GR

ORIGINAL SUBMISSION

Nagase ChemteX

1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

August 17, 2006

Via Mail

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

RECD AUG 31 2006

Re: GRAS Notification-Exempt claim for a phospholipase A_2 preparation produced by *Streptomyces violaceoruber* expressing the gene encoding the phospholipase A_2 from *Streptomyces violaceoruber*

Dear Sir or Madam:

Pursuant to the proposed 21 CFR § 170.36(c), we are hereby submitting information for GRAS notification of Nagase ChemteX's (NCX's) phospholipase A_2 enzyme preparation produced by *Streptomyces violaceoruber* expressing the gene encoding a phospholipase A_2 from *Streptomyces violaceoruber*. We have determined by publicly available information or by scientific procedures that this enzyme preparation is Generally Recognized as safe for use in producing enzyme-treated egg yolk or enzyme-hydrolyzed lecithin added to foods.

In accordance with proposed regulation, the following information is provided.

Proposed § 170.36(c)(1)(i): The name and address of the notifier:

Nagase ChemteX Corporation
1-1-17, Shinmachi, Nishi-ku,
Osaka 550-8668, Japan

Proposed § 170.36(c)(1)(ii): The common or usual name of the notified substance:

Phospholipase A_2 enzyme preparation produced by a genetically modified *Streptomyces violaceoruber*.

Proposed § 170.36(c)(1)(iii): The applicable conditions of use of the notified substance:

The Phospholipase A_2 enzyme preparation produced by *Streptomyces violaceoruber* is intend to be used as a processing aid at minimum levels necessary, in accordance with current Good Manufacturing Practices for the preparation of enzyme-treated (or modified) egg yolk or enzyme hydrolyzed (or modified) lecithin, which can then be added to food such as mayonnaise to achieve the desired effect. The purpose of the enzyme is to hydrolyze the fatty acid at the 2nd position of the lecithin or lecithin that present in egg yolk.

Proposed § 170.36(c)(1)(iv): The basis for the GRAS determination:

Nagase ChemteX
1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

This GRAS determination is based on publicly available information and scientific procedures.

Proposed § 170.36(c)(1)(v): Availability of information:

A summary of the data and information supporting this GRAS notification is attached. References and other information that are the basis for this determination are available for review at our facilities in Japan. Please contact the following person by direct telephone at +81-78-992-0195 or direct fax at +81-78-992-1050 or the e-mail address below, if you have any questions or require additional information.

Xiaoli Liu (PhD), xiaoli.liu@ncx.nagase.co.jp
2-2-3 Murotani, Nishi-ku, Kobe 651-2241, Japan
Product Development Section 1
Bio/Fine Chemicals Division
Nagase ChemteX Corporation

Yours sincerely,

Hitoshi Morimoto
Director, Bio/Fine Chemicals Division
Nagase ChemteX Corporation

(Enclosure 3 binders)

Nagase ChemteX

1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

A phospholipase A_2 preparation produced by *Streptomyces violaceoruber* expressing the gene encoding phospholipase A_2 from *Streptomyces violaceoruber*

Nagase ChemteX
1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

TABLE OF CONTENTS

	PAGE
1. GENERAL INTRODUCTION	3
2. PRODUCTION MICROORGANISM	3
2.1 Production Strain	3
2.2 Recipient Organism	4
2.3 Phospholipase Expression Plasmid	4
2.4 Stability of the Introduced Genetic Sequences	5
2.5 Antibiotic Resistance Gene	5
2.6 Absence of the production organism in product	6
3. ENZYME IDENTITY	6
3.1 Enzyme and protein chemical characteristics	6
3.2 Amino acid sequence	7
4. MANUFACTURING PROCESS	7
4.1 Raw Materials	7
4.2 Fermentation Process	7
4.3 Recovery Process	8
4.4 Quality Control of Finished Product	8
5. COMPOSITON AND SPECIFICATIONS	8
5.1 Quantitative Composition	9
5.2 Specifications	9
6. APPLICATION	9
6.1 Mode of Action	9
6.2 Use Levels	10
6.3 Enzyme Residues in the Final Food	10
7. SAFETY EVALUATION	10
7.1 Safety of the Production Strain	10
7.2 Safety of the Donor Organism	11
7.3 Safety of the Phospholipase Enzyme	11
7.4 Safety of the Manufacturing Process	11
7.5 Safety Studies	12
7.6 Estimates of Human Consumption and Safety Margin	13
7.7 Results and Conclusion	14
8. LIST OF APPENDICES	15
9. LIST OF REFERENCES	23

000005

1. GENERAL INTRODUCTION

The subject is a phospholipase A_2 preparation produced by submerged fermentation of a *Streptomyces violaceoruber* microorganism carrying the gene coding for phospholipase A_2 from *Streptomyces violaceoruber*. The trade name for this enzyme preparation is "PLA2 Nagase". The enzyme preparation is intend to be used as a processing aid in producing enzyme-treated (or modified) egg yolk or enzyme-hydrolyzed (or modified) lecithin, which is then used as an emulsifying agent in food such as mayonnaise.

The enzyme is a phospholipase A_2 (EC3.1.1.4) that specifically acts on the fatty acid in 2nd position of phospholipids. When used as a processing aid in treatment of egg yolk or lecithin, it is added to the egg yolk or the lecithin suspension and kept at a constant temperature for a certain period of time. After the treatment, the temperature of the yolk is raised in order to inactivate the enzyme, or in the case of lecithin, followed by further purification procedures. The treated egg yolk and lecithin have improved emulsifying properties. Phospholipases including the phospholipase A_2 activity are found in most cells and tissues including animal tissues that are historically consumed by human.

The information provided in the following sections is the basis for our determination of general recognition of safety of this enzyme preparation. Our safety evaluation in Section 7 includes an evaluation of the production strain, the donor strain, the introduced genetic material, the enzyme preparation and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (ref.1, 2). The production organism for this phospholipase A_2, *S. violaceoruber*, is discussed in Sections 2 and 7. The production organism is a well-characterized bacterium, nontoxic and nonpathogenic as demonstrated by a thorough literature review. An enzyme preparation, functionally the same phospholipase A_2 produced by this organism has been favored as GRAS by notification, which filed by Genecor Internationals (GRN000145).

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material (ref.1, 4-5). The genetic modifications used to construct the production microorganism are well defined and are described in Section 2.

The safety of the enzyme preparation has been discussed in Section 7.5 based on the toxicological data comprising of a 13-week subacute oral toxicity in rats, an Ames test and an in vitro human lymphocyte cytogenesis assay, as well as an acute oral LD_{50} estimation in rats.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

000006

The *S. violaceoruber* strain, designated AS-10, was constructed by plasmid transformation of the recipient strain, designated *S. violaceoruber* 1326 (see Section 2.2). This genetically modified production organism complies with the OECD (organization for Economic Co-operation and

Development) Criteria for GILSP (Good Industrial large Scale Practice) microorganisms (ref.5). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (ref.1) and other expert groups (ref.3).

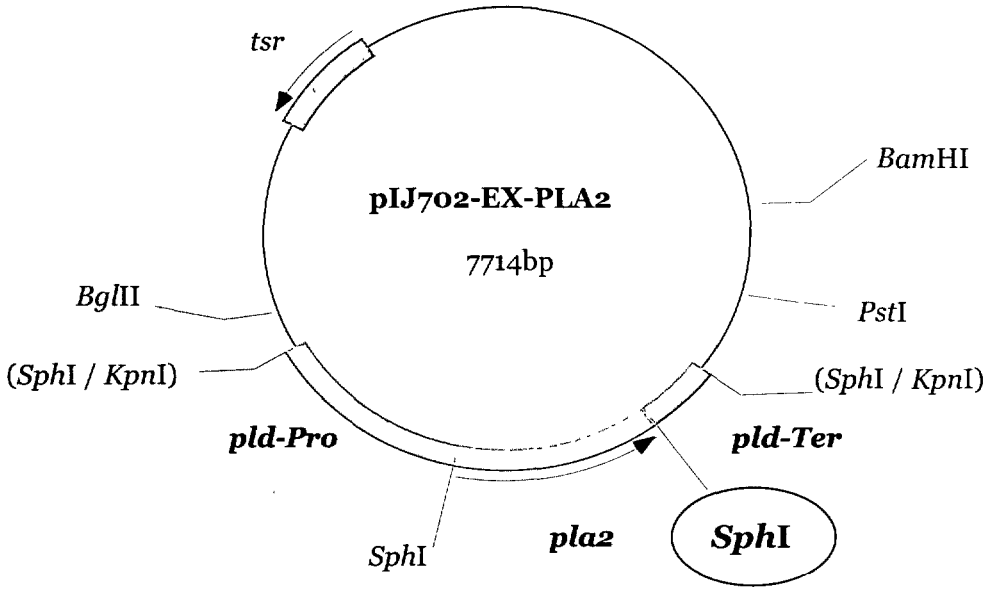
The phospholipase A_2 expression plasmid, pIJ702-EX-PLA$_2$ used in the strain construction contains strictly defined Streptomyces chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include: a gene encoding a phospholipase A_2 enzyme from *S. violaceoruber* IFO 15146; a well-characterized regulatory sequences including a *S. cinnamoneum* originated phospholipase D promoter and terminator, a 5681bp fragment from Streptomyces cloning vector pIJ702 (ref.6) and a synthetic marker (see also section 2.3). Detailed information on the construction of pIJ702-EX-PLA$_2$ is described in Appendix 1.

2.2 Recipient Organism (Host)

The recipient microorganism, designated *Streptomyces violaceoruber* 1326, used in the construction of the phospholipase A_2 production strain is a well-known nontoxic and nonpathogenic type strain. The strain was obtained from the John Innes Center.

