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ORIGINAL SUBMISSION

KELLER AND HECKMAN LLP
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1001 G Street, N.W.
Suite 500 West
Washington, D.C. 20001
tel. 202.434.4100
fax 202.434.4646

Writer's Direct Access
Melvin S. Drozen
(202) 434-4222
drozen@khlaw.com

September 2, 2015

Via FedEx (Tracking No. 774421499511)

Antonia Mattia, Ph.D.
Director, Division of Biotechnology and
GRAS Notice Review (HFS-225)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
4300 River Road
College Park, Maryland 20740

Re: GRAS Notification for THERMOASE C100 (thermolysin enzyme preparation)

Dear Dr. Mattia:

We respectfully submit the attached GRAS Notification on behalf of our client, Amano Enzyme, Inc. (Amano) for the thermolysin enzyme preparation, THERMOASE C100, 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. THERMOASE C100 – which contains up to 39% thermolysin protein and 86% diluents and stabilizers – will be used at levels ranging from 0.002 to 0.08% in the production of the foods specified above. More detailed information regarding product identification, intended use levels, and anticipated levels in finished foods appears in the attached GRAS Notification. We have included three (3) hard copies of the GRAS Notification for your review.

Amano has determined that THERMOASE C100 is GRAS based on scientific procedures in accordance with 21 C.F.R. § 170.30(b) and in conformance with the guidance issued by the Food and Drug Administration (FDA) under proposed 21 C.F.R. § 170.36, 62 Fed. Reg. 18938 (Apr. 17, 1997). Therefore, the use of THERMOASE C100 in food as described in this GRAS Notification is exempt from the requirement of premarket approval as set forth in the Federal Food, Drug, and Cosmetic Act.

The analytical data, published studies, and information that are the basis for this GRAS determination are available for FDA review and copying at reasonable times at Keller and Heckman LLP, 1001 G Street, NW, Suite 500W, Washington, DC 20001, or will be sent to FDA upon request.

We look forward to FDA's review of this submission and would be happy to provide Agency officials with any information they may need to complete their assessment. Thank you for your attention to this matter.

Sincerely,

(b) (6)

Melvin S. Drozen

Enclosure

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*
☒ New ☐ Amendment to GRN No._____ ☐ Supplement to GRN No. _____

2. ☐ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Hiromichi Yoshida	Quality Assurance Department

Company *(if applicable)*: Amano Enzyme, Inc.

Mailing Address *(number and street)*: 27 Hanno, Kunotsubo

City	State or Province	Zip Code/Postal Code	Country
Kita-Nagoya	Aichi Prefecture	481-8533	Japan

Telephone Number	Fax Number	E-Mail Address
c/o 202-434-4222		hiromichi_yoshida@amano-enzyme.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position
Melvin S. Drozen	Partner

Company *(if applicable)*: Keller and Heckman LLP

Mailing Address *(number and street)*: 1001 G Street NW, Suite 500W

City	State or Province	Zip Code/Postal Code	Country
Washington	District of Columbia	20001	United States of America

Telephone Number	Fax Number	E-Mail Address
(202) 434-4222	(202) 434-4646	drozen@khlaw.com

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PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

THERMOASE C100 (thermolysin enzyme preparation)

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☐ Electronic files on physical media with paper signature page

☒ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes 1

Total number of pages 30

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for determination of GRAS status *(Check one)*

☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, see attached Designation of Confidential Information

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

THERMOASE C100 will be used 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.

THERMOASE C100 will be used at levels ranging from 0.002 to 0.08% in the production of the foods specified above. More detailed information regarding use levels and anticipated levels in finished foods appears in Table 1 of the GRAS Notification.

Foods and ingredients produced using THERMOASE C100 will be consumed by the general population, i.e., adults and children (1 year and older).

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☒ No

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PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	THERMOASE C100 (thermolysin enzyme preparation)	CAS	9073-78-3	*Geobacillus stearothermophilus*	
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

THERMOASE C100, a powdered protease preparation, contains up to 39% thermolysin protein and 86% diluents and stabilizers, such as sodium chloride (NaCl), calcium chloride (CaCl2), disodium phosphate (Na2HPO4), calcium hydroxide (Ca (OH)2), and carbohydrate. 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. Each of the diluents and stabilizers used in the production of THERMOASE C100 has an appropriate FDA regulatory status, as detailed in the GRAS Notification.

3. Synonyms

Provide as available or relevant:

1	
2	
3	

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PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☐ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☐ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that Amano Enzyme, Inc. *(name of notifier)*

has concluded that the intended use*(s)* of THERMOASE C100 (thermolysin enzyme preparation) *(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ Amano Enzyme, Inc. *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

Amano Enzyme, Inc. *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

Keller and Heckman LLP, 1001 G Street NW, Suite 500W, Washington, DC 20001 *(address of notifier or other location)*

Amano Enzyme, Inc. *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

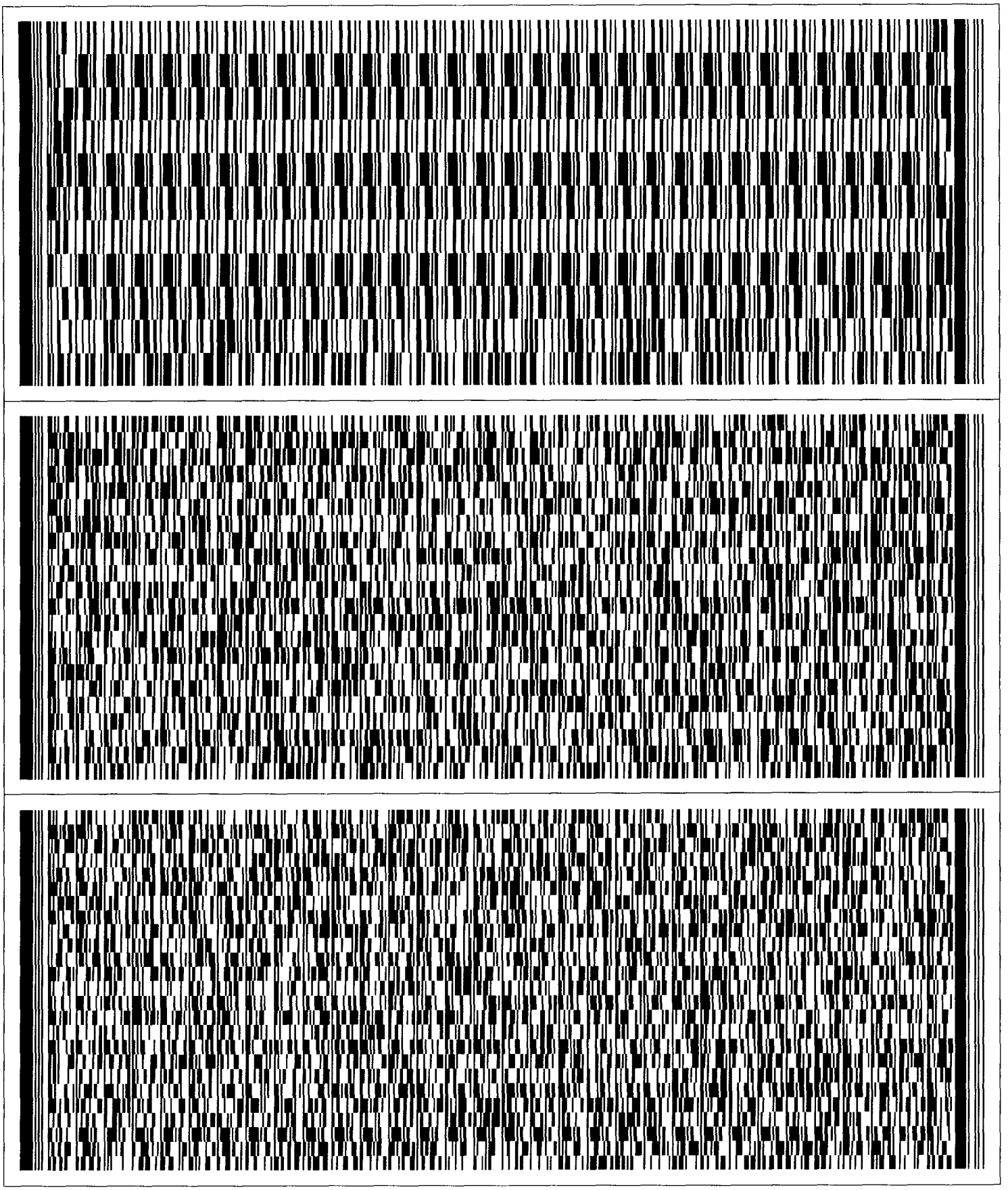
☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ---------- *(GRAS Affirmation Petition No.)*

