groups was similar to that of the control group at all times.

Ophthalmological examinations revealed no differences between treated and control rats. Any findings that were noted are among those commonly seen in rats of this age and strain. No clinical signs of a toxic reaction to treatment were noted. Brown staining of the fur on the head of 13 (13/20) females in the top dose group [1000 mg/kg (390 mg/kg thermolysin protein)] was noted from week 10 onwards. As no blood from skin damage or bloody urine or feces were observed, the brown stain was assumed to have been caused by the brown pigmented test sample, which may have leaked or transferred from mouth to fur. No evidence suggests that the staining represented a toxic effect of the test article. Incidental findings noted during the course of the study included hair loss, encrustations, slight ulceration of the skin, chromodacryorrhea, salivation, rough coat, and swelling of the forepaws. These were considered incidental and not treatment-related.

Hematology and clinical biochemistry results revealed no treatment-related differences between the treated and control groups. The parameters are summarized in Table 1. A slight but statistically significant decrease in red blood cell numbers, haematocrit values, platelets, and numbers of large unstained cells was noted in males of the top dose group [1000 mg/kg (390 mg/kg thermolysin protein)] when compared to controls. A statistically significant decrease in platelet levels was noted in males of the mid dose group [200 mg/kg (78 mg/kg thermolysin protein)], when compared to controls. However, these and other statistically significant differences were found sporadically and were considered to be due to normal animal variation. In addition, there were no treatment-related macroscopic or microscopic changes in any dose groups suggestive of toxicity in rats.

There were no treatment-related differences in organ weights in any group. Organ weight and relative organ weight results are summarized in Tables 2 and 3, respectively. A statistically significant increase in relative adrenal weights was observed in treated males. However, the significant difference was observed only between the high, mid, or low dose group and controls and there was no dose-response relationship. Furthermore, the adrenal weights were within the range of values normally expected for rats of this age and strain, which was between 0.059 and 0.083 g. Therefore, the increased adrenal weights in males are not suggestive of a treatment-related effect.

Other statistically significant changes in organ weights were sporadic, including a decrease in liver weights among males in the 200 mg/kg (78 mg/kg thermolysin protein) group. These differences were considered to reflect incidental biological variation because of the lack of a dose-response relationship and the absence of any corresponding, treatment-related changes in these organs.

Treatment of rats with thermolysin for at least 91 days produced no observable signs of toxicity at 1000 mg/kg (390 mg/kg thermolysin protein) per day. As a result, a no observed effect level (NOEL) of 1000 mg/kg (390 mg/kg thermolysin protein) per day was established.

3.4. Chromosomal aberration test in cultured chinese hamster cells

In the cell-growth inhibition test, cell-growth inhibition that exceeded 50% was observed at 78.1 $\mu\text{g/mL}$ (24.1 $\mu\text{g/mL}$ thermolysin protein) in the short-term and continuous treatments. The 50% cell-growth inhibition concentration (approximate value) was calculated to be 57.5 and 64.8 $\mu\text{g/mL}$ in the short-term treatment with and without metabolic activation, respectively. It was calculated to be 56.9 and 53.7 $\mu\text{g/mL}$ in the 24- and 48-h continuous treatments, respectively. Based on these results and the guideline that “the highest dose level should be set at the level at which cell growth of at least 50% of the cells is inhibited, if cytotoxicity is observed in at least 50% of the cells,” 80.0 $\mu\text{g/mL}$ (24.7 $\mu\text{g/mL}$ thermolysin protein) was set as the highest concentration. A total of six dose concentrations were diluted with a common ratio of 1.2, namely, 80.0, 66.7, 55.6, 46.3, 38.6, and 32.2 $\mu\text{g/mL}$ for the subsequent test, corresponding to 24.7, 20.6, 17.2, 14.3, 11.9, and 9.9 $\mu\text{g/mL}$ thermolysin protein. The results from the chromosomal aberration test are summarized in Table 4. They indicate that thermolysin did not induce structural or numerical chromosome aberration. In the short-term treatment (6–18 h), with metabolic activation, cell ablation and cell death were observed at 55.6 $\mu\text{g/mL}$ (17.2 $\mu\text{g/mL}$ thermolysin protein) and above. The incidence of cells with structural or numerical (polyploidy) chromosome aberrations was 0% at tested doses 46.3, 38.6, and 32.2 $\mu\text{g/mL}$, corresponding to 14.3, 11.9, and 9.9 $\mu\text{g/mL}$ thermolysin protein.