2.3 Phospholipase A_2 Expression Plasmid

The 7714bp expression plasmid, pIJ702-EX-PLA$_2$, used to transform the *Streptomyces violaceoruber* 1326 strain, contains the gene elements outlined below.



	pld-Pro	*Streptomyces cinnamoneum* originated phospholipase D promoter (1370bp)
	pla2	phospholipase A2 gene from *Streptomyces violaceoruber* (456bp)
	pld-Ter	*Streptomyces cinnamoneum* originated phospholipase D terminator (201bp)
	pIJ702	fragment Digested with *SphI* and blunted (5681bp)
SphI		Synthetic Marker (6bp)

The complete nucleotide sequence of the plasmid has been determined (data is available upon request). All the genetic elements are from Streptomyces species.

2.3.1 database search for unintended open reading frames

The database search was performed on DDBJ (DNA Data Bank of Japan), which has been functioning as the international nucleotide sequence database in collaboration with EBI/EMBL and NCBI/GenBank. The search was done by two approaches:

1) The complete nucleotide sequence of pIJ702-EX-PLA2 was searched for homology within the database with the option of "bacteria" and no homology with any known bacterial toxins was found. Besides, homology with Streptomyces tyrosinase gene, plasmid *pIJ101* and *pSB24.2*, *tsr* gene and phospholipase A2 gene were detected, which either belong to the vector itself or the intended gene being introduced.

2) A sequence-analyzing program called GENETYX-WIN was first used to search for open reading frames with the option for initiation codons "ATG" and "GTG", termination codons "TAA","TAG" and "TGA". (These codons are frequently used codons of Streptomyces). All open reading frames within or overlapping the inserted DNA was then searched on DDBJ database for homology detections and no unintended open reading frames coding for known toxins were found.

2.3.2 the pleiotropic effects

Literature search with the topic of "pleiotropic effect of bacterial phospholipases", "pleiotropic effect in Streptomyces" and "pleiotropic genes of Streptomyces" were carried out and no pleiotropic effect was reported with the genes originated from the vector *pIJ702* and the inserted gene of Phospholipase A_2. Combined the results with the above-mentioned 2.3.1 and the expression products compared with the original host as described in Appendix 4, we concluded that there is unlikely a pleiotropic effect in this recombinant strain. Other support data include growth speed and colony formed on agar plates compared between the host and the recombinant microorganisms.

2.4 Stability of the Introduced Genetic Sequences

As described by Kieser T. et al (ref.6-7), the pIJ702 is a nontransferable, nonconjugative plasmid, which retains in the cell at its autonomous form. Therefore the frequency of mobilization of the inserted vector or genetic transfer capability can be estimated to be extremely low.

The stability of the introduced genetic sequences was also tested by subsequent cultivation of the production strain for several generations including that cultivated under no selective pressures, and the enzyme productivity was tested for each generation. The enzyme productivity was constant between tests.

2.5 Antibiotic Resistance Gene

The introduced DNA contains an antibiotic (thiostrepton) resistance gene, *tsr*, which is indigenous to *Streptomyces azureus* (Mol Gen Genet 1985, 199:26-36), meets the GILSP criteria and is listed in GILSP recombinant microorganism automatic list issued by the Ministry of Economy, Trade and Industry of Japan. The *Streptomyces lividans*-pIJ101 and its derivatives such as pIJ702 are certified host-vector systems by NIH (ref: http://www4.od.nih.gov/oba/rac/guidelines_02/).

According to Pariza and Johnson (ref.1), the concern of the antibiotic resistance gene is limited in if it is transferable or not. The *tsr* gene, even in its plasmid form, is non-transferable.

The Joint FAO/WHO Expert Consultation on Biotechnology and Food Safety has suggested that "if the recombinant microorganism does not enter the food product or if the organism is not deliberately released to the environment, then the presence of antibiotic resistance genes should not be a concern." This is because the expression products of such genes do not add toxic components to the food supply and, more importantly, the genes themselves will not be transferred to other organisms. In many cases, like that with PLA2 Nagase, the recombinant microorganism is used in a contained fermentation facility to produce an enzyme or other food ingredient. The recombinant microorganism is then removed from the commercial product (ref: Regulatory Toxicology and Pharmacology 12, S122-S123, 1990). It is standard operation procedure to remove the microorganism in producing the enzyme preparation of PLA2 Nagase.

The FDA scientists have evaluated the existence of antibiotic resistance marker genes in transgenic plants and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of man or animal, and in the environment is remote, which is the similar case with the *tsr* gene in pIJ702 because of the immobility of the plasmid. Furthermore it has been concluded that the existence of antibiotic resistance gene does not raise a safety concern. "This is because, most DNA is degraded in the gut and thus, would be unavailable for transfer, and even if some DNA survived and was available for transfer into these cells, it would not be integrated and expressed due to lack of selective pressure. Additionally, because these cells are continuously sloughed off and replaced by new cells, a cell that incorporated an antibiotic resistance gene would not be long-lived and present a safety hazard with respect to compromising therapy with antibiotics." (ref: http://www.cfsan.fda.gov/).

Although the antibiotic, thiostrepton, is used as a topical medicine for animals, it is unlikely to enter the human intestinal tract to provide enough selective pressure. We calculated the possible amount of the antibiotic gene in the final product of PLA2 Nagase to be less than 2 ng by a southern blotting test (data not shown), based on a worst-case estimation. This amount is absolutely not of any concern.

2.6 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food.

3. ENZYME IDENTITY

3.1 Enzyme and protein chemical characteristics

Classification:	phospholipase A_2 (generic name)
IUB nomenclature:	phosphatidylcholine 2-acylhydrolase
IUB No.	3.1.1.4
Trade name:	PLA2 Nagase
CAS No.	9001-84-7
Specificity:	hydrolyses the sn-2 ester bond of diacylphospholipids to form 1-acyl-2-lysophospholipid and free fatty acid.

000009

3.2 Amino acid sequence

The total nucleotide and amino acid sequences have been determined and the amino acid sequences of the enzyme produced from the production strain, *Streptomyces violaceoruber* AS-10, is identical to that of the donor strain, *Streptomyces violaceoruber* IFO 15146 (see Appendix 3).

The enzyme properties such as optimum temperature, optimum pH and stability profiles are shown in Appendix 2.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for PLA2 Nagase that follows standard industry practices (ref.8-9). The quality management system used in the manufacturing process for the PLA2 Nagase complies with the requirements of ISO 9001 and the requirements for manufacturing food-additives by the Japanese Ministry of Health, Labor and Welfare.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for PLA2 Nagase are standard ingredients used in the enzyme industry (ref. 8-9 as well as the Food sanitary law by the Japanese Ministry of Health, Labor and Welfare).

4.1.1 Fermentation Medium

The ingredients used in fermentation are corn steep liquor, fish extract, glycerol, dipotassium hydrogen phosphate, sodium chloride, sodium hydroxide and polyoxyalkyleneglycol (antifoam)

The maximum use level of antifoams in the fermentation is less than 1%.

Thiostrepton was not used in the fermentation medium.

4.1.2 Purification Process

The materials used in the purification process are calcium chloride, disodium hydrogenphosphate, sodium hydroxide, pulpwood (filter aid), ammonium sulfate and diatomaceous earth (filter aid).

4.1.3 Formulation

The ingredients used in formulation are sorbitol, salt and potassium sorbate.

4.2 Fermentation Process

000010

The phospholipase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *Streptomyces violaceoruber* AS-10 described in section 2. All equipments is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analysis are done to ensure absence of foreign

microorganisms and confirm strain identity.

4.2.1 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *Streptomyces violaceoruber* AS-10, as described in section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermentor and the main fermentor before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for enzyme productivity and microbiological contamination by microscopy. In case any infection is observed in 2 or more samples, the fermentation is regarded as "contaminated" and is rejected.

4.3 Recovery Process

The recovery process is a multi-step operation that starts immediately after the fermentation process and consists of both the purification and the formulation processes.

4.3.1 Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1. Primary Separation- vacuum drum filtration
2. Purification by precipitating with ammonium sulfate
3. Re-suspension with water
4. Separation to remove any insoluble – vacuum drum filtration
5. Germ Filtration- for removal of residual production strain organisms and as a general precaution against microbial degradation
6. Preservation and stabilization of the liquid enzyme concentrate

4.3.2 Formulation and Standardization Processes

The stabilized concentrate is blended with water, sorbitol, and preserved with potassium sorbate and salt. The product is standardized according to the product specification.

4.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in section 5.

5. COMPOSITION AND SPECIFICATIONS

000011

PLA2 Nagase enzyme preparation is presently available in a formula for use in egg yolk treatment and in producing enzyme hydrolyzed lecithin.

5.1 Quantitative Composition

PLA2 Nagase has the following typical composition:

Enzyme Solids (TOS)	approx. 1%
Water	approx. 60%
Sorbitol	approx. 30%
Ash (mainly sodium chloride and ammonium sulfate)	approx. 9%
Potassium sorbate	approx. 0.1%

5.2 Specifications

PLA2 Nagase complies with the purity criteria recommended for enzyme preparations as described in Food Chemicals Codex, 4th edition, 3rd supplement, 2001. In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications.

The following specifications have been established:

Enzyme activity	according to declaration
Heavy metals	not more than 30 ppm
Lead	not more than 5 ppm
Arsenic	not more than 3 ppm
Total viable count/g	not more than 10,000
Total coliforms/g	negative by test
Salmonella/25g	negative by test
Antibiotic activity	negative by test
Production organism	negative by test

Antibiotic activity is analyzed at regular intervals.

PLA2 Nagase has a typical activity of 10,000 PLA2UN/ml. The enzyme activity (PLA2UN) is measured by hydrolyzing lecithin under constant pH and temperature. The activity is determined as the rate of the production of the liberated fatty acid (refer to Appendix 2).