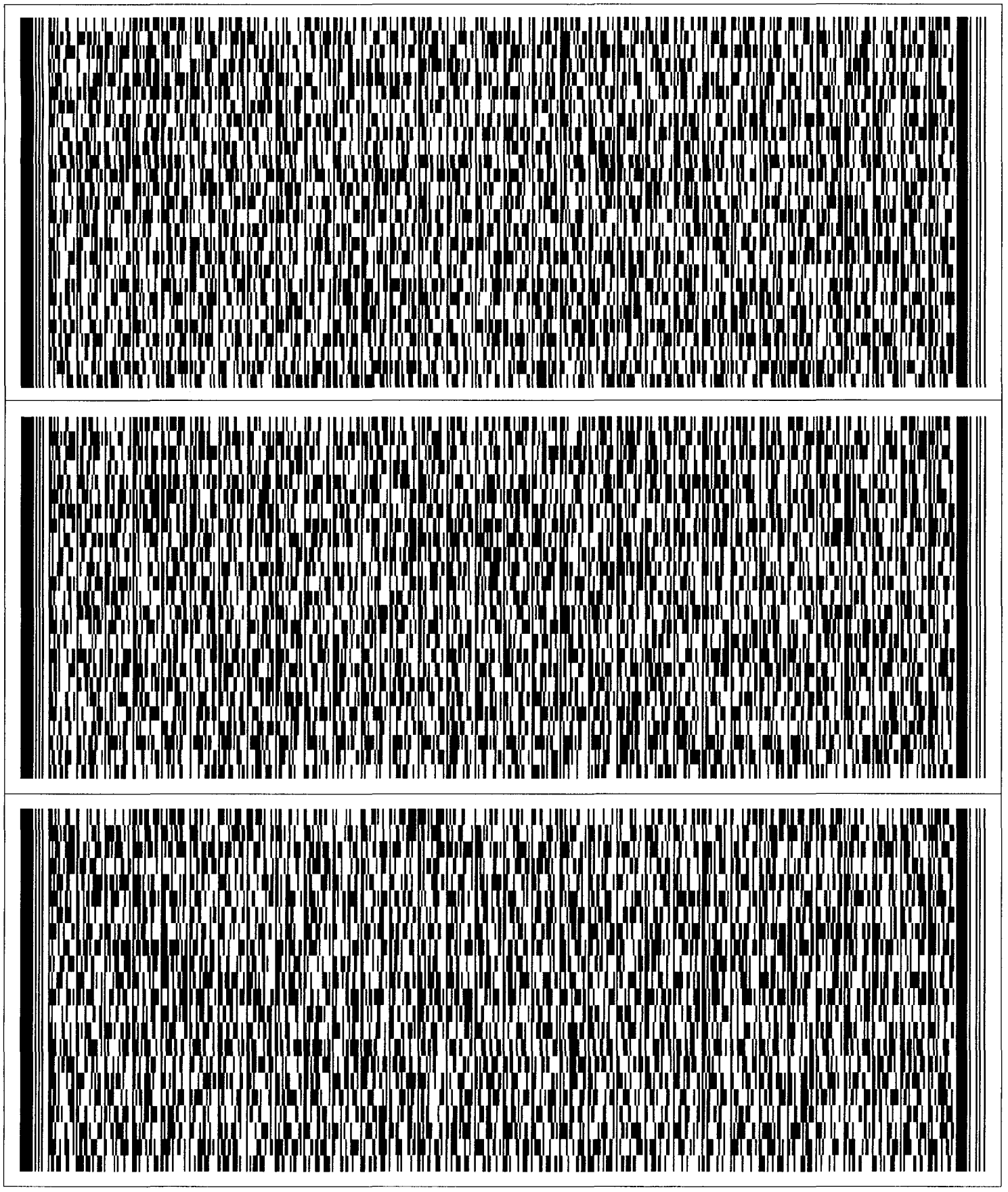
3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
(b) (6)	Melvin S. Drozen, Partner	09/01/2015

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Cover Letter to Antonia Mattia	N/A
	GRAS Notification for THERMOASE C100	1-21
	Appendices to GRAS Notification for THERMOASE C100	22-30





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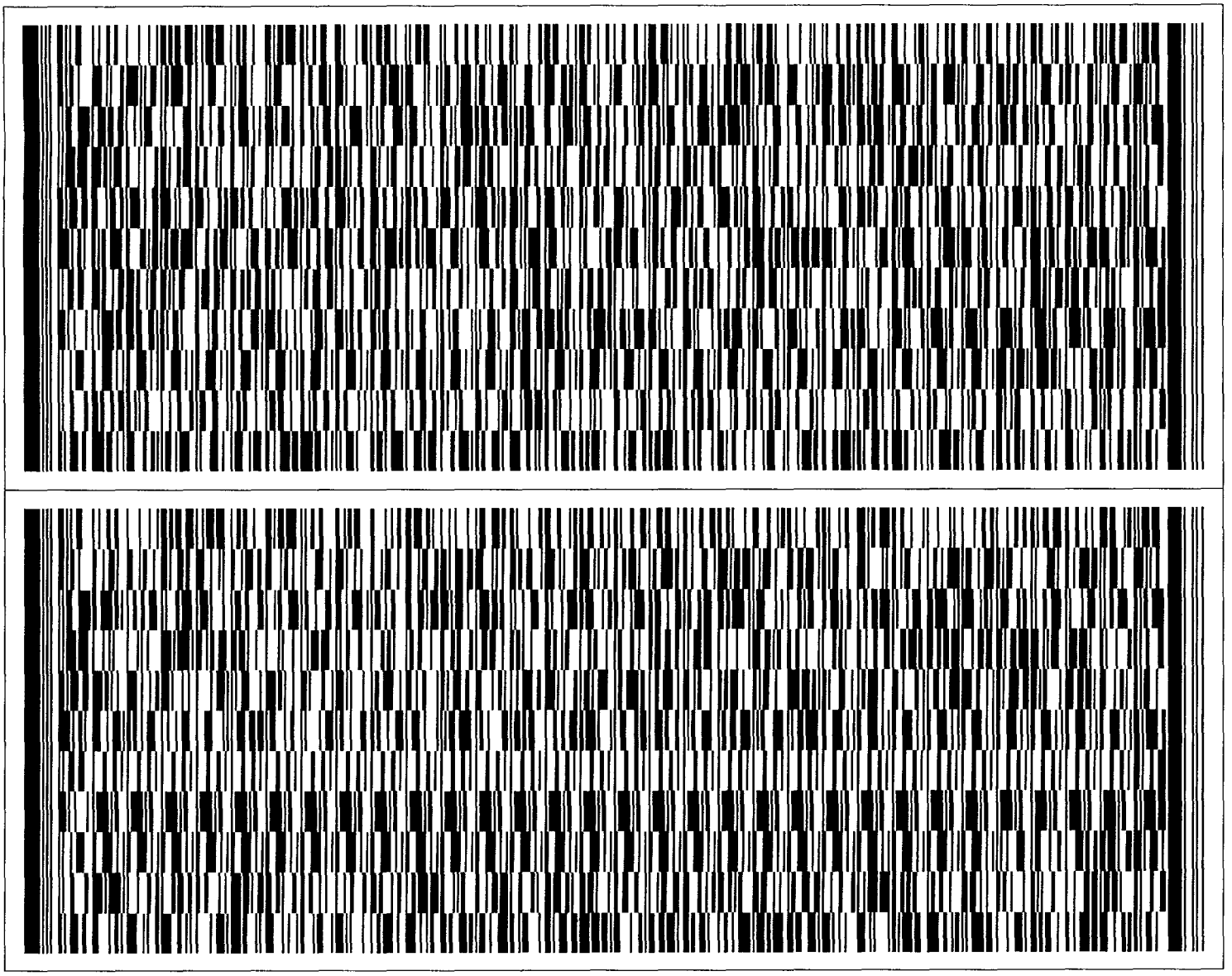