Without metabolic activation, cell ablation and cell death were observed at 66.7 $\mu\text{g/mL}$ (20.6 $\mu\text{g/mL}$ thermolysin protein) and above; cell suspension and changes in cell shape were observed

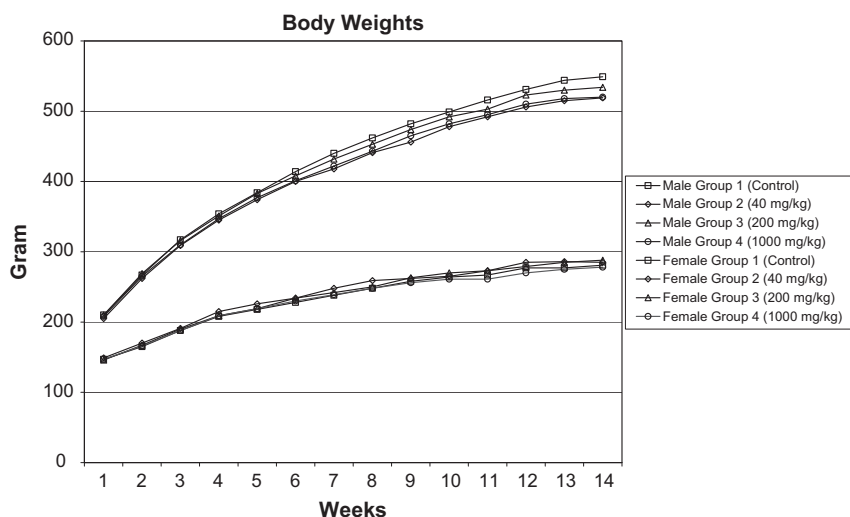


Fig. 1. Mean body weight of rats administered thermolysin for 91 days.

Table 1

Selected hematology and clinical chemistry parameters in rats treated with thermolysin for 91 days.