6. APPLICATION

6.1 Mode of Action

The active enzyme in PLA2 NAGASE is a phospholipase (EC3.1.1.4), which specifically acts on the fatty acid in 2nd position of the phospholipid substrates. It catalyzes the hydrolysis of sn-2 ester bond of diacylphospholipids to form 1-acyl-2-lysophospholipid and free fatty acid.

The PLA2 Nagase is used as a processing aid in egg yolk treatment or in hydrolyzing lecithin, where it is added to the egg yolk or lecithin suspension. The treated egg yolk or hydrolyzed lecithin has improved emulsifying properties and such result in improved taste when added in food like mayonnaise and/or a longer shelf life of the product.

6.2 Use levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect. The recommended dosage of PLA2 Nagase is up to 20 PLA2UN (0.02 mg TOS) for 1 g egg yolk or 200 PLA2UN (0.2 mg TOS) for 1 g lecithin.

6.3 Enzyme Residues in the Final Food

The enzyme is added to the egg yolk or lecithin, after treatment the enzyme is denatured by raising the temperature to 60-65℃ or removed by further purification process. The egg yolk or hydrolyzed lecithin is then added at 5-10% or 0.5-1% respectively to food such as mayonnaise. It is estimated that the enzyme residues left in food is not more than 0.002 mg TOS/ g.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (ref, 1-2). If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices is safe to consume (ref.10). Pariza and Foster (1983) define a nontoxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". We concluded that *Streptomyces violaceoruber* meets these criteria for nontoxigenicity and nonpathogenicity by the following evaluations.

7.1.1 Literature search

We did a though search on Scifinder, copyright owned by American Chemical society, which covers extensive scientific journals, reports and books dated from 1907. Searching on research topics of "Streptomyces", we retrieved 34319 articles. To refine the search on topics of "pathogenic Streptomyces", totally 231 articles were retrieved. To further refine the search on topics of "*Streptomyces violaceoruber*", "*Streptomyces lividans*", "*Streptomyces coelicolor*" have given us 11articles, 0articles and 9 articles respectively. (The reason we did the search on these three species is because they are considered as a single taxon; and the synonym name for *Streptomyces violaceoruber* is *Streptomyces lividans*). These articles reported some plant pathogens of *Streptomyces*, such as the well-known *Streptomyces scabies* and *S. acidiscabies*, which produce phytotoxin thaxtomin A, and none of animal pathogens are reported. The following two reviews have suggested *Streptomyces lividans* as a good host for expressing heterologous proteins because of its non-pathogenic characteristics, efficient secretion capacity and well-established fermentation technology (ref.12-13).

We also complete a search on STN search engine with search command: (*Streptomyces violaceoruber* or *Streptomyces lividans*) and (toxin or pathogenicity) and reviewed the literatures found. Besides those in repeats, we found totally 49 related, among which 14 has reported on using this bacterium as cloning host for various genes and 32 as a study subject which are not relevant. 7 reported the organism as non-pathogenic and one reported it as a plant pathogen that is likely mistaken.

7.1.2 Reviews: Pathogenity of *Streptomycetes*

A Book titled "Practical Streptomyces genetics" (ed. by T. Kieser, MJ Bibb, MJ Buttner, KF Chater and DA Hopwood, chapter 1) has reviewed the pathogenity of *Streptomycetes* and indicated that human diseases caused by actinomycetes are largely due to infections by Mycobacterium, Actinomudura, Nocardia and Actinomyces species. Only one case, *Streptomyces somaliensis* has been demonstrated as human pathogen (personal communications). This is also reported in "The Prokaryotes", A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, and Applications. Edited by A.Balows and so on (1991) p937-941.

In the GRAS notification of GRN 000145, a phospholipase A_2 from the same microorganism *Streptomyces violaceoruber* has been demonstrated as safe for use in food. The PLA2 Nagase's production strain is originated from the same species, *Streptomyces violaceoruber* with some genetic modifications as described in section 2. Because the genetic modifications are well characterized and specific, and an extensive homology search has indicted that the introduced DNA does not encode and express any known harmful or toxic substances, in addition, no pleiotropic effects were found, the phospholipase A_2 enzyme preparation derived from the genetically modified *Streptomyces violaceoruber* should be considered safe.

7.2 Safety of the Donor Organism

The donor for the phospholipase A_2 enzyme is *Streptomyces violaceoruber*. The donor for the regulatory genes such as the promoter and the terminator is *Streptomyces cinnamoneum,* which also meets the criteria for nontoxigenicity and nonpathogenicity.

7.3 Safety of the Phospholipase A_2 Enzyme

PLA2 Nagase is functionally the same as the enzyme of the subject of the GRAS Notice No. GRN 000145 and GRN 000183. This enzyme activity is found in most cells and tissues including animal tissues such as porcine pancreas that are traditionally consumed by man before Jan. 1st, 1958.

7.4 Safety of the Manufacturing Process

PLA2 Nagase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 4, the PLA2 Nagase preparation is produced in accordance with current good manufacturing practices, using only ingredients that are acceptable for general uses in foods in the U.S., except the antifoam, which will be evaluated later, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (ref. 8-9).

The antifoam used in the fermentation process is polyoxyalkyleneglycol (Product name: Colorin 102) of Sanyo Chemicals Industries, Ltd. This chemical is generally used as antifoam in producing food enzymes in Japan and is considered safe for use in cosmetics in the U.S. (ref.14). We evaluated the safety of this chemical in the PLA2 Nagase enzyme preparations as follows.

The molecular distribution of polyoxyalkyleneglycol is ranged from 191-4072 with about 1% of fractions that has a molecular weight probably lower than 1000, as estimated by the manufacturer. In general, substances having molecular weights exceeding 1000 Daltons are not of toxicity concern because they are not absorbed in the gastrointestinal tract. The antifoam used in the fermentation process is less than 1%. Much, if not all, of the antifoam is separated from the enzyme during the purification steps because the substance is soluble, however, in a worst-case estimation the carryover amount of the antifoam in the enzyme is 1% in the TOS. At this level, the dietary intake of that in concern can be calculated as 0.82 ppb (refer to 7.6.1: daily intake of PLA2 Nagase from mayonnaise is 0.06mgTOS/day/person; daily intake of PLA2 Nagase from lecithin is 0.104mgTOS/day/person; therefore the total intake can be estimated as 0.164mg/day/person. Presuming a person's daily food consumption is 2kg, the dietary intake of the antifoam in concern can be calculated as 0.164mg×1% ÷2kg=0.00082mg/kg=0.82ppb). There is absolutely no safety concern with intake at this level (ref. 15). Furthermore, the toxicity studies of the substance indicated a LD_{50} of approximately 9760mg/kg in rat, as presented by the manufacture, which strongly support the safety at this intake level.

7.5 Safety Studies

This section describes the studies performed to evaluate the safety of the use of PLA2 Nagase.

7.5.1 Description of the test Material

The safety studies described below were conducted on a powdered PLA2 Nagase Concentrate, which was prepared according to the description given in Section 4.3.1, from operation 1 through 5 and freeze-dried without formulating (operation 6). The powdered PLA2 Nagase concentrate was dispersed in appropriate media before applied to tests.

The enzyme solids (TOS) in the PLA2 Nagase concentrate is approximately 25%.

7.5.2 Studies

The following studies were performed:

- -Test for reverse mutagenic activity (Ames test)
- -Acute Oral LD_{50} in Rats
- -In vitro chromosome aberration study using Chinese hamster lung fibroblast (CHL/IU) cells
- -13-week subacute oral toxicity in rats

These tests are described in Appendix 5. The conclusion of these tests is that the enzyme preparation is categorized as GHS category 5, which has a LD_{50}>2000mg/kg, is considered non-cytotoxic and does not induce gene mutations in bacteria and mammalian cells under the conditions of the study. The results of the 13-week subacute toxicity indicate the enzyme preparation has a NOAEL (no observed adverse effect level) of 200mg/kg/day for male rat and 40mg/kg/day for female rat, equivalent to 50mgTOS/kg/day for male rat and 10mgTOS/kg/day for female rat. Since bacterial toxins are known acute toxins, these data are considered sufficient for supporting the safety of the enzyme preparation of PLA2 Nagase.

7.6 Estimates of Human Consumption and safety Margin

As stated in section 6, PLA2 Nagase is used in egg yolk treatment and in hydrolyzing lecithin. The enzyme activity is largely removed or heat inactivated during the process, however, in order to illustrate a "worst case" situation, the following calculation is made assuming that all enzyme activity is retained in the final food product.

7.6.1 Estimates of human consumption

PLA2 Nagase has a typical activity of 10000 PLA2UN/ml and an approximate content of 0.001mgTOS per PLA2UN (TOS: Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

7.6.1.1 In the case of enzyme treated egg yolk for mayonnaise

The average yearly consumption of mayonnaise is estimated at 7g/person/day (g/p/d) and mayonnaise consumption by the 90th percentile consumer was 14g/p/d. in the U.S. according to Pao, E.M., and et al. 1982 (ref: Consumer Nutrition Center, Human Nutrition Information Service, USDA. Home Economics Research Report Number 44 reviewed and approved for reprinting February 1990). In order to demonstrate a worst-case calculation, an exaggerated human intake is estimated using the following assumptions.

a) The calculation is made assuming that all TOS originating from PLA2 Nagase remains in the mayonnaise. PLA2 Nagase contains 0.001mgTOS/PLA_2UN.
b) It is assumed that all mayonnaise is produced using PLA2 Nagase as a processing aid during the production process, and that it is used at the highest recommended dosage. And the daily maximum consumption of the mayonnaise is 30g/day/person.
c) The treated egg yolk added to the mayonnaise is over estimated as 10%.

The maximum recommended dosage of PLA_2 Nagase is 20PLA_2UN per g egg yolk, that is 0.02mgTOS per g egg yolk=0.2mgTOS per 100g mayonnaise. Based on the highest average daily intake per person of mayonnaise (30g), this corresponds to an intake of 30g×0.2mg÷100g=0.06mg TOS per person per day. For an average person weighing 60kg this corresponds to 0.001mgTOS per kg-body weight per day (TOS/kg/day).