GRAS Notification for THERMOASE C100

Prepared for:

U.S. Food and Drug Administration
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740-3835

Prepared by:

Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001

Date:

September 2, 2015

Table of Contents

Tables

Figures

Appendices

I. Introduction

Keller and Heckman LLP submits the enclosed information on behalf of our client, Amano Enzyme, Inc. (Amano), in support of this Notification that the thermolysin enzyme preparation, THERMOASE C100, is generally recognized as safe (GRAS) for use in multiple food applications.

THERMOASE C100 is intended to be used as a processing aid in the manufacture of yeast extract; 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 ingredients. These ingredients are intended to be used in processed foods such as seasonings, soups, sauces, prepared foods, snack foods, and meat-derived foods (e.g., sausages). THERMOASE C100 also is intended to be used as a tenderizer in the production of cooked fish to provide a desirable, soft texture, along with optimal elasticity and moisture.

We submit information in the following areas:

- The identity and specifications for THERMOASE C100;
- Conformity of THERMOASE C100 to JEFCA and FCC specifications for enzyme preparations;
- The manufacturing process for THERMOASE C100;
- Toxicological studies conducted on thermolysin enzyme preparations;
- The intended uses and a consumption estimate for THERMOASE C100; and
- Supportive evidence from the long history of safe use of enzymes in food.

It is our expectation that FDA will concur that the information presented fully supports the determination that THERMOASE C100 as produced by Amano is GRAS for use in the intended food applications.

II. Administrative Information

A. Claim Regarding GRAS Status

Amano has determined that THERMOASE C100 is GRAS based on scientific procedures in accordance with 21 C.F.R. § 170.30(b) and in conformance with the guidance issued by FDA under proposed 21 C.F.R. § 170.36, 62 Fed. Reg. 18938 (Apr. 17, 1997). The analytical data, published studies, and information that are the basis for this GRAS determination are available for FDA review and copying at reasonable times at Keller and Heckman LLP, 1001 G Street, NW, Suite 500W, Washington, DC 20001, or will be sent to FDA upon request.

B. Name and Address of the Notifier

Amano Enzyme, Inc.
2-7, 1-Chome, Nishiki
Naka-ku, Nagoya, Japan 460-8630

All communications on this matter are to be sent to Counsel for the Notifier:

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, NW Suite 500W
Washington, DC 20001
Telephone: (202) 434-4222
Facsimile: (202) 434-4646
Email: Drozen@khlaw.com

C. Common or Usual Name of GRAS Substance

The common or usual name for the GRAS ingredient is "thermolysin enzyme preparation." Amano's product will be marketed under the trade name "THERMOASE C100." For ease of reference, we refer to the commercial product as THERMOASE C100 throughout this Notification.

D. Conditions of Use

THERMOASE C100 will be used in the production of the following products, most of which are ingredients intended for use in the preparation of a range of finished foods, as specified in **Table 1** below:

Table 1. Intended Use Levels of THERMOASE C100 in Ingredient Production and Levels of Incorporation of Ingredients into Finished Foods

Intended Use	Use Level	Typical Categories of Processed and Prepared Foods in which Ingredient will be Used	Estimated Use Level of Ingredient in Finished Foods
Yeast extract	0.02-0.05%	Seasonings	2%
		Soups, soup mixes, and sauces	2%
		Snack foods	2%
		Meat-derived foods (e.g., sausages)	5%

Cooked fish	0.002-0.065%	N/A	100%
Egg white hydrolysates	0.01-0.08%	Condiments and relishes, including seasoning sauces (e.g., mayonnaise, dressing, aioli, hollandaise sauces)	1-20%
		Desserts and mousses (e.g., ice cream, mousses, yogurts, jellies)	1-20%
		Baked goods (e.g., cakes, breads, pastries, cream puffs, cookies)	1-20%
		Beverages	1-20%
		Soups, curries, stews, and snack foods	1-20%
		Meat-derived products (e.g., ham, sausages) and fish products (e.g., boiled fish paste products, tube-shaped fish cakes)	1-20%
Enzyme-modified dairy ingredients	0.01-0.05%	Processed cheese	5%
		Soups, soup mixes, and sauces	2-5%
		Snack foods	2-5%
		Condiments and relishes (e.g., dressing)	2%
Protein hydrolysates (soy, wheat gluten, milk protein, fish)	0.01-0.05%	Snack foods	2%
		Meat-derived products (e.g., sausage)	5%

		Seasonings	2%
		Soup, soup mixes, and sauces	2%

E. Self-Limiting Levels of Use

THERMOASE C100 will be used at levels no higher than necessary to achieve its intended effect in the production of the ingredients and foods specified in **Table 1** above.

III. Product Identity and Specifications

A. Product Identification

THERMOASE C100, a powdered protease preparation, contains up to 39% thermolysin protein and 86% diluents and stabilizers, such as sodium chloride (NaCl), calcium chloride ($CaCl_2$), disodium phosphate (Na_2HPO4), calcium hydroxide ($Ca(OH)_2$), and carbohydrate. Each of the diluents and stabilizers used in the production of THERMOASE C100 has an appropriate FDA regulatory status, as discussed further in **Table 5** below. In the future, should Amano choose to modify the diluents and stabilizers used in the production of THERMOASE C100, the Company will ensure that all such components are safe and suitable for their intended use.

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'Donohue 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) previously was classified as *Bacillus stearothermophilus* and subsequently was transferred to a new thermophilic genus, *Geobacillus*, due to a phenotypic and 16S rRNA gene sequence analysis (Nazina et al., 2001). The production strain in this Notification was confirmed by a third party laboratory to be *Bacillus sterothermophilus* (now known as *G. stearothermophilus*) based on gram staining, nutrient usage, morphology, ability to produce spores, motility, ability to grow at various temperatures and conditions, and ability to produce certain compounds.[1] These criteria are commonly used to classify bacteria.

The thermolysin produced by *G. stearothermophilus* is a 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. Additionally, the three

[1] Appendix 1: Leatherhead Food RA: Identification Report.

dimensional structure of thermolysin was determined through X- ray crystallography (Matthews et al., 1972). Thermolysin has the potential for commercial applications in fields such as the food industry because 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).

B. Product Specifications

Table 2 provides the chemical, lead, and microbiological specifications for THERMOASE C100 and their associated test methods. **Table 3** provides the results of a five-batch analysis for THERMOASE C100. **Table 4** provides the results of a three-batch analysis of the heavy metal content of a concentrated protease product that is used to formulate THERMOASE C100.