Parameters	Males				Females			
	0 mg/kg	40 mg/kg	200 mg/kg	1000 mg/kg	0 mg/kg	40 mg/kg	200 mg/kg	1000 mg/kg
RBC ($10^6/\mu\text{L}$)	9.03 $\pm$ 0.28	8.74 $\pm$ 0.38	8.74 $\pm$ 0.34	8.67 $\pm$ 0.27*	7.57 $\pm$ 0.17	7.59 $\pm$ 0.29	7.68 $\pm$ 0.24	7.53 $\pm$ 0.31
HB (mmol/L)	9.7 $\pm$ 0.3	9.6 $\pm$ 0.4	9.5 $\pm$ 0.3	9.4 $\pm$ 0.2	8.9 $\pm$ 0.3	9.1 $\pm$ 0.4	9.1 $\pm$ 0.3	8.9 $\pm$ 0.3
HCT (L/L)	0.5 $\pm$ 0.01	0.49 $\pm$ 0.02	0.49 $\pm$ 0.01	0.48 $\pm$ 0.1*	0.45 $\pm$ 0.01	0.45 $\pm$ 0.02	0.45 $\pm$ 0.01	0.44 $\pm$ 0.02
MCV (fL)	56 $\pm$ 2	56 $\pm$ 2	56 $\pm$ 1	55 $\pm$ 1	59 $\pm$ 1	60 $\pm$ 2	59 $\pm$ 2	59 $\pm$ 1
MCH (fmol)	1.1 $\pm$ 0.0	1.1 $\pm$ 0.0	1.1 $\pm$ 0.0	1.1 $\pm$ 0.0	1.2 $\pm$ 0.1	1.2 $\pm$ 0.0	1.2 $\pm$ 0.0	1.2 $\pm$ 0.0
MCHC (mmol/L)	19 $\pm$ 1	20 $\pm$ 1	20 $\pm$ 1	20 $\pm$ 0	20 $\pm$ 0	20 $\pm$ 0	20 $\pm$ 0	20 $\pm$ 0
RDW (%)	14.8 $\pm$ 1.1	14.7 $\pm$ 0.9	14.7 $\pm$ 0.5	14.9 $\pm$ 0.8	12.8 $\pm$ 0.5	12.5 $\pm$ 0.4	12.7 $\pm$ 0.4	12.9 $\pm$ 0.3
WBC ($10^3/\mu\text{L}$)	14.5 $\pm$ 3.1	14.9 $\pm$ 2.8	16.4 $\pm$ 3.0	14.5 $\pm$ 1.9	8.3 $\pm$ 2.1	10.0 $\pm$ 3.7	8.2 $\pm$ 1.9	7.7 $\pm$ 1.5
EO (%)	0.8 $\pm$ 0.2	0.9 $\pm$ 0.4	0.7 $\pm$ 0.2	0.9 $\pm$ 0.3	1.3 $\pm$ 0.4	1.0 $\pm$ 0.2	1.4 $\pm$ 0.7	1.2 $\pm$ 0.4
BASO (%)	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
LYMPH (%)	74.6 $\pm$ 6.3	74.0 $\pm$ 8.4	77.0 $\pm$ 6.0	75.7 $\pm$ 2.9	76.2 $\pm$ 5.5	72.3 $\pm$ 13.3	76.5 $\pm$ 4.1	73.2 $\pm$ 7.8
NEN (%)	0.1 $\pm$ 0.3	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
PLATELETS ($10^3/\mu\text{L}$)	979 $\pm$ 139	834 $\pm$ 232	792 $\pm$ 101*	770 $\pm$ 101*	847 $\pm$ 91	818 $\pm$ 162	767 $\pm$ 179	859 $\pm$ 138
GLUCOSE (mmol/L)	7.2 $\pm$ 1.1	7.3 $\pm$ 1.1	7.3 $\pm$ 0.9	7.1 $\pm$ 0.8	6.3 $\pm$ 0.9	7.8 $\pm$ 1.3*	6.7 $\pm$ 0.9	6.9 $\pm$ 1.1
UREA (mmol/L)	5.0 $\pm$ 1.0	5.0 $\pm$ 0.7	5.2 $\pm$ 0.5	5.2 $\pm$ 0.5	6.2 $\pm$ 0.7	5.5 $\pm$ 0.7	6.0 $\pm$ 0.6	6.1 $\pm$ 1.2
CREATINE ($\mu\text{mol/L}$)	41 $\pm$ 2	41 $\pm$ 3	40 $\pm$ 3	40 $\pm$ 2	42 $\pm$ 4	48 $\pm$ 4*	46 $\pm$ 3	46 $\pm$ 3
ASAT (GOT) ($\mu\text{kat/L}$)	1.73 $\pm$ 0.20	1.52 $\pm$ 0.26	1.61 $\pm$ 0.17	1.58 $\pm$ 0.31	1.30 $\pm$ 0.24	1.17 $\pm$ 0.28	1.30 $\pm$ 0.18	1.22 $\pm$ 0.26
ALAT (GPT) ($\mu\text{kat/L}$)	0.47 $\pm$ 0.05	0.51 $\pm$ 0.08	0.49 $\pm$ 0.07	0.46 $\pm$ 0.08	0.49 $\pm$ 0.09	0.43 $\pm$ 0.07	0.48 $\pm$ 0.15	0.70 $\pm$ 0.32*
CHLORIDE (mmol/L)	101 $\pm$ 1	100 $\pm$ 1*	101 $\pm$ 1	101 $\pm$ 1	103 $\pm$ 2	102 $\pm$ 1	101 $\pm$ 1	102 $\pm$ 1
PROTEIN T. (g/L)	70 $\pm$ 3	68 $\pm$ 3	68 $\pm$ 2	69 $\pm$ 3	72 $\pm$ 4	72 $\pm$ 3	70 $\pm$ 4	74 $\pm$ 5
ALBUMIN (g/L)	36 $\pm$ 1	35 $\pm$ 2	34 $\pm$ 1	35 $\pm$ 1	40 $\pm$	40 $\pm$ 2	39 $\pm$ 2	42 $\pm$ 3