7.6.1.2 In the case of enzyme hydrolyzed lecithin

000016

Unlike in the case of hydrolyzing egg yolk, where the enzyme has little chance to be removed from the system, the hydrolysis of lecithin is usually performed using solvent systems and enzyme is usually removed afterwards. Therefore we presumed there is less risk than that with the egg yolk treatment. An estimation of the human consumption in this case is calculated as the following:

1) According to Freedonia's report on the lecithin demand 1992-2012 (report number 1699), the yearly consumption in U.S. for soy lecithin in year 2002 is estimated at 0.417 lb/person, equivalent to approximately 0.52g per person per day. In order to demonstrate a worst-case situation, all lecithin consumed is presumed to be PLA2 Nagase modified.
2) The highest dosage for hydrolyzing soy lecithin is estimated at 200PLA_2UN/g and we presume they are remained in the lecithin product fully.

Then, the daily consumption will be: 0.52g×200PLA2UN/g×0.001mgTOS/PLA_2UN /day=0.104mg

TOS/day/person. For a 60kg adult the daily intake will be: 0.104mgTOS/day÷60kg=0.00173mgTOS per kg-body weight per day(TOS/kg/day). This estimation is highly exaggerated because; the enzyme is usually removed from the lecithin during processing. Additionally, it is unlikely that all lecithin is enzymatically-hydrolyzed; much less solely processed using PLA2 Nagase.

7.6.2 Safety margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The conservative NOAEL dose level in the 13-week oral toxicity studies in rats for PLA2 Nagase preparations was determined as 10 mgTOS/kg/day (refer to 7.5.2).

The estimated human consumption is 0.001mgTOS/kg/day in the case of enzyme treated egg yolk, or 0.00173mgTOS/kg/day in the case of hydrolyzed lecithin.

The safety margin can thus be calculated to be:	10mg/0.001mg =10000	(for egg yolk)
Or:	10mg/0.00173mg=5780	(for lecithin)

Both cases demonstrated a worst-case calculation and they meet well over the required 100 for determining the safety of enzyme preparations for food, as suggested by Pariza and Johnson (ref.1&2). Therefore it can be concluded that the enzyme preparation of PLA2 Nagase is regarded safe for human consumption under the current consuming conditions, i.e. for producing enzyme treated egg yolk or hydrolyzed lecithin.

7.7 Results and Conclusion

On the basis of the evaluation contained in Section 7, with the published information confirming the lack of any safety concern with regard to the production strain and plasmid, the limited and well defined nature of the genetic modifications, the appropriate manufacturing process and the estimated human consumption well within the range of NOAEL which is determined through systematic toxicity studies on the enzyme preparation, we, Nagase ChemteX Corporation has concluded that the PLA2 Nagase enzyme preparation intend to use in treatment of egg yolk and in hydrolyzing lecithin should be deemed generally regarded as safe (GRAS).

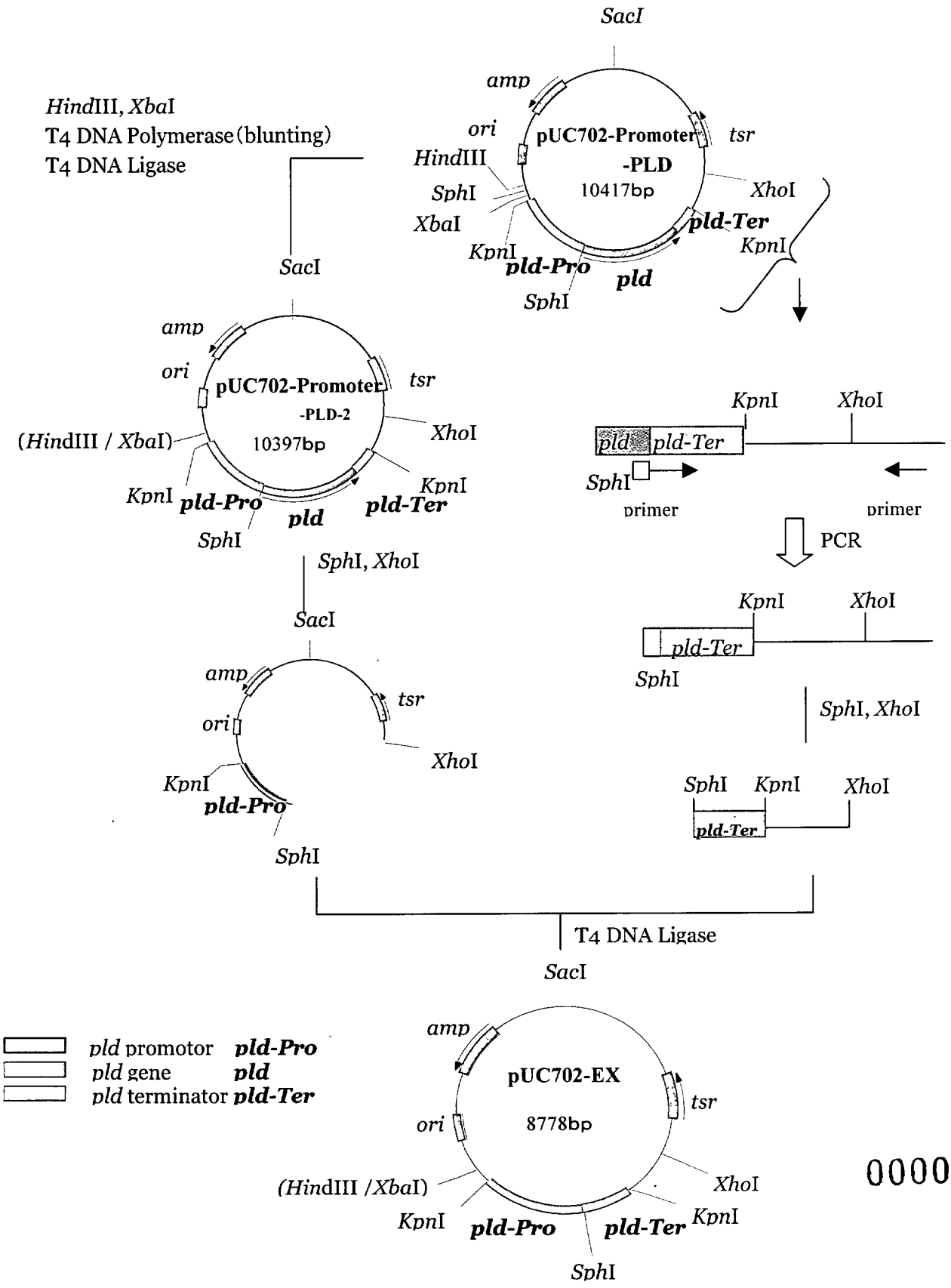
Nagase ChemteX
1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

LIST OF APPENDICES

Nagase ChemteX
1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

Appendix 1. Construction of pIJ702-EX-PLA_2

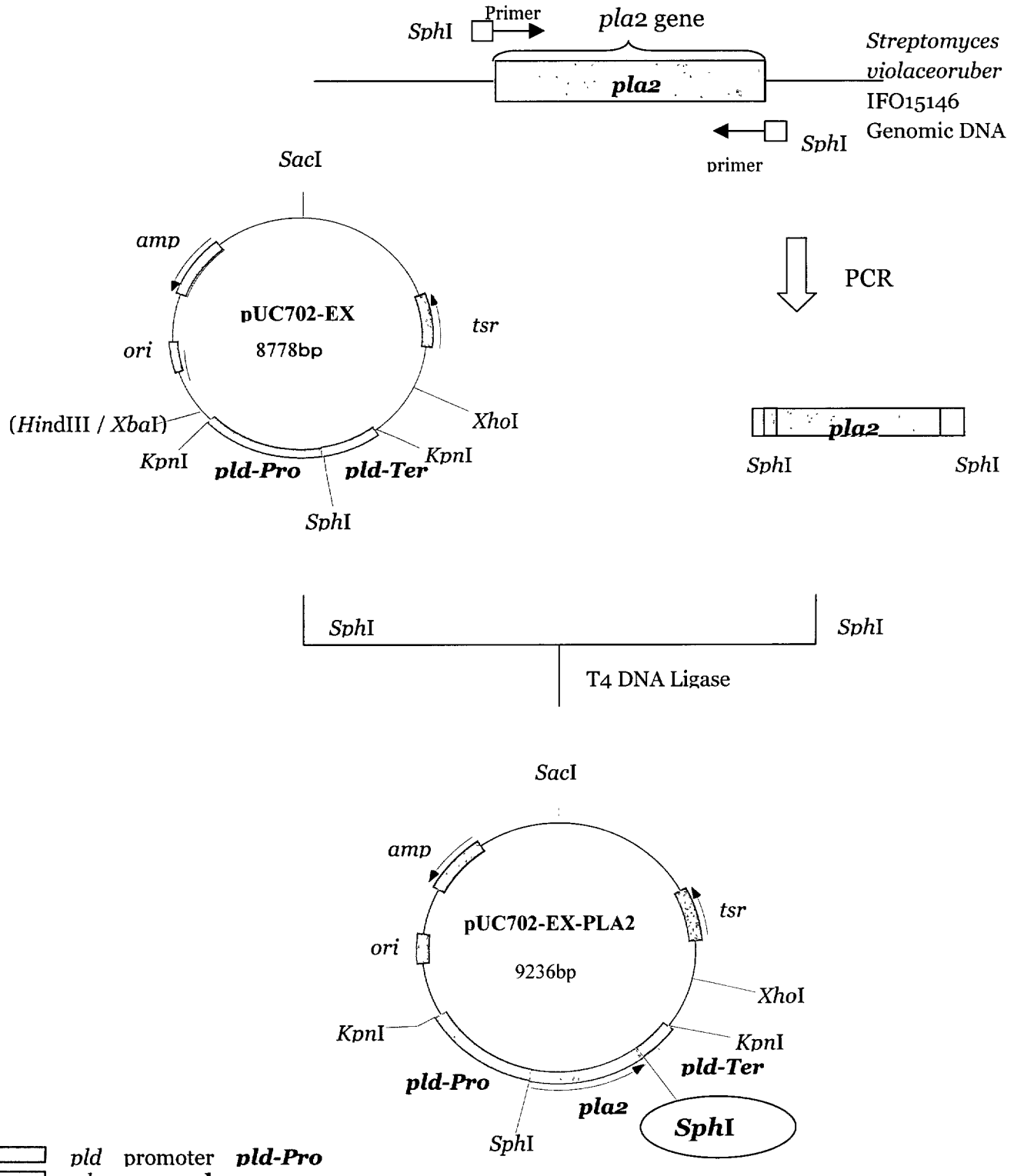
Step1: ***Construction of pUC702-EX, which contains the promoter and terminator of phospholipase D from Streptomyces cinnamoneum***