The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) has established purity standards for enzymes derived from microbial sources for use in food processing, including standards for lead (< 5 mg/kg), *Salmonella* (absent in 25 g sample), total coliform (<30 CFU/g), *Escherichia coli* (absent in 25 g sample), and antimicrobial activity (absent). As summarized in **Table 3** and as described in Section V.D. of this Notification ("Absence of Antibiotics from the THERMOASE C100 Product"), THERMOASE C100 meets all the JEFCA purity standards for enzymes used in food processing.

The Food Chemicals Codex (FCC) also has specifications for enzyme preparations, including standards for lead (≤ 5 mg/kg), coliform (≤30 CFU/g), and *Salmonella* (negative in 25 g). Enzyme preparations should be cultivated using Good Manufacturing Practices (GMP) and using a pure culture fermentation of non-pathogenic and non-toxigenic strains under controlled fermentation to prevent the introduction of microorganisms that could be the source of toxic materials and other undesirable substances. As described in **Table 3**, THERMOASE C100 meets FCC standards for lead, coliform, and *Salmonella*. This enzyme is manufactured in accordance with quality standards that are certified under the International Organization for Standardization (ISO) 9001.[2] Section IV of this Notification ("Manufacturing Process") describes the controlled fermentation conditions that minimize the introduction of microorganisms and Section VI.A ("Safety of the Source Microorganism") documents the non-pathogenic and non-toxigenic status of *G. stearothermophilus*.

[2] Appendix 2: HP ISO9001 Certificate 2014. ISO 9001 is compared to GMPs and is cited favorably in FDA's Guidance titled "Good Manufacturing Practices (GMPs) for the 21st Century - Food Processing" (August 2004), available at: http://www.fda.gov/Food/GuidanceRegulation/CGMP/ucm110877.htm.

Table 2. Product Specifications for THERMOASE C100

	Specification	Method
Activity (Protease activity)	More than 900,000 units/g	Folin method
Loss on drying	< 6%	1g, 105°C, 4hrs
Lead	< 5 ppm	FCC method
Total viable aerobic count	< 10,000 CFU/g	SCD agar plate method
Coliforms	< 30 CFU/g	FDA-BAM method*
E. coli	Negative/25 g	FDA-BAM method*
Salmonella	Negative/25 g	FDA-BAM method*

* U.S. Food and Drug Administration-Bacteriological Analytical Manual

Table 3. Batch Analysis for THERMOASE C100

	Lot and date assayed					Specification
	THJ1150801	THJ0550606	DINH 052609	DTHI1052608	DTHI0852003	
	11.14.2011	5.12.2011	11.1.2010	11.1.2010	8.26.2010	
Protease activity (units/g)	1,070,000	1,060,000	1,060,000	1,070,000	1,090,000	> 900,000
Loss on drying (%)	0.69	0.65	0.60	1.00	0.30	< 6.00
Lead (ppm)	< 5	< 5	< 5	< 5	< 5	< 5
Total viable aerobic count (CFU/g)	< 10,000	< 10,000	< 10,000	< 10,000	< 10,000	< 10,000
Coliforms (CFU/g)	< 30	< 30	< 30	< 30	< 30	< 30

E. coli (in 25g)	Negative	Negative	Negative	Negative	Negative	Negative
Salmonella (in 25g)	Negative	Negative	Negative	Negative	Negative	Negative

Heavy metal analysis was performed on three lots of the concentrated protease product (referred to as TP(SDD-D)) that is subsequently formulated into the final THERMOASE C100 product as described in Section III.A. ("Product Specifications"). Therefore, the final heavy metal concentrations in THERMOASE C100 are expected to be lower than those found in the concentrated protease product, presented in **Table 4** below. This analysis supports the safety of the manufacturing and purification process with respect to heavy metal levels.

Table 4. Heavy Metal Batch Analysis for Protease Concentrate

Heavy Metal (mg/kg)	**Lot and date assayed**		
	20043780	**20043781**	**20043782**
	5.21.2013	**5.21.2013**	**5.21.2013**
Arsenic	0.199	0.338	0.390
Cadmium	0.140	0.209	0.181
Lead	0.007	0.060	0.005
Mercury	<0.001	0.004	<0.001

IV. Manufacturing Process

Figure 1 below provides a step-by-step illustration of the manufacturing process for THERMOASE C100, which is conducted in accordance with an ISO-certified quality system.[3]

Manufacturing starts with a cultivation step, followed by several filtration and purification steps. The cultivation step involves the inoculation of a pure culture of *G. stearothermophilus* under controlled fermentation conditions to minimize microbial contamination. All raw materials and processing aids used in the enzyme production process are listed in **Table 5** below. Each raw material is a food ingredient or a food substance with an appropriate regulatory status in the U.S. In the future, should Amano choose to modify the raw materials or processing aids used in the production of THERMOASE C100, the Company will ensure that all such components are safe and suitable for their intended use.

[3] See Appendix 2: HP ISO90001 Certificate 2014.

At the end of the aerobic fermentation stage, the production strain is removed through the use of consecutive filter-presses and a final filtration apparatus. The protease preparation is recovered from the culture via filtration, concentration, crystallization, and spray drying to convert it into powder form. The concentrates of the protease preparation are standardized to about 90,000 units/g by the addition of sodium chloride. Heavy metal and microbial analyses are undertaken on the final enzyme product to ensure that product specifications are met.

Daiwa Kasei K.K. (a subsidiary of Amano) manufactures the THERMOASE C100 enzyme preparation up to the spray drying stage, after which Amano finalizes the manufacturing process. In both facilities, the final product is manufactured under Quality Assurance Certification, as indicated in the certificate that appears in Appendix 2. Each lot is manufactured in accordance with Amano's specifications (see **Table 2** above).