Values are the mean of rats $\pm$ SD. RBC, erythrocyte count; $10^6/\mu\text{L}$, $10^6/\text{microliter}$; HB, haemoglobin; mmol/L, millimoles/liter; HCT, haematocrit; L/L, liter/liter; MCV, mean corpuscular volume; fL, femtoliters (10^{-15} L); MCH, mean corpuscular haemoglobin; fmol, femtomol (10^{-15} mol); MCHC, mean corpuscular haemoglobin concentration; mmol/L, millimoles/liter; RDW, red cell distribution width; WBC, total leucocyte count; $10^3/\mu\text{L}$; EO, eosinophils; BASO, basophils; LYMPH, lymphocytes; NEN, nucleated erythrocytes; PLATELETS, platelet count; $10^3/\mu\text{L}$, micromoles/liter; ASAT/GOT, aspartate aminotransferase; $\mu\text{kat/L}$, microkatal/liter; ALAT/GPT, alanine aminotransferase; PROTEIN T., total protein.

* Statistically identified difference from control $p < 0.05$.

Table 2

Selected organ weight results in rats administered thermolysin for 91 days.

Organ weights (g)				
	0 mg/kg	40 mg/kg	200 mg/kg	1000 mg/kg
<i>Males</i>				
Body W.	552 $\pm$ 64	524 $\pm$ 58	536 $\pm$ 44	524 $\pm$ 42
Liver	20.60 $\pm$ 3.72	19.02 $\pm$ 3.35	18.19 $\pm$ 2.39*	18.73 $\pm$ 2.44
Kidneys	3.78 $\pm$ 0.46	3.62 $\pm$ 0.50	3.65 $\pm$ 0.45	3.59 $\pm$ 0.36
Adrenals	0.064 $\pm$ 0.008	0.069 $\pm$ 0.012	0.073 $\pm$ 0.009*	0.070 $\pm$ 0.013
Testes	3.41 $\pm$ 0.21	3.41 $\pm$ 0.21	3.44 $\pm$ 0.54	3.38 $\pm$ 0.48
<i>Females</i>				
Body W.	277 $\pm$ 29	289 $\pm$ 25	284 $\pm$ 20	277 $\pm$ 18
Liver	8.90 $\pm$ 1.06	9.51 $\pm$ 0.91	8.67 $\pm$ 1.03	9.34 $\pm$ 1.08
Kidneys	2.10 $\pm$ 0.22	2.14 $\pm$ 0.31	2.15 $\pm$ 0.18	2.16 $\pm$ 0.22
Adrenals	0.071 $\pm$ 0.012	0.078 $\pm$ 0.011	0.071 $\pm$ 0.012	0.071 $\pm$ 0.010

at 55.6 $\mu\text{g/mL}$ (17.2 $\mu\text{g/mL}$ thermolysin protein) and above. The incidence of cells with structural aberrations was 0%, 0%, 0.5%, and 0% at tested doses 55.6, 46.3, 38.6, and 32.2 $\mu\text{g/mL}$ (17.2, 14.3, 11.9, and 9.9 $\mu\text{g/mL}$ thermolysin protein), respectively; and that with numerical aberrations was all 0% at the above tested doses. The same result was obtained from the 24 h treatment. In the 48 h treatment, the incidence of cells with structural aberrations was 0.5%, 0%, 0%, and 1.0% at tested doses 55.6, 46.3, 38.6, and 32.2 $\mu\text{g/mL}$ (17.2, 14.3, 11.9, and 9.9 $\mu\text{g/mL}$ thermolysin protein), respectively; and that with numerical aberrations was all 0% at the above tested doses.