000019

Nagase ChemteX
1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

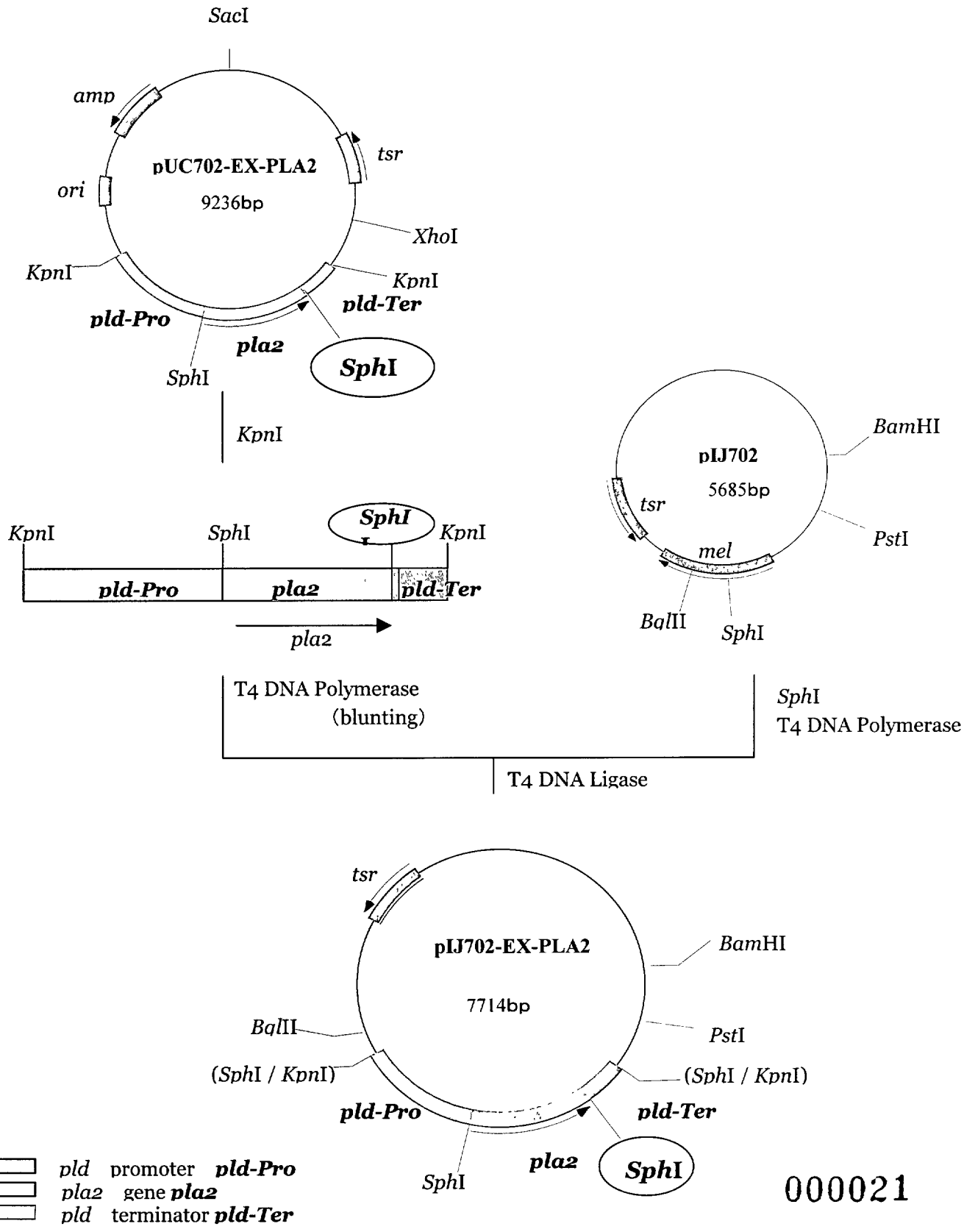
Step 2: ***Insert the pla2 gene into a vector pUC702-EX***



000020

Nagase ChemteX
1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

Step 3: ***Construction of expression plasmid pIJ702-Ex-PLA2***



000021

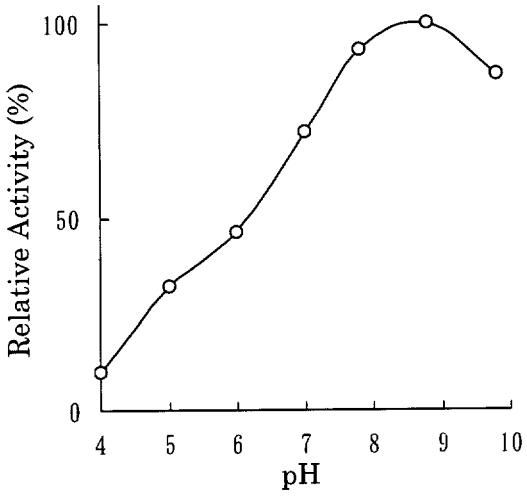
Phospholipids - hydrolytic enzyme

PLA2 NAGASE

PLA2 NAGASE is a purified phospholipase A_2 preparation manufactured from liquid culture of a genetically modified strain of ***Streptomyces violaceoruber***. PLA2 NAGASE is stable in wide pH range and can be used for hydrolysis of phospholipids in food industry.

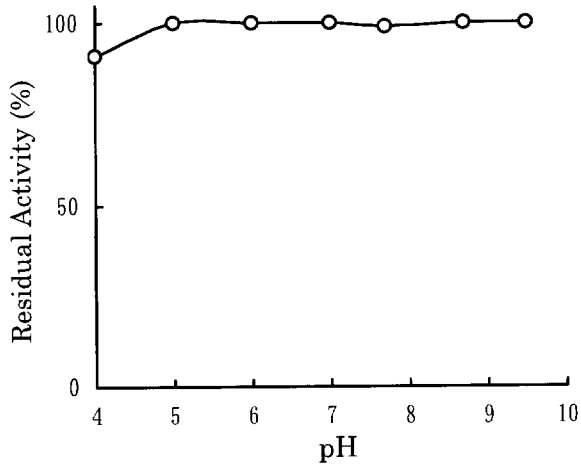
○General Properties of PLA2 NAGASE

(1) pH Activity



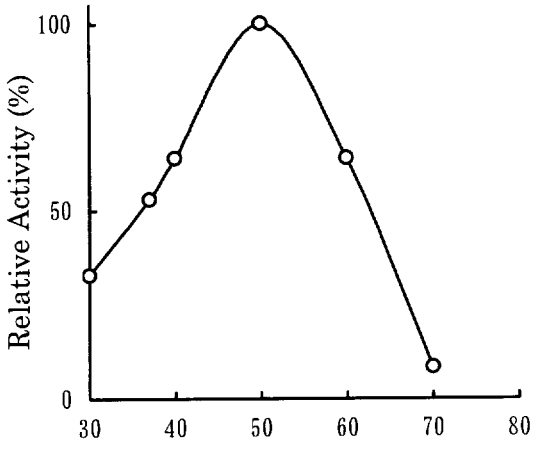
Substrate: 1% soy lecithin

(2) pH Stability



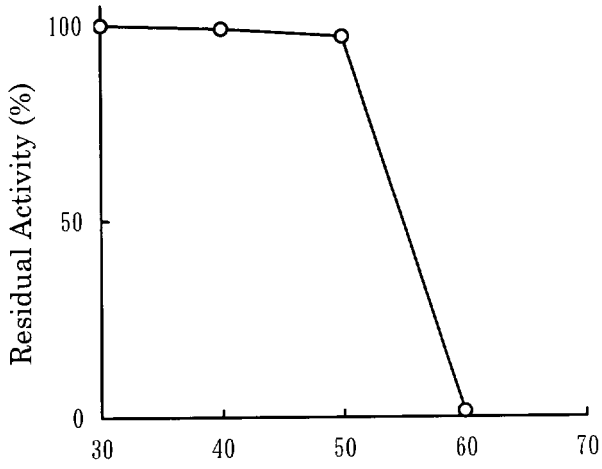
40℃, 30 min-Treatment

(3) Temperature Activity



Temperature (℃)
pH 8.0, 10min-Reaction

(4) Thermal Stability



Temperature (℃)
pH 8.0, 30min-Treatment

000022

○Definition of Activity

1 PLA2UN is defined as the amount of enzyme producing 1 μg of free fatty acid per minute when the enzyme solution is reacted at 37℃ with 1% L-a-phosphatidylcholine solution (pH 8.0, 0.1M Tris-HCl buffer contained 5mM $CaCl_2$).