Figure 1. Manufacturing Process Flow Chart for THERMOASE C100

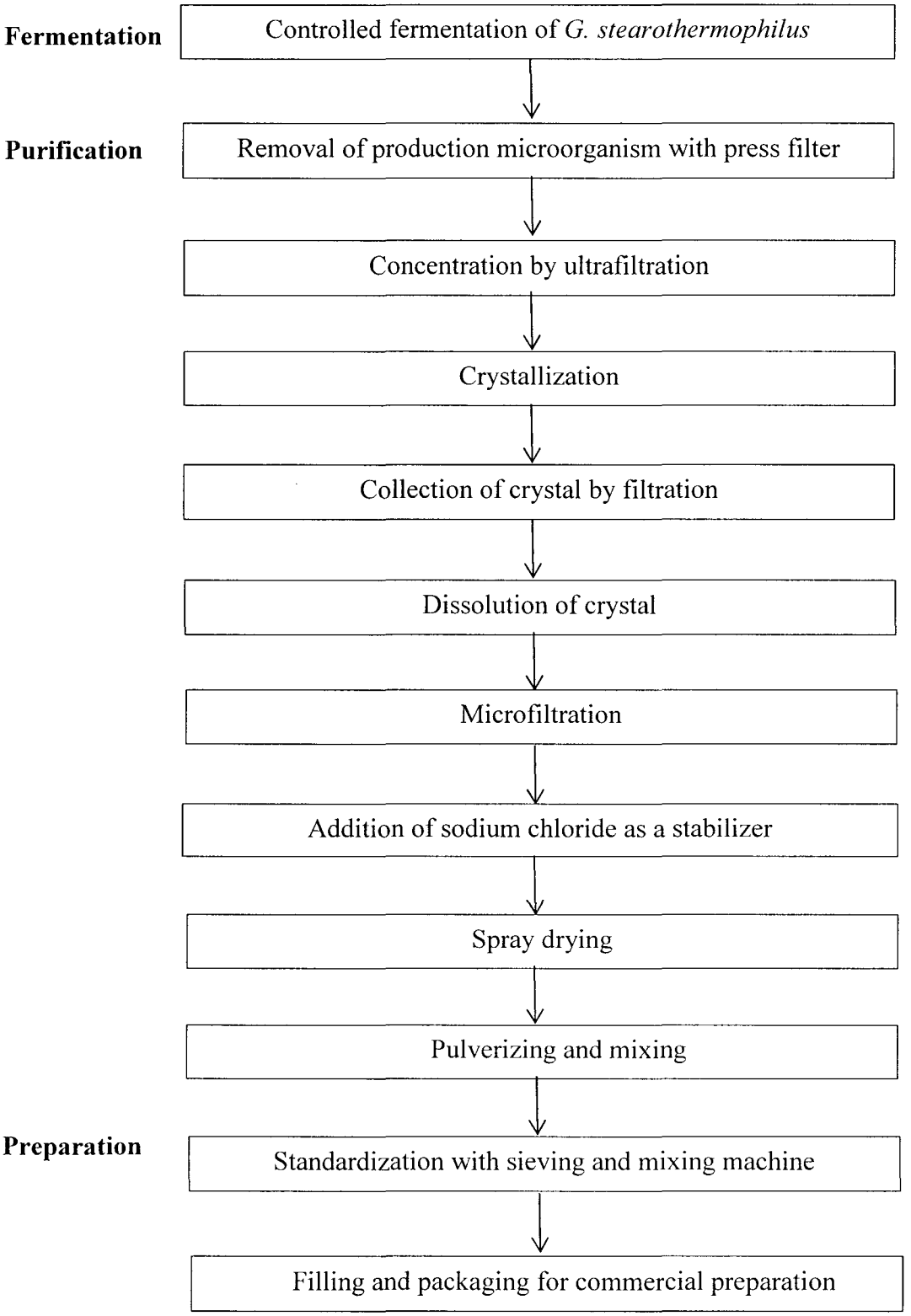

Table 5. Materials Currently used in the Production of THERMOASE C100

Name of Process	Raw Material, Processing Aid, Diluent, or Stabilizer[4]
Fermentation	Potato starch Defatted soybean Casein Corn steep liquor* Diammonium hydrogen phosphate Calcium carbonate Magnesium sulfate Disodium carbonate
Purification	Sodium phosphate dibasic anhydrous Calcium chloride Calcium hydroxide Sodium hydroxide Acetic acid Sodium chloride Activated carbon** Diatomaceous earth
Preparation	Sodium chloride Calcium chloride Disodium phosphate Calcium hydroxide Carbohydrate

* Corn steep liquor is identified as a component of a color additive allowed in chicken feed (21 C.F.R. § 73.275 ("Dried algae meal")). Corn steep liquor is a viscous liquid mixture consisting entirely of the water soluble components of corn steeped in water. All constituents are naturally occurring nutritive materials such as crude proteins, amino acids, vitamins, reducing sugars (e.g., dextrose), organic acids (e.g., lactic acid), minerals, and other elemental nutrients. No ecological, mammalian, or human toxicity would be expected from these natural nutritive materials.

** Activated carbon is used by the food and beverage industries for the removal of impurities and decolorization of liquid food ingredients and food. Activated carbon functions by the adsorption of impurities and color bodies from the food onto the porous surface of its substrate. Activated carbon is not present in the THERMOASE C100 product.

[4] Each ingredient has a clear FDA regulatory status for use in food; several are subject to specific GRAS regulations. *See* 21 C.F.R. § 184.1141b (ammonium phosphate, dibasic); 21 C.F.R. § 184.1191 (calcium carbonate); 21 C.F.R. § 184.1443 (magnesium sulfate); 21 C.F.R. § 184.1742 (sodium carbonate); 21 C.F.R. § 182.1778 (sodium phosphate dibasic anhydrous); 21 C.F.R. § 184.1193 (calcium chloride); 21 C.F.R. § 184.1205 (calcium hydroxide); 21 C.F.R. § 184.1763 (sodium hydroxide); and 21 C.F.R. § 184.1005 (acetic acid). In the future, should Amano choose to modify the raw materials, processing aids, diluents, or stabilizers used in the production of THERMOASE C100, the Company will ensure that all such components are safe and suitable for their intended use.

V. Consideration of Potential Toxins and Contaminants

A. Absence of the Source Microorganism from the THERMOASE C100 Product

Each batch of the protease preparation is filtered for sterility to ensure the absence of the production strain, *G. stearothermophilus*. Next, the enzyme is highly purified by crystallization and microfiltration (0.2μm). The source microorganism and other microorganisms larger than 0.2 μm thus are completely eliminated from the final enzyme preparation. All manufacturing steps are subject to stringent controls under the certified quality control system.

B. Absence of Enzymatic Activity of THERMOASE C100 in the Final Food Product

THERMOASE C100 is a thermostable protease that can be used at higher temperatures (60-70°C), which is advantageous for maintaining product sterility. The hyper-thermostability of THERMOASE C100 requires us to address the concern that enzymatic activity might occur in the finished food product. To address this concern, Amano tested the enzymatic activity of THERMOASE C100 in acid casein, a representative protein; the result showed that THERMOASE C100 is fully inactivated during the manufacturing process.[5]

C. Absence of Toxins from the THERMOASE C100 Product

G. stearothermophilus is not known to produce any bacterial toxins, which is why it is a common production organism for food processing enzymes. As the species *G. stearothermophilus* is a bacterium, it does not produce any mycotoxins. Nevertheless, to confirm the absence of contamination derived from raw materials, fungal contamination, or during manufacturing, Amano tested for the presence of mycotoxins such as Aflatoxin B1, Ochratoxin A (OTA), Sterigmatocystin, Zearalenone, and T-2 Toxin and confirmed their absence from the THERMOASE C100 product. The method of analysis in each assay follows methods described by Patterson and Roberts (1979).

D. Absence of Antibiotics from the THERMOASE C100 Product

As no antibiotics were added to the fermentation broth, no antibiotic activity is expected in the enzyme preparation. Nevertheless, Amano confirmed the absence of antibiotic activity in three lots of THERMOASE C100 using a JECFA method.[6]

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[5] Appendix 3: Demonstration of the Absence of Enzyme Activity of THERMOASE C100 in Finished Products.

[6] FAO JECFA Monographs, Combined Compendium of Food Additive Specifications, Vol. 4, Analytical methods, test procedures, and laboratory solutions used by and referenced in the food additive specifications (2006), available at: ftp://ftp.fao.org/docrep/fao/009/a0691e/a0691e.pdf (See page 154/331 of *pdf version for antibiotic assay).

VI. Safety of THERMOASE C100 Enzyme Preparation

A. Safety of the Source Microorganism

The production strain, *G. stearothermophilus*, is a non-toxigenic and non-pathogenic Gram positive bacterium that is used in food processing as a safe source of native enzymes (Pariza and Johnson, 2001; Olempska-Beer et al., 2006). For 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 which is the subject of a GRAS Notice (GRN 405) submitted to FDA. FDA subsequently issued a letter indicating that the Agency had no questions about the submitter's GRAS conclusion.[7]

Given that *G. stearothermophilus* has a history of safe use in the food industry as described both in the review articles on food processing enzymes from recombinant microorganisms (Olempska-Beer et al., 2006) and in the 1,4-α-glucan branching enzyme GRAS Notice referenced above, it is not anticipated to have any unintended pleiotropic effects that would lead to the production of toxins or other unwanted metabolites – a point of consideration commonly used to assess the safety of enzymes (Pariza and Johnson, 2001). An analysis using the Pariza and Johnson decision tree was performed, which concluded that the test article was accepted.[8]

The final production strain of *G. stearothermophilus* for THERMOASE C100 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 to be unaffected (i.e., the nucleotide sequence remained unchanged) by the NTG treatment. Therefore, the thermolysin gene and subsequent thermolysin enzyme produced from the mutagenized *G. sterothermophilus* production strain is identical to the thermolysin produced from the wild type parental strain.

Microbially derived enzymes have a long and established history of safe use in the food industry. Enzymes are ubiquitous in living organisms and therefore are consumed on a regular basis. Enzymes consumed as part of the normal human 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 therefore are digested and metabolized like other types of dietary proteins (Olempska-Beer et al., 2006). Proteases in general have been used in the food industry for over seventy years (García-Carreño, 1991). Thermolysin (EC 3.4.24.27) from a closely related bacterial species, *Bacillus thermoproteolyticus* Rokko, has already been used to synthesize N-carbobenzoxy L-Asp-L-Phe methyl ester (ZDFM), which is a precursor for the artificial sweetener aspartame, on a commercial scale (Inouye et al., 2007). To confirm the

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[7] Letter from FDA to Ezaki Glico Co., Ltd. Re: GRAS Notice No. GRN000405 (Nov. 1, 2012), available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=405.

[8] Appendix 4: Safety Evaluation of THERMOASE C100 derived from *Geobacillus stearothermophilus* using the Pariza and Johnson Decision Tree.

safety of THERMOASE C100, Amano has conducted a series of standard toxicological studies including acute toxicity feeding studies in rats and mice, a 91-day oral gavage toxicity study in rats, chromosomal aberration in cultured Chinese hamster cells, and a micronucleus test in mice, as detailed below (Ke et al., 2013).

B. Toxicological Studies with the Enzyme Preparation

1. Acute Toxicity Study in Rats

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 hrs, 15 hrs, 24 hrs, 48 hrs, 72 hrs, and 168 hrs 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.

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 hrs after the administration. Excretion returned to normal by 15-24 hrs. No abnormalities were observed in the main organs. The oral LD_{50} 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.

2. Acute Toxicity Study in Mice

Groups of 10 ddY-N stain mice 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 hrs, 15 hrs, 24 hrs, 48 hrs, 72 hrs, and 168 hrs 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.

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. 91-Day Oral Gavage Toxicity Study in Rats

Groups of 20 Sprague-Dawley CD 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.

All control and treated animals survived the course of the study. There were no statistically significant differences identified in body weight between treated and control groups. Food intake of treated groups was similar to that of the control groups at all times.

Ophthalmological examinations revealed no differences between treated and control rats. Any findings noted were among those commonly seen in rats of this age and strain. No clinical signs of a toxic reaction to treatment were noted. Hematology and clinical biochemistry results revealed no treatment related differences between the treated and control groups. 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. 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. Therefore, the increased adrenal weights in males are not suggestive of a treatment-related effect. Other statistically significant changes in organ weights were sporadic, which included 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 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.

4. Chromosomal Aberration Test in Cultured Chinese Hamster Cells

The potential of the enzyme preparation to induce chromosome aberration was tested using Chinese hamster lung fibroblast (CHL/IU) cells.

In the cell-growth inhibition test, cell-growth inhibition that exceeded 50% was observed at 78.1 μg/mL (24.1 μg/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 μg/mL in the short-term treatment with and without metabolic activation, respectively. It was calculated to be 56.9 and 53.7 μg/mL in the 24- and 48-hour 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,"[9] 80.0 μg/mL (24.7 μg/mL thermolysin protein, due to the 30.9% thermolysin protein content of the test article) 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 μg/mL for the subsequent test, corresponding to 24.7, 20.6, 17.2, 14.3, 11.9, and 9.9 μg/mL thermolysin protein. The results indicate that thermolysin did not induce structural or numerical chromosome aberration. In the short-term treatment (6-18 hrs), with metabolic activation, cell ablation and cell death were observed at 55.6 μg/mL (17.2 μg/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 μg/mL, corresponding to 14.3, 11.9, and 9.9 μg/mL thermolysin protein.

Without metabolic activation, cell ablation and cell death were observed at 66.7 μg/mL (20.6 μg/mL thermolysin protein) and above; cell suspension and changes in cell shape were observed at 55.6 μg/mL (17.2 μg/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 μg/mL (17.2, 14.3, 11.9, and 9.9 μg/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 hr treatment. In the 48 hr 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 μg/mL (17.2, 14.3, 11.9, and 9.9 μg/mL thermolysin protein), respectively; and that with numerical aberrations was all 0% at the above tested doses.

On the other hand, 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 to be not genotoxic in this *in vitro* chromosomal aberration assay.

5. Micronucleus Test in Mice

Groups of Male ICR mice [Crj: CD-1 (ICR)], 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, and 3250 mg/kg, respectively. The oral treatment was administered in a single dose, and the animals were sacrificed 30 hours 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

[9] OECD Guidelines for the Testing of Chemicals, Test No. 473: In Vitro Mammalian Chromosomal Aberration Test.

tests contained approximately 14% 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 hours later served as positive controls.

The results from the micronucleus test 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 negative controls. As a result, thermolysin enzyme preparation was considered to be negative in the mouse bone marrow micronucleus test.

6. Allergenicity Analysis

In addition to the toxicological studies described above, the amino acid sequence for thermolysin (http://www.ncbi.nlm.nih.gov/nuccore/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 introduced as an option on the FARRP site, both the literature and FARRP 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.

C. Estimated Human Exposure

The proposed use level of THERMOASE C100 in the production of specific ingredients, as well as the intended level of incorporation of these ingredients into finished foods is provided in **Table 1** above.

A conservative dietary exposure estimate was developed using the "Budget method," (Douglass et al., 1997) as detailed below:

Based on the Budget method model:

1. A conservative estimate of food intake is 25 g per kg body weight per day, of which processed food comprises 50% of the total intake.

2. A conservative estimate of beverage (non-milk) intake is 100 ml per kg body weight per day, of which processed beverages (soft drinks) comprise 25% of the total beverage intake.

3. Based on information regarding the use levels of THERMOASE C100 in the production of food ingredients and the information regarding levels of incorporation of those ingredients into finished foods (provided in **Table 1** above), the highest-level (and thus, "worst-case") use of THERMOASE C100 in food is reflected in the egg white hydrolysate category. THERMOASE C100 is used at a level of up to 0.08% in the production of these ingredients, and these ingredients are used at levels of up to 20% in certain food categories.

4. As a "worst-case" estimate, it is assumed that the above-referenced processed foods and beverages contain 20% egg white hydrolysates (which were produced using THERMOASE C100 at a level of up to 0.08%). The calculation below assumes that all enzyme protein remains in the final product.

5. THERMOASE C100 may contain up to 39% thermolysin protein.

Based on the "worst-case" assumptions given above, the amount of thermolysin protein per kg body weight (bw) per day is calculated as follows:

Food Intake:

Food intake according to Budget method: 25 g food/kg bw/day

50% is processed food: 12.5 g processed food/kg bw/day

Processed food contains 20% egg white hydrolysates:
2.5 g egg white hydrolysates/kg bw/day

Egg white hydrolysates contain (max) 0.08% THERMOASE C100:
2 mg THERMOASE C100/kg bw/day

THERMOASE C100 contains 39% thermolysin protein:
0.78 mg protein/kg bw/day

Beverage Intake:

Beverage intake (non-milk) according to Budget method:
100 ml/kg bw/day

25% is processed beverages (soft drinks): 25 ml = 25 g processed beverages/kg bw/day

Processed beverages contain 20% egg white hydrolysates:
5 g egg white hydrolysates/kg bw/day

Egg white hydrolysates contain (max) 0.08% THERMOASE C100:
4 mg THERMOASE C100/kg bw/day

THERMOASE C100 contains 39% thermolysin protein:
1.56 mg protein/kg bw/day

Total thermolysin protein in food and beverage intake:
0.78 mg + 1.56 mg
= 2.34 mg protein/kg bw/day
(Total Theoretical Maximum Daily Intake)

The margin of safety is calculated as the dose level with the no observed effect level (NOEL) divided by the estimated human consumption level. The NOEL from the 91-day oral toxicity study in rats is 1000 mg/kg bw/day (390 mg protein/kg bw/day).

The margin of safety in the "worst-case" situation described above is thus calculated to be:
390/2.34 = 167

Where the margin of safety is greater than 100, it suggests that the available toxicological data support the proposed uses and application rates. Therefore, even with the extremely conservative assumptions described above, there is still an adequate safety margin. We further note that as discussed above in Section V.B ("Absence of Enzymatic Activity of THERMOASE C100 in the Final Food Product"), no enzymatic activity from the presence of THERMOASE C100 in food ingredients is detectable, which further diminishes the risk of any toxicity.

VII. Summary of Basis for GRAS Determination

Amano has determined that THERMOASE C100 is GRAS based on the following:

- The identity and specifications for THERMOASE C100;
- Conformity of THERMOASE C100 to JEFCA and FCC specifications for enzyme preparations;

- The manufacturing process for THERMOASE C100;
- Toxicological studies conducted on thermolysin enzyme preparations;
- The intended uses and a consumption estimate for THERMOASE C100; and
- Supportive evidence from the long history of safe use of enzymes in food.

VIII. Conclusion

Based on the documentation provided in this GRAS Notification, and as discussed above, Amano has concluded that THERMOASE C100 is GRAS via scientific procedures for use in the proposed food categories.

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Appendix 1
Leatherhead Food RA:
Identification Report

THE INTERNATIONAL
CENTRE FOR INFORMATION,
FOOD SCIENCE AND
TECHNOLOGY

LEATHERHEAD FOOD RA,
RANDALLS ROAD, LEATHERHEAD,
SURREY KT22 7RY
Tel: (0372) 376761 Telex: 929846
Fax: (0372) 386228

YOUR REF: FRA 3417.93 E001 Date 15 Feb 1994
OUR REF:

MICROBIOLOGY MEMBERS SERVICES LABORATORY

Report on : Suspect *Bacillus* culture

For the attention of: Mamoru Eda

Received from : DAIWA KASEI K.K.
7-12 UEHONMACHI 5-CHOME
TENNOJI-KU
Osaka
543

Date sample received: 25/10/93

Sample

One vial of lyophilised culture.

Examination

Upon receipt, the lyophiled culture was rehydrated according to the protocol send with the vial. After incubation, the resulting culture was streaked for purity onto a Tryptone Soya Agar (TSA : Unipath UK Ltd code CM131) plate, and incubated at 55°C for 24 hours. From this culture, the Gram reaction, catalase reaction, oxidase reaction, cell morphology and motility was ascertained then, one colony from this culture was then subcultured into 200ml Brain Heart Infusion (BHI : Unipath UK Ltd code CM225) and incubated at 55°C for 24 hours, to obtain a pure broth culture of the organism. This broth was then used to inoculate the following tests :-

Growth at different temperatures - The broth culture was streaked onto seven plates of BHI agar and incubated at 25°C, 30°C, 37°C, 42°C, 44°C, 50°C and 55°C for upto two weeks. Plates were checked for growth on a daily basis. Additionally, the culture was subcultured into 100 ml BHI and incubated at 65°C for the same length of time.

Growth anaerobically - One BHI agar plate was streaked with the culture and incubated at 55°C for 24 hours in anaerobic conditions. Growth on the plate indicated anaerobic growth.

000035

Growth at pH 5.7 - the culture was streaked onto a plate of Sabouraud Dextrose Agar (SDA : Unipath code CM41), incubated at 55°C for 24 hours. Growth indicated ability to grow at pH 5.7.

Growth in various sodium chloride concentrations - the culture was subcultured into 100 ml BHI broths containing 0%, 1%, 3%, 5%, 7% and 10% sodium chloride. Broths were incubated at 55°C for upto 2 weeks being checked on a daily basis.

Utilisation of Starch - the culture was streaked onto starch agar [1], and incubated at 55°C for 24 hours

Utilisation of Caesin - the culture was streaked onto Caesin agar [1], and incubated at 55°C for 24 hours

Utilisation of Citrate 0 the culture was subcultured onto a slope of Kosers Citrate Agar [1] and incubated at 55°C for 24 hours

Fermentation of Carbohydrates - the culture was subcultured into duplicate tubes of Purple Broth Base (BBL UK Ltd code 11558) containing the carbohydrate under study at a concentration of 0.5-1.0%. Sterile paraffin oil was added to one set of the tubes (called the closed tube) to study if the carbohydrates were attacked fermentatively. The other tube was left "open" to study if the carbohydrates were attacked oxidatively. The carbohydrates which were studied were Glucose, Lactose, Raffinose, Mannose, Maltose, Trehalose, Xylose, Rhamnose, Sucrose, Sorbitol and Mannitol.

Voges-Proskauer test - the culture was subcultured into glucose phosphate broth [1] and incubated at 55°C for 24 hours. After incubation the pH of the culture was measured and noted, then the culture tested for acetylmethylcarbinol by mixing in 40% sodium chloride and creatine.

Production of Bacterial Endospores - The culture was subcultured into BHI containing 0.1% manganese sulphate, and incubated at 55°C for upto three weeks, checking the culture on a daily basis for the presence of spores microscopically.

Reduction of Nitrate - The culture was subcultured into nitrate broth [1] and incubated at 55°C for 24 hours. Nitrite produced from nitrate, was detected using sulphanilic acid, dimethyl-α-naphthylamine and 5N-acetic acid.

Production of indole from tryptophan - The culture was subcultured into 1% tryptone broth [1] and incubated at 55°C for 24 hours. Production of indole was detected using 5% para-dimethylaminobenzaldehyde in acidified iso-amyl alcohol.

Results

The results for all the tests carried out, and the final identification of the culture may be found in Table I.

If you require further information please contact the undersigned.

(b) (6)

Simon Blaber
Food Microbiology

Reference

1 - The Genus *Bacillus*. 1973. Agriculture Handbook No 427 by Gordon R. E. , W. C. Haynes and C Hor-Nay Pang. Agricultural Research Service, United States Department of Agriculture.

Gram Reaction	+
Catalase	+
Oxidase	-
Growth anaerobically	-
Growth aerobically	+
Cell Shape	rod
Spores	terminal swelling sporangium
Motility	-
Growth at 25°C	-
Growth at 30°C	-
Growth at 37°C	+w
Growth at 42°C	+
Growth at 44°C	+
Growth at 50°C	+
Growth at 55°C	+
Growth at 65°C	+
Growth at pH 5.7	-
Growth in 0% sodium chloride	+
Growth in 1.0% sodium chloride	+
Growth in 3.0% sodium chloride	-
Growth in 5.0% sodium chloride	-
Growth in 7.0% sodium chloride	-
Growth in 10.0% sodium chloride	-
Utilisation of Starch	+
Utilisation of Caesin	+
Utilisation of Citrate	-
Utilisation of Glucose	+ fermentatively
Utilisation of Lactose	- fermentatively
Utilisation of Raffinose	-
Utilisation of Mannose	-
Utilisation of Trehalose	+ fermentatively
Utilisation of Xylose	-
Utilisation of Rhamonose	-
Utilisation of Sucrose	-
Utilisation of Sorbitol	+ fermentatively
Utilisation of Mannitol	+ fermentatively
pH in Glucose Phosphate broth	<7.0
production of acetylmethylcarbinol	-
Reduction of Nitrate to Nitrite	+
Production of indole from tryptophan	-

Final Identification - *Bacillus stearothermophilus*

000038

Appendix 2
HP ISO9001 Certificate (2014)

Quality Management System

CERTIFICATE OF CONFORMITY

Certificate No. :	JCQA-0043	Registration Date :	1995.3.27
Renewal Date :	2013.3.27	Expiry Date :	2016.3.26

Name of Registered Organization: **Amano Enzyme Inc.**
Head Office et al.

Address of Registered Organization: **2-7, Nishiki 1-chome, Naka-ku, Nagoya-shi, Aichi, 460-8630, Japan et al.**

JAPAN CHEMICAL QUALITY ASSURANCE LTD. certifies that the Quality Management System of the above organization specified in the appendix, has been assessed and verified to be in accordance with the requirements of the quality management system standards as shown below:

Quality Management System Standards: **JIS Q 9001:2008**
ISO 9001:2008

The Date of Issue: 2014.3.3

JAPAN CHEMICAL QUALITY ASSURANCE LTD.(JCQA)
Nittochi-Uchisaiwaicho BLDG. 7F. 1-2-1, Uchisaiwaicho, Chiyoda-ku, Tokyo, 100-0011, JAPAN
(b) (6)
Tadanori Tamada
PRESIDENT REPRESENTATIVE DIRECTOR

Quality Management System

APPENDIX TO CERTIFICATE OF CONFORMITY

Certificate No. :	JCQA-0043	Registration Date :	1995.3.27
Renewal Date	2013.3.27	Expiry Date :	2016.3.26
Extention Date :			

Name of Registered Organization: **Amano Enzyme Inc.**
Head Office et al.

Scope of Activity:

The Design/Development, Manufacture and Sales of Enzymes

Address of Registered Organization & Scope of Activity:

Head Office:
2-7, Nishiki 1-chome, Naka-ku, Nagoya-shi, Aichi, 460-8630, Japan
The Sales of Enzymes

Gifu R&D Center:
6, Technoplaza 1-chome, Kakamigahara-shi, Gifu, 509-0109, Japan
The Design/Development of Enzymes

Nagoya Plant:
27, Hanno, Kunotsubo, Kitanagoya-shi, Aichi, 481-8533, Japan

Yoro Plant:
3600-1, Makita, Kamiishizu-cho, Ogaki-shi, Gifu, 503-1602, Japan

Shiga Plant:
4-19, Hie-cho, Konan-shi, Shiga, 520-3203, Japan
The Design/Development, Manufacture of Enzymes

The Date of Issue: 2014.3.3

JAPAN CHEMICAL QUALITY ASSURANCE LTD.(JCQA)
Nittochi-Uchisaiwaicho BLDG. 7F, 1-2-1, Uchisaiwaicho,
Chiyoda-ku, Tokyo, 100-0011, JAPAN

(b) (6)

Tadanori Tamada
PRESIDENT REPRESENTATIVE DIRECTOR

Appendix 3

Demonstration of the Absence of Enzyme Activity of THERMOASE C100 In Finished Products

Purpose

THERMOASE C100 is an useful thermostable protease, it can be used under higher temperature (60-70℃) during protein degradation phase in the manufacture.
Besides its advantage, hyper thermostability leads to the concern that the enzyme activity might remain in the product.
The purpose of this document is to demonstrate the disappearance of the enzyme activity under a practically inactivation condition in the manufacture.
In this demonstration, acid casein was used as the representative of protein.

Materials & Method

1. Test procedure

1-1. Mixed solutions bellow;

5.5% acid casein solution	4.0mL
THERMOASE C100 solution (0.01% against the protein)	0.5mL
0.8% Sodiun azide solution*	0.5mL

**: added as antiseptic to prevent contamination of the mixture during long incubation period (37℃ for 20hrs) of the afterward enzyme activity assay.*

1-2. Heated above mixture under conditions bellow;

80℃: 10, 20, 30 and 45min.
90℃: 10, 20, 30 and 45min.
95℃**: 10, 20, 30 and 45min. ***: Boiling state*

1-3. Assayed remaining protease activity by Azo-casein method using kit (*PRORAZYME AK, Megazyme International Ireland Ltd.*).

(1) Mixed & solved following ingredients;

Sample solution (mixture)	1mL
0.1M Phosphate buffer	1mL
PRORAZYME AK	1 tablet

↓

(2) Incubated at 37℃ for 20hrs.

↓

(3) Added 10mL of Na_3PO_4 solution (2%(w/v))

↓

(4) Left it stationary at room temperature for 5min.

↓

(5) Filtration (No.131, ADVANTEC MFS,INC.)

↓

(6) Measured absorption of the filtrate (590nm)

1-4. Assay of the enzyme activity without heat treatment

At 1-3-(1), using 1mL of non-heated mixture instead of heated mixture, the same procedure was conducted from (2) through (6).

1-5. Blank

The same procedure was conducted only without incubation step of 1-3-(2). Subsequent procedure was immediately conducted.

2. Results & Discussion

Results were indicated in the table. Enzyme activity was expressed by △OD/hr value.
In the case of non-heat tretement, distinct enzyme activity was obserbed.
However in the case of heat-treated, activity was not observed in all temperatures and treatment periods except the trace value in the case of 10minutes treatment at 80℃.
These results indicated that the enzyme was inactivated under these treatment conditions.
In the literature[1], it has been reported that approximately 50% protease activity of the enzyme remained after the incubation at 80℃ for 60min. It has been clarified that calcium bound polypeptide chain and yielded strong thermo-stability to the enzyme[2].
The experiment described in the literature has been conducted under ideal conditions such as enough amount of calcium ion addition (2 mM $CaSO_4$). On the other hand, the condition in this document was more practically because the supply of calcium ion from the outside did not exist.
Therefore, it can be concluded that THERMOASE C100 is fully inactivated under practically manufacturing process.

Treatment (℃)	Period (min.)	Activity (△OD/hr)
80	10	0.02
	20	0.00
	30	0.00
	45	0.00
90	10	0.00
	20	0.00
	30	0.00
	45	0.00
95	10	0.00
	20	0.00
	30	0.00
	45	0.00
Non-heated	—	0.47

△ OD=OD(sample)-OD(blank)

Reference

1) Endo S. (1962). Studies on Protease Produced by Thermophilic Bacteria. J. Ferment. Technol. (Japanese). 40: 346-353

2) Tajima M, Urabe I. et al. (1976). "Role of calcium ions in the thermostability of thermolysin and Bacillus subtilis var. amylosacchariticus neutral protease". Eur. J. Biochem. 64 (1): 243–247

Appendix 4

Safety Evaluation of THERMOASE C100 derived from *G. stearothermophillus* using the Pariza and Johnson Decision Tree

1. *Is the production strain genetically modified?*

G. stearothermophillus has undergone classical mutagenesis with the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG)..

YES
If yes, go to 2. If no, go to 6.

2. *Is the production strain modified using rDNA techniques?*

NO
If yes, go to 3. If no, go to 5.

5. *Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects, which may result in the synthesis of toxins or other unsafe metabolites, will not arise due to the genetic modification method that was employed?*

The base production strain is non-pathogenic and non-toxigenic. Genetic analysis was performed on the thermolysin gene after mutagenesis and the nucleotide sequence determined to be the same as wild type (*i.e.*, the thermolysin gene was unaffected by the mutagen). The final enzyme product is highly purified, which is intended to remove potential contaminants from the production organism. Furthermore, traditional toxicological studies have been performed on the enzyme that support the safety of the product.

YES
If yes, go to 6. if no, go to 7
If yes, the test article is **ACCEPTED.**

SUBMISSION END