Cultures treated with the positive control chemicals (i.e., cyclophosphamide or mitomycin C) had significantly higher incidences of abnormal cells in all assays. Based on these results, it was concluded that the test article had no potential to induce structural or numerical chromosome aberration; therefore, thermolysin was considered not to be genotoxic in this *in vitro* chromosomal aberration assay.

Table 3

Selected relative organ weights (grams per 100 g of body weight) results in rats administered thermolysin for 91 days.

Organ/body weight ratios				
	0 mg/kg	40 mg/kg	200 mg/kg	1000 mg/kg
<i>Males</i>				
Body W.	552 $\pm$ 64	524 $\pm$ 58	536 $\pm$ 44	524 $\pm$ 42
Liver	3.72 $\pm$ 0.34	3.62 $\pm$ 0.41	3.38 $\pm$ 0.27**	3.57 $\pm$ 0.35
Kidneys	0.69 $\pm$ 0.05	0.69 $\pm$ 0.08	0.68 $\pm$ 0.05	0.69 $\pm$ 0.06
Adrenals	0.012 $\pm$ 0.001	0.013 $\pm$ 0.002*	0.014 $\pm$ 0.002**	0.013 $\pm$ 0.002*
Testes	0.63 $\pm$ 0.08	0.66 $\pm$ 0.07	0.64 $\pm$ 0.11	0.65 $\pm$ 0.10
<i>Females</i>				
Body W.	277 $\pm$ 29	289 $\pm$ 25	284 $\pm$ 20	277 $\pm$ 18
Liver	3.22 $\pm$ 0.27	3.29 $\pm$ 0.22	3.05 $\pm$ 0.26	3.38 $\pm$ 0.30
Kidneys	0.76 $\pm$ 0.05	0.74 $\pm$ 0.11	0.76 $\pm$ 0.05	0.78 $\pm$ 0.07
Adrenals	0.026 $\pm$ 0.004	0.027 $\pm$ 0.004	0.025 $\pm$ 0.005	0.026 $\pm$ 0.004

* Statistically identified difference from control $p < 0.05$.

** Statistically identified difference from control $p < 0.01$.

3.5. Micronucleus test in mice

The results from the micronucleus test are summarized in Table 5, which showed that thermolysin was negative in the mouse bone marrow micronucleus test. No treatment-related clinical signs were noted in any animals during the course of the study. There were no statistically significant increases in the frequencies of micronucleated polychromatic erythrocytes (MNPCE), micronucleated normochromatic erythrocytes (MNNCE), or micronucleated erythrocytes (MNE) in groups treated with the test article as compared to the negative controls. The positive control animals showed a significant increase in the frequency of MNPCE and MNE as compared to the negative controls. There were no statistically significant differences in the percent polychromatic erythrocytes (PCE) in groups treated with the test materials while the value from the positive control was significantly lower than the

Table 4

Chromosome aberration in cultured Chinese hamster cells treated with thermolysin.

Treatment duration	Without S-9 activation			With S-9 activation		
	Dose (μg/mL)	% Polyploid	% Cells with aberrations	Dose (μg/mL)	% Polyploid	% Cells with aberrations
6–18 h	0	0.5	0.5	0	0.0	0.5
	32.2	0.0	0.0	32.2	0.0	0.0
	38.6	0.0	0.5	38.6	0.0	0.0
	46.3	0.0	0.0	46.3	0.0	0.0
	55.6	0.0	0.0	55.6	0.0	0.0
	66.7	0.0	0.0	66.7	0.0	0.0
	80.0	0.0	0.0	80.0	0.0	0.0
	MMC	0.0	31.0	CP	0.0	58.0
Treatment duration	Without S-9 activation					
	Dose (μg/mL)	% Polyploid	% Cells with aberrations			
24 h	0	0.0	0.5			
	32.2	0.0	0.0			
	38.6	0.0	0.0			
	46.3	0.0	0.5			
	55.6	0.0	0.0			
	66.7	0.0	0.0			
	80.0	0.0	0.0			
	MMC	0.0	30.5			
48 h	0	0.0	0.5			
	32.2	0.0	1.0			
	38.6	0.0	0.0			
	46.3	0.0	0.0			
	55.6	0.0	0.5			
	66.7	0.0	0.0			
	80.0	0.0	0.0			
	MMC	0.0	47.5			

Positive controls were mitomycin C (MMC, 0.0025 mg/mL) and cyclophosphamide (CP, 0.70 mg/mL).

