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May 7, 2010

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Division of
Biotechnology and
GRAS Notice Review

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U.S. Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Attention:
Dr. Robert L. Martin, Deputy Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Mail code: HFS-255
Building: CPK 2 Room 2045

RE: GRAS Exemption Claim Notification – Polyglutamic Acid

Dear Dr. Martin:

Attached please find three copies of a revised GRAS Exemption Claim Notification for the food ingredient Polyglutamic Acid that has been determined to be Generally Recognized As Safe by scientific procedures. The attached copies of the GRAS Exemption Claim Notification for Polyglutamic Acid have been edited to address suggested revisions discussed in our telephone conversation of last month, including that of a more detailed report of the deliberations of the independent Expert Panel who opined that PGA is GRAS for the intended conditions of use. This communication is a letter of transmittal for that submission.

If there are questions or a need for additional information, please do not hesitate to contact me [phone (202) 457-0285, email burseyb@ajiusa.com].

Personal regards,
(b) (6)

Robert G. Bursey, Ph.D.
President, Ajinomoto Corporate Services, LLC

3 Attachments

GRAS EXEMPTION CLAIM FOR POLYGLUTAMIC ACID (PGA)

Summary of Data Relating to the Safety and GRAS Determination of Polyglutamic Acid for Use as a Food Ingredient

Submitted to:

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

Submitted by:

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GRAS EXMPTION CLAIM FOR POLYGLUTAMIC ACIDS

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VIII-2	Estimated Intakes of Regular and Low Sodium Salt for The US Population – Exponent Report, 2008
IX-1	A Single Oral Dose Toxicity study of PGA in Rats – Nippon Experimental Medical Research Institute, 2001
IX-2	Mutagenicity Study of PGA in *Salmonella Typhimurium* and *Escherichia coli*- Ajinomoto, 1999
IX-3	English Translation of Mutagenicity Test of *Bacillus subtilis* Gum –(PGA) (see Appendix IX-4)
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IX-8	Micronucleus Test with Rodents – Noriyuki Hachiya, 1996 (in Japanese)
IX-9	English Translation of Japanese Publication, "A 90 Day Subchronic Oral Toxicity Study of *Bacillus subtilis* Gum in F344 Rats"(see Appendix IX-11)
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Appendices (cont.)

IX-11	Subchronic Toxicity Study with Rats (90 days) – H. Nakamura et al., 1999 (in Japanese)
IX-12	Safety Study of the PGA-producing Strain (Acute Toxicity/Toxin Productivity) – Kitasato Institute, 2001
IX-13	Identification of the PGA-producing Strain
IX-14	Letter from Dr. Pariza on Safety of the PGA-producing Strain
XI-1	English Abstract of Arerugi (in Japanese), 55, 832, 2