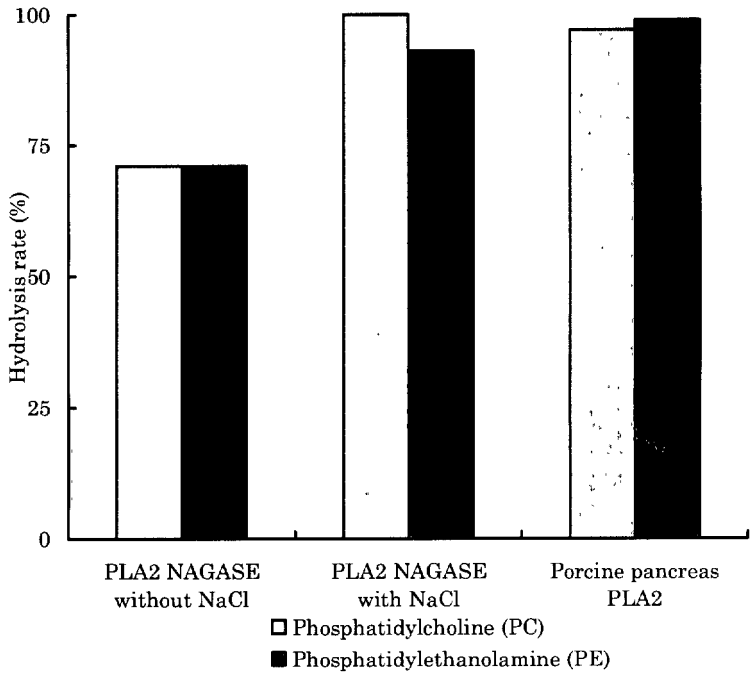
○Grade

PLA2 NAGASE 10,000 – 13,000 PLA2UN/mL (Liquid)

○Applications

Hydrolysis of egg yolk

1) Stir egg yolk well and filter it through gauze.
2) Add 5% water and 10% NaCl and adjust pH to 8.5.
3) Add 0.2% PLA2 NAGASE and react it at 45℃ for 5 – 6 hours.
4) After reaction, heat-treat it at 60℃ for 10 minutes to inactivate the enzyme (for reference only).
   (Conditions for inactivating the enzyme vary with virtual situations)



This information is accurate to the best of our knowledge and experience without warranty. We can not accept responsibility or liability for any damage, loss or patent infringement from the use of this information.

Nagase ChemteX Corporation
1-52, Osadano-cho, Fukuchiyama-shi, Kyoto, 620-0853, Japan
TEL +81-773-27-3495
FAX +81-773-27-2040

M301-0602

Nagase ChemteX
1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

Appendix 3
The total nucleotide and amino acid sequences of PLA_2 enzymes

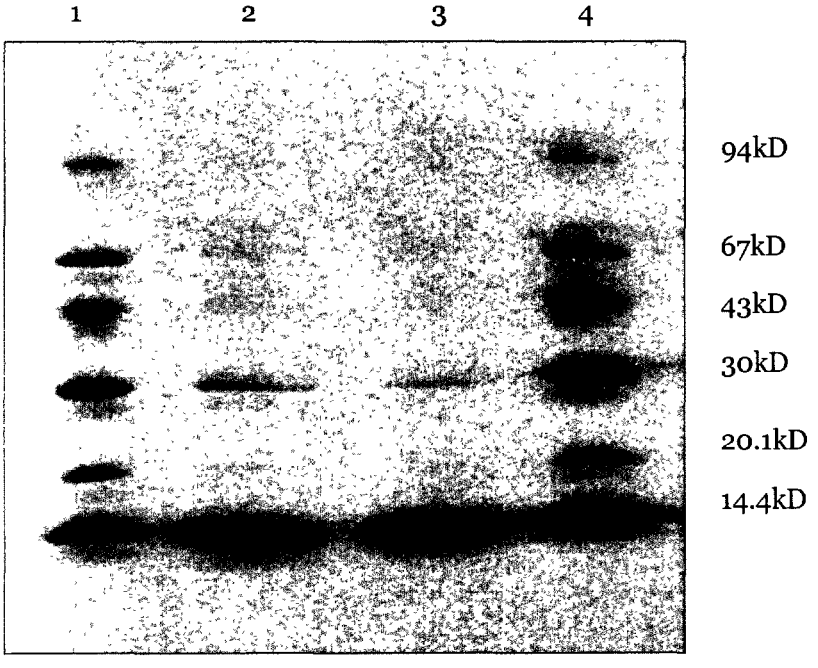
Up: phospholipase A_2 from *Streptomyces violaceoruber* AS-10 (pIJ702-EX-PLA_2)
Down: phospholipase A_2 from *Streptomyces violaceoruber* IFO15146

```
  1' MRTTTRTRTT LAAVGAALAL GVAAAPAQAA PADKPQVLAS FTQTSASSQN AWLAANRNQS
     ********** ********** ********** ********** ********** **********
  1" MRTTTRTRTT LAAVGAALAL GVAAAPAQAA PADKPQVLAS FTQTSASSQN AWLAANRNQS

 61' AWAAYEFDWS TDLCTQAPDN PFGFPFNTAC ARHDFGYRNY KAAGSFDANK SRIDSAFYED
     ********** ********** ********** ********** ********** **********
 61" AWAAYEFDWS TDLCTQAPDN PFGFPFNTAC ARHDFGYRNY KAAGSFDANK SRIDSAFYED

121' MKRVCTGYTG EKNTACNSTA WTYYQAVKIF G
     ********** ********** ********** *
121" MKRVCTGYTG EKNTACNSTA WTYYQAVKIF G
```

Nagase ChemteX
1-1-17, Shinmachi, Nishi-ku, Osaka, 550-8668, Japan
Phone: +81-6-6535-2582 Fax: +81-6-6535-2174

Appendix 4

Protein pattern of the production strain, *Streptomyces violaceoruber* AS-10, compared with that of the donor strain, *Streptomyces violaceoruber* IFO15146 shown in a SDS-PAGE



Lane 1 & 4: molecular weight markers

Lane 2: phospholipase A2 from the donor strain, *Streptomyces violaceoruber* IFO 15146
Concentrated at 4.24mg/ml protein. Loading amount: 4ul

Lane 3: phospholipase A2 from the production strain, *Streptomyces violaceoruber* AS-10
Protein concentrate: 4.77mg/ml protein. Loading amount: 4ul

Both enzyme preparations show a similar protein pattern, with the main protein being the 14kDa Phospholipase A_2.

The sensitivity of the SDS-PAGE is estimated as 5ng-100ng per band. Therefore, the undetected protein can be estimated at less than 5ng-100ng/4ul enzyme preparations, i.e. 5ng-100ng/40U, equivalent to 5ng-10ng/0.04mgTOS. According to section 7, the maximum human exposure to this enzyme preparation is 0.164mgTOS/day/person. Presuming a person's daily food intake is 2kg, the maximum exposure of undetected protein, if any existed, would be (0.164mgTOS÷0.04mg)×10ng÷2kg=41ng/2kg=0.0000205mg/kg=0.0205ppb. This amount is unlikely hazard-driven.

Appendix 5
Safety studies of the enzyme preparation

1. Acute Oral LD_{50} in Rats
   (Bozo Research Center, test No. B-5474, June 9th, 2005)

The enzyme preparation (Batch No.04-3t-01) was given as a single limit test at 2000mg/kg body weight to a group of 10 rats per sex. All animals were observed for mortality and toxicity for 14 days after treatment. There were no mortalities and the oral LD_{50} was determined as >2000mg/kg body weight. Based on the LD value, the enzyme preparation could be classified as non-toxic by ingestion.

2. Reverse Mutation Assay
   (B.M.L Corp., test No. 10057, May 24th, 2005)

The mutagenic potential of the enzyme preparation was assessed by using *Salmonella typhimurium* TA100, TA1535, TA98, TA1537, and *Escherichia coli* WP2 *uvrA*. In this study, the number of revertant colonies after treatment with the test article was less than twice that of the negative control and there was no dose-dependency for either the base-pair substitution type or frameshift type bacterial strains irrespective of the presence or absence of metabolic activation of the test article. Thus, it is judged that the enzyme preparation is not mutagenic under the conditions of this study.

3. Chromosome Aberration Test in CHL/IU cells
   (Bozo Research Center, test No. M-1193, June 13, 2005)

The objective of this study is to examine whether or not the enzyme preparation induces chromosome aberration in cultured mammalian cells (CHL/IU cells). First, a cell-growth inhibition test was conducted and the highest dose concentrations in the chromosome aberration test were determined. In the chromosome aberration test, in both the short-term and continuous for 24 hr and 48 hr treatment, there was neither increase in the incidence of structural chromosome aberrations nor increases in the incidence of polyploid cells, which indicated that the PLA_2 Nagase Concentrate had no chromosome aberration inducibility under the study conditions.

4. 13-week Oral (Gavage) Toxicity study in the Rat
   (Bozo Research center, test No. B-5475, Feb. 22, 2006)

The toxic changes by the repeated administration of PLA2 Nagase Concentrate were examined in rats. It was administered orally by gavage to Sprague-Dawley strain SPF rats at 6 weeks of age for 90 days at dose levels of 0, 40, 200, and 1000 mg/kg/day. No death occurred during the administration period of all doses, and no test article-related changes in clinical signs, detailed clinical signs, body weight, food consumption, manipulative test, grip strength or spontaneous movement. Ophthalmological examination and urinalysis showed no toxic signs. No test article-related effects were observed in hematological or blood chemistry examinations. In pathological examination, hyperplasia of the limiting ridge of stomach was observed in males in the 1000 mg/kg group and females in the 200 mg/kg or higher dose groups. There were gastric mucosa hyperplasia in males and females in the 1000 mg/kg group, globule leukocyte infiltration in females in the 1000 mg/kg group, and mucosal hyperplasia of cecum in males in the 1000 mg/kg group and females in the 200 mg/kg or higher dose groups. Therefore, the NOAEL of PLA2 Nagase Concentrate under the conditions of this study was estimated to be 200 mg/kg/day for males and 40 mg/kg/day for females, equivalent to 50mgTOS/kg/day for males and 10mgTOS/kg/day for females.

LIST OF REFERENCES

1. Pariza, M.W. and Johnson, E.A. (2001). Evaluating the safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. *Reg. Tox. And Pharm.* 33: 173-186.
2. Pariza, M.W. and Foster, E.M. (1983). Determining the Safety of Enzymes Used in Food Processing. *Journal of Food Protection* 46:5:453-468.
3. Enzyme Preparations Used in Food Processing (as compiled by the ETA members).
4. FAO/WHO. Biotechnology and Food Safety, Report of a Joint FAO/WHO Consultation (1996). *FAO Food and Nutrition* Paper 61. Rome. Italy.
5. Organization for Economic Co-operation and Development, Safety Considerations for Biotechnology (1992).
6. Katz E. Thompson C.J. & Hopwood D.A.(1983). Cloning and Expression of the Tyrosinase Gene from *Streptomyces antibioticus* in *Streptomyces lividans*.
7. Kieser T. et al. (1982). *Mol. Gen. Genet.* 185, pp 223-238.
8. Aunstrup, K., Andersen,O., Falch, E.A., and Nielsen, T.K.(1979). *Production of Microbial Enzymes in Microbial Technology*, 2nd ed., Vol.I, Eds. Peppler, H.J. and Periman, D., Chapter 9, pp.282-309.
9. Aunstrup, K. (1979). Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, *Enzyme Technology*, Eds. Wingard, L.B., Katchalski-Katzir, E. And Goldstein, L, pp.28-68.
10. Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms *Regulatory Toxicology and Pharmacology* 12, S114-S128, (1990).
11. Cheeseman, M.A., Machuga, E.J., and Bailey, A.B. (1999). A tiered approach to threshold of regulation. *Fd. Chem. Toxicol.* 37, 387-412.
12. Anne, J.; Van Mellaert, L.; Lammertyn, E.; Joris, B.; Sablon, E.; Van Broekhoven, A.; Eyssen, H. (1995). Factors influencing secretion of heterologous proteins in Streptomyces lividans using mouse tumor necrosis factor alpha as a model protein. *Biotekhnologiya*. Volume 0, Number 7-8, pp55-60.
13. Recent Research Developments in Microbiology (1999), 3(Pt.2), 425-440.
14. "Final Report on the Safety Assessment of Propylene Glycol and Polypropylene Glycols," *J. Am. Coll. Toxicol.* 13(6), 437-491 (1994).
15. Cheeseman, M.A., Machuga, E.J., and Bailey, A.B. (1999). A tiered approach to threshold of regulation. *Fd. Chem. Toxicol.* 37, 387-412.

SUBMISSION END

Merker, Robert I

From: xiaoli.liu@ncx.nagase.co.jp
Sent: Wednesday, February 28, 2007 6:55 PM
To: Merker, Robert I
Cc: Edwards, Alison
Subject: RE: antifoams and DNA
Attachments: file1.xls; file2.xls

Dear Dr. Merker,

We have evaluated the amount of antibiotic *tsr* gene that possibly remains in our product using a more sensitive approach.
The details are described in the attached file1. The outlines and the evaluation results are written below:

1. Procedure
Firstly, we determined the PCR conditions for detecting 1 molecule of the *tsr* gene in the presence of the enzyme product (as positive control).
Secondly, using the same conditions to do PCR on 0.6 ul samples of the enzyme products that are diluted 10 times and 100 times.
Thirdly, the PCR products was run on agar gel for detection. if the corresponding band shows on the gel, there are at least 1 molecule existing in the sample.
If not, less than 1 molecule could possibly exist.

2.Results
The result show negative with 100-fold diluted product, but positive with 10-fold diluted product.

3.Conclutions
From the results, it can be concluded that less than 1 molecule *tsr* gene in 0.6 ul of 100-fold diluted enzyme product might exist, corresponding to 1 molecule
per 0.09U of the enzyme product.

4.Estimation of the daily intake
Based on the following facts, the daily possible intake of the antibiotic gene is estimated:
(1)The mass of 1 molecule *tsr* gene encoded on the plasmid template is calculated to be 3.96×10^{-18} g.
Since the molecular weight of the plasmid template is 2384493.6, which can be calculated from the DNA sequence, the mass of 1 molecule *tsr* gene encoded
on the plasmid template is calculated to be $2384493.6/6.02\times10^{23}$ 3.96×10^{-18}g.
(2)The daily maximum intake of the enzyme product is estimated at 0.06mg TOS, i.e. 60U (Refer to section 7.6)
(3)A rational estimation for food intake at 2000g/day

Then, the possible maximum intake of the antibiotic gene can be estimated at $60U/0.09U\times3.96\times10^{-18}g/2000g$ $1.32\times10^{-18}g/g$ $1.32\times10^{-9}ppb$.
By molecule in the form of the circular plasmid, the daily intake can be estimated at less than 60U/0.09U=667 molecules.

5. Risk evalution
(1) because the amount of the antibiotic gene that could possibly be taken is very trivial, and most of the circular DNA is likely digested by the tract, the possibility
of the antibiotic gene could mobilize into the gut microbial can be estimated extremely low.
(2)Even if there seems a lot of molecules (667) taken, most of these molecules are not in the circular form as plasmid (see attached file 2).

On a worst case estimation, even if these molecules are in circular form, plasmid pIJ702 can only be transfered in very special conditions, with a very low efficiency
(Ref: Rafii F. & Crawford D.L. 1988, Applied and Environmental Microbiology, vol54(6): 1334-1340).

Through the above scientific procedures, we believe it is reasonable to conclude the amount of antibiotic genes left over in the enzyme product is not likely harmful.

Sincerely,
Xiaoli Liu (PhD)
Nagase ChemteX Corporation
Tel: 078-992-0195
Fax:078-992-1050

--Original Message-----
From: Merker, Robert I [mailto:robert.merker@fda.hhs.gov]
Sent: Wednesday, February 14, 2007 2:05 AM
To: li.
Cc: Edwards, Alison
Subject: RE: antifoams and DNA

Dear Dr. Liu:

It has been several months since we have received your initial response (below) and we haven't received a final reply from you with information. Dr. Edwards, our chemistry on this review has provided the following comments and questions that would assist us in completing our response to this notice.

On p. 6 of your GRAS notice, you state that "We calculated the possible amount of the antibiotic gene in the final product of PLA2 Nagase to be less than 2 ng by a southern blotting test (data not shown), based on a worst-case estimation. This amount is absolutely not of any concern."

We had previously noted the following in an email dated November 10, 2006: "On page 6, you note that using Southern blotting you calculated a "possible amount " of the antibiotic resistance gene in the final product of PLA2 Nagase to be less than 2 ng, based on a "worst case estimation." It isn't completely clear what you mean by this, in that neither the amount of product in which the approximate 2 ng occurs nor whether the 2 ng levels represent intact *tsr* DNA or fragments is stated. Further, it is unclear what assumptions were used to calculate a "worst case" estimate. Please provide the amount of the PLA2 Nagase in which you found 2 ng of the *tsr* DNA and calculate the concentration of the *tsr* DNA in the enzyme preparation. Please also clarify if the measured DNA represents the intact, full-length *tsr* gene or includes gene fragments. As an alternative to measuring the *tsr* DNA, you may use an appropriate transformation assay to show that PLA2 Nagase does not contain the transformable *tsr* gene. Please also clarify the assumptions used to prepare the estimate of exposure to antibiotic resistance gene."

In your response, you noted the following: "As you have suggested, we are testing if the transformable tsr gene is present in our product using a different approach."

Do you have the results of this additional testing?

If not, perhaps you could still provide clarification of the original statements made in the GRAS notice. In what amount of product did you measure 2 ng of antibiotic resistance gene? Was the gene actually detected or does 2 ng reflect the detection limit of the assay? If the gene was detected, did the measured DNA represent the intact, full-length *tsr* gene or gene fragments?

We expect that this information is readily available to you and should not require substantial data collection and preparation. If you choose to respond by post we would appreciate a copy of the response either by email (robert.merker@fda.hhs.gov) or fax (301-436-2964) so that we may

proceed with the evaluation of your notice as soon as possible. If you have any questions about our request, or if you believe you will not be able to respond within approximately two weeks, please feel free to contact me either by email or by phone during FDA business hours at 301-436-1226.

Thank you.

Sincerely,

Robert I. Merker, Ph.D.
Food and Drug Administration
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review

Mailing Address:
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740

Phone: 301-436-1226
FAX: 301-436-2964
e-mail: Robert.Merker@fda.hhs.gov

From: xiaoli.liu@ncx.nagase.co.jp [mailto:xiaoli.liu@ncx.nagase.co.jp]
Sent: Tuesday, November 14, 2006 12:02 AM
To: Merker, Robert I
Cc: Edwards, Alison
Subject: RE: antifoams and DNA

Dear Dr. Merker,

We have recieved the following e-mail regarding our GRAS notice No. GRN000212.
As you have suggested, we are testing if the transformable tsr gene is present in our product using a different approach.
Could you kindly give us some time? Is there a deadline for answering your inquiries?

Sincerely,
Xiaoli Liu (PhD)
Product Development Section 1
Enzyme division
Nagase ChemteX Corp.
Tel:078-992-0195
Fax:078-992-1050

-----Original Message-----
From: Merker, Robert I [mailto:robert.merker@fda.hhs.gov]
Sent: Friday, November 10, 2006 5:34 AM
To: li.
Cc: Edwards, Alison
Subject: antifoams and DNA

Dear Dr. Liu:

In its review of Nagase ChemteX's GRAS Notice on phospholipase A2 from *Streptomyces violaceoruber* expressing the gene encoding phospholipase A2 from *S. violaceoruber*, the

Office of Food Additive Safety would appreciate some clarification on a few points.

On page 7 and page 11-12 of your notice, you note the use of polyoxyalkyleneglycol (antifoam) and that you note that with the exception of the antifoam, ingredients are acceptable for general uses in foods in the U.S. On pages 11-12 of the submission you go on to note that the antifoam is used in producing food enzymes in Japan and is considered safe for cosmetics use in the U.S. and you note that the amount of antifoam that would be consumed in foods would be very low. However, the information you have provided on the identity and safety of the antifoam is somewhat vague. Is the antifoam you describe a mixture of polymers? A single polymer? If available, please provide information such as the Chemical Abstracts Service Registry Number and molecular formula for the antifoam you describe. Antifoams that are suitable for use in enzyme manufacture are either GRAS or are regulated as food additives. Is the antifoam you describe in compliance with existing regulations in the U.S.?

On page 6, you note that using Southern blotting you calculated a "possible amount " of the antibiotic resistance gene in the final product of PLA2 Nagase to be less than 2 ng, based on a "worst case estimation." It isn't completely clear what you mean by this, in that neither the amount of product in which the approximate 2 ng occurs nor whether the 2 ng levels represent intact *tsr* DNA or fragments is stated. Further, it is unclear what assumptions were used to calculate a "worst case" estimate. Please provide the amount of the PLA2 Nagase in which you found 2 ng of the *tsr* DNA and calculate the concentration of the *tsr* DNA in the enzyme preparation. Please also clarify if the measured DNA represents the intact, full-length *tsr* gene or includes gene fragments. As an alternative to measuring the *tsr* DNA, you may use an appropriate transformation assay to show that PLA2 Nagase does not contain the transformable *tsr* gene. Please also clarify the assumptions used to prepare the estimate of exposure to antibiotic resistance gene.

Robert Merker, Ph.D. Consumer Safety Officer and Alison Edwards, Ph.D. Chemistry Reviewer

Robert I. Merker, Ph.D.
Food and Drug Administration
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review

Mailing Address:
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740

Phone: 301-436-1226
FAX: 301-436-2964
e-mail: Robert.Merker@fda.hhs.gov

Determination of the possible amount of *tsr* gene in PLA2 Nagase

Method
1.Prepare samples: PLA2 Nagase 10 times diluted &100 times diluted (two sets)
2. Add 1 molecule of plasmid template containing tsr gene to one set of the above samples
3. Perform PCR, (negative control: no samples in 1 & 2 added)
4. Run the PCR products on Agar gels

Procedure
1. PCR *TaKaRa LA Taq* with GC Buffer (TaKaRa)

		□diluted PLA2 Nagase	□ 1 molecule plasmid+diluted PLA2 Nagase(positive control)	Negative Control
	1st PCR			
	TaKaRa LA Taq (5 units/μL)	0.1 μL	0.1 μL	0.1 μL
	2×GC Buffer I	5 μL	5 μL	5 μL
	dNTP mixture (2.5 mM each)	1.6 μL	1.6 μL	1.6 μL
*1	Template(1 molecule)	0 μL	0.6 μL	0 μL
	Primer□final. 2μM)	each0.5 μL	□0.5 μL	□0.5 μL
*2	TSR-F			
*3	TSR-R			
	10 times, 100 times diluted PLA2 Nagase	0.6 μL	0.6 μL	0 μL
	D.W.	1.7 μL	1.1 μL	2.3 μL
	Total	10 μL	10 μL	10 μL

PCR Conditions

94□	5min	
94□	30sec	□□40cycles
55□	30sec	
72□	2min	
72□	5min	
4□	∞	

2nd PCR	
TaKaRa LA Taq (5 units/μL)	0.2 μL
2×GC Buffer I	10 μL
dNTP mixture (2.5 mM each)	3.2 μL
Template(1st PCR products)	1 μL
Primer□final. 2μM)	each1 μL
TSR-F	
TSR-R	
D.W.	3.6 μL
Total	20 μL

PCR Conditions

94□	5min	
94□	30sec	40cycles
55□	30sec	
72□	2min	
72□	5min	
4□	∞	

*1 Template Preparation
1.plasmid extraction

□□)Inoculate *E.* JM109/pUC702 in 5mL LB (50μg/mL Amp) and culture at 37□ overnight
□□)Use QIAPrep Spin Miniprep Kit(□□) (QIAGEN) to extract
□□)Use nano drop to determine the amount

A_{260} = 6.132

□□)Determine the concentration based on sequence information

sequence□9511 b¡ number of bases: A 1720, C 3112, G 3048, T 1630, N 1

$E_{260} = A\times\varepsilon_A+C\times\varepsilon_C+G\times\varepsilon_G+T\times\varepsilon_T+N\times\varepsilon_N$

□□ =1720×15300+3112×7400+3048×11800+1630×9300+1×10950

□□ =100481150

A_{260}/E_{260} = 6.132/100481150

= 61.02 nM

M.W.=A×313.2+C×289.2+G×329.2+T×304.2+N×309.0

=1720×313.2+3112×289.2+3048×329.2+1630×304.2+1×309.0

= 2938251

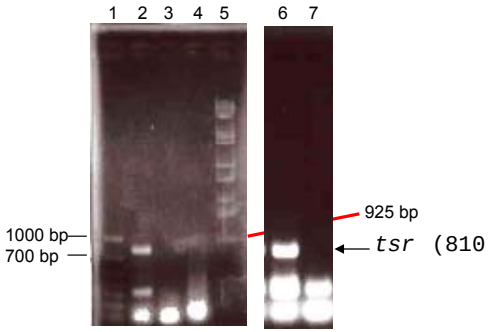
1 molecule = 2938251/6.02×10^{23}
□□□□= 4.88×10^{-18} g

61.02 nM×2938251 = 179.3 g/L
179 g/L/4.88×10^{-18} g/molecule = 3.674×10^{19}molecules/L

□□)Delute to add to PCR with TE buffer until final concentration at 1 molecule

*2□TSR-F: 5'- ATG ACT GAG TTG GAC ACC ATC GCA A -3' □□Invitrogen)
*3□TSR-R: 5'- TTA TCG GTT GGC CGC GAG ATT CCT G-3'□□Invitrogen)

2. Agar gel



Lane1□1000 bp Ladder marker (CAMBREX)□□1000 bp□700 bp□525 bp□500 bp□400 bp□300 bp□200 bp□100 bp□50 bp)
Lane2□100 times diluted PLA2 Nagase with 1 molecule plasmid (positive Control)
Lane3□negative control (no template)
Lane4 & 7□100 times diluted PLA2 Nagase
Lane5□λ□*Eco*T14 I digest □□□□ (19329 bp□7743 bp□6223 bp□4254 bp□3472 bp□2690 bp□1882 bp□1489 bp□925 bp□421 bp□74 bp)
Lane6□10 times diluted PLA2 Nagase

3. Result

with 100-fold diluted PLA2 nagase, no PCR products were detected correspond to *tsr* gene ; However, with 10-fold diluted PLA2 Nagase, PCR products showed positive.

4. Conclusion

In 0.6 ul of 100-fold diluted PLA2 nagase, there is less than 1 molecules of *tsr*.
PLA2 Nagase used has 15000U/ml activity, therefore, 15000U/ml/100×0.6ul×10^{3}ml=0.09U enzyme contains less than 1 molecule *tsr*.
As discussed in section 7.6, the maximum intake of the enzyme can be calculated as 60U, therefore, the daily maximum intake of the *tsr* is:
60/0.09=667 molecules
*4 The mass of 1 molecule of plasmid can be calculated as 3.96×10^{18}
Therefore, the daily intake amount of *tsr* as cirdular plasmid is: 3.96×10^{18}g/molecule×667molecules=2.64×10^{15}g

*4□Determination of the mass of 1 molecule circular plasmid ;pIJ702Ex-PLA2

Sequence:□7714 bp numbers of bases: A 1050, C 2689, G 2751, T 1223, N 1
M.W.=A×313.2+C×289.2+G×329.2+T×304.2+N×309.0
=1050×313.2+2689×289.2+2751×329.2+1223×304.2+1×309.0
= 2384493.6
1 molecule's mass = 2384493.6/6.02×10^{23}=3.96×10^{-18} g

DNA Size distribution in PLA Nagase

Method 1.Purification of DNA from PLA2 Nagase

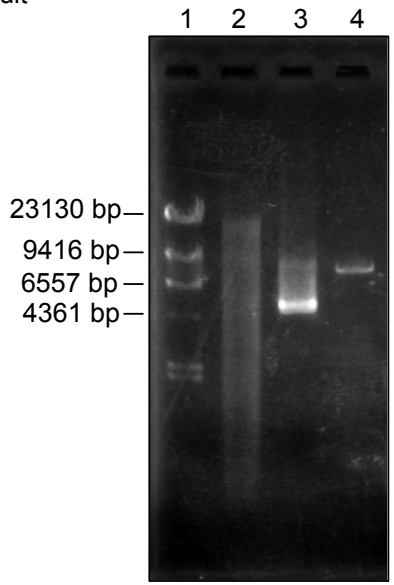
□Use QIAquick Nucleotide Removal Kit (QIAGEN) to purify the DNA from 1 ml PLA2 Nagase (lot.05-50t-01-1)

2.Use nanodrop to determine the amount

□Result: 159.6 ng/μL

3. Run on 0.6□ Agar gel

Result



Lane1□λ□HindIII digest Marker (23130 bp□9416 bp□6557 bp□4361 bp□2322 bp□2027 bp□564 bp□125 bp)

Lane2□ 100 ng DNA purified from PLA2 Nagase

Lane3□ 100 ng pIJ702Ex-PLA2 plasmid

Lane4□ 10 ng pIJ702Ex-PLA2 digested with *Sac* I digest (7714 bp□

Conclusion

Most of the DNA purified fron PLA2 Nagase has a size smaller than the circular plasmid.