Table 5

Frequencies of micronucleated polychromatic erythrocytes (% MNPCE), MNNE, MNE, and PCE.

Dose (mg/kg/day)	% MNPCE (range)	% MNNE (range)	% MNE (range)	% PCE (range)
0	0.18(0.0–0.5)	0.04(0.0–0.1)	0.14(0.0–0.4)	57.52(52.0–61.3)
1250	0.14(0.0–0.3)	0.04(0.0–0.1)	0.14(0.0–0.3)	55.10(50.2–61.8)
2500	0.16(0.0–0.4)	0.06(0.0–0.1)	0.10(0.0–0.3)	46.88(32.9–62.6)
5000	0.12(0.1–0.2)	0.12(0.1–0.3)	0.14(0.0–0.3)	48.54(35.2–65.4)
MMC	6.22 ^{**} (2.8–9.1)	0.1(0.0–0.2)	2.48 ^{**} (0.7–3.8)	39.40 [*] (28.3–46.7)

Values represent 5000 cells counted per animal. Positive control was mitomycin C (MMC, 2.0 mg/mL (i.p.)). MNPCE, micronucleated polychromatic erythrocytes; MNNE, micronucleated normochromatic erythrocytes; MNE, micronucleated erythrocytes; PCE, polychromatic erythrocytes.

^{*} Significantly different from negative control at $p < 0.05$ (t -test).^{**} Significantly different from negative control at $p < 0.01$ (binominal test).

negative controls. As a result, under the experimental conditions used, thermolysin enzyme preparation was considered to be negative in the mouse bone marrow micronucleus test.

3.6. Allergenicity sequence analysis

In addition to the toxicological studies, the amino acid sequence for thermolysin (<http://www.ncbi.nlm.nih.gov/nucore/M21663>) was compared against the Food Allergy Research and Resource Program (FARRP) protein allergen database using AllergenOnline (<http://www.allergenonline.org/databasefasta.shtml>) to determine allergenicity potential. FARRP maintains and regularly updates a comprehensive database of proven or putative allergens (food, airway, venom/saliva, and contact). No positive matches were found (data not shown) when the amino acid sequence was subjected to a full-length Fast-Alignment (FASTA) search and an 80 amino acid sliding window analysis through the FARRP search algorithm.

Although the 8 amino acid exact analysis was recently introduced as an option on the FAARP site, both the literature and FAARP acknowledge that the use of such a short sequence is not a reliable method for determining allergenicity potential and is likely to generate false positives (Ladics, 2008). FARRP has been

unable to identify any proteins in which a 6–8 amino acid sequence match was found between cross-reactive proteins that did not also have a positive match in the 80 amino acid sliding window analysis, and therefore previously did not include an 8 amino acid exact match option due to the lack of scientific support for such an analysis. However, the option was recently offered as some countries still require an 8 amino acid exact match analysis “even in the lack of evidence demonstrating a positive predictive value” (<http://www.allergenonline.org/about.shtml>). An 8 amino acid exact analysis of the thermolysin protein was performed and did show one 8 amino acid positive match to an aspartic protease inhibitor 11 (Sola t 2), which is derived from potato (Lehesranta et al., 2006). However, the absence of an 80 amino acid sliding window sequence match supports the conclusion that the 8 amino acid positive result is most likely a false positive. Therefore, no conclusive evidence suggests that thermolysin is an allergen.

4. Discussion

To evaluate the safety of the enzyme preparation, thermolysin was subjected to standard toxicological experiments designed to

assess its potential acute and subchronic oral toxicity and genotoxicity. The thermolysin powder contains a small amount of carbohydrate left over from the fermentation media, as well as minerals such as NaCl, CaCl₂, Na₂HPO₄, Ca(OH)₂, Ca(CH₃COO)₂, and CH₃COONa. As these are commonly used food ingredients that are of low or no toxicity, we believe that the carbohydrates and minerals did not affect the results of the toxicity tests on thermolysin protein, especially considering that no significant adverse effects were observed in these tests.

In the acute oral toxicity in both rats and mice, all treated and control animals survived the experimental period in general good health. The oral LD₅₀ of thermolysin was determined to be more than 18,000 mg/kg (2520 mg/kg thermolysin protein) in rats and more than 24,000 mg/kg (3360 mg/kg thermolysin protein) in mice.

In a 91-day oral toxicity study, all animals survived during the course of the study in general good health. No differences were observed in body weight or food consumption between control and treated rats. There were no differences between treated and control animals from the ophthalmological examinations. Brown staining of the fur on the head of females in the top dose group [1000 mg/kg (390 mg/kg thermolysin protein)] was considered not to be treatment-related, but rather related to the physical nature of the test substance. Incidental findings such as brown staining of the fur on the head, hair loss, encrustations, slight ulceration of the skin, chromodacryorrhea, salivation, rough coat, and swelling of forepaws, were considered not to be treatment-related. Results from hematology and clinical biochemistry revealed no treatment-related differences between the treated and control groups. The variations observed in some of the parameters measured could not be attributed to the treatment as they were within a normal range. Although small but statistically significant increases in relative adrenal weights and decreases in liver weights were observed in treated males, these differences were considered not to be treatment-related. They reflected incidental biological variation because there was a lack of a dose–response relationship and the absence of any treatment-related changes in these organs. Furthermore, the response was not consistent between sexes. Based on these results, the highest dose tested, 1000 mg/kg/day (390 mg/kg thermolysin protein), was established as the NOEL for thermolysin in rats.

In addition, thermolysin was shown to have no genotoxic potential when subjected to chromosomal aberration assay in the cultured Chinese hamster cells and micronucleus assay in mice. Allergenicity sequence analysis did not reveal any evidence suggesting that thermolysin is an allergen.

One of the considerations for the evaluation of an enzyme intended for the food industry is the safety of the production strain (Pariza and Johnson, 2001). *G. stearothermophilus* is a non-pathogenic gram positive bacteria. It is ubiquitous in nature, and its species distribution includes food items such as sugar and turmeric (Richmond and Fields, 1966). *G. stearothermophilus* is commonly described as a typical thermophilic spoilage bacteria found in low-acid food (Richmond and Fields, 1966). *G. stearothermophilus* (previously known as *Bacillus stearothermophilus*) has an established history of safe use for the production of alpha and maltogenic amylases (Pariza and Johnson, 2001) and aspartame (Yagasaki and Hashimoto, 2008). Native enzymes derived from *G. stearothermophilus* have been identified to be safe for use in food (Pariza and Johnson, 2001).

In addition, *G. stearothermophilus* has been assessed by the European Food Safety Authority (EFSA) and assigned Qualified Presumption of Safety (QPS) status. The risk classification of the European Community (Directive 2000/54/EC) is group 1, which is designated for biological agents most unlikely to cause human disease. The American Type Culture Collection (ATCC) has given this strain a biosafety level 1 designation, which means that the

organism is not known to consistently cause disease in healthy human adults.

5. Conclusions

The lack of toxicological findings directly related to thermolysin is consistent with the history of safe consumption of enzymes derived from *G. stearothermophilus* as described above. Consequently, it may properly be concluded that thermolysin enzyme preparation is safe for use in food production.

Disclosure statement

Amano Enzyme, Inc. manufactures and produces Thermoase, the thermolysin enzyme that is the subject of this manuscript. Co-author Masashi Minoda is an employee of Amano Enzyme, Inc. (Amano) and played a role in the development of Thermoase. Co-author Hiromichi Yoshida is an employee of Amano.

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Conflict of Interest

The authors declared no conflicts of interest, other than the foregoing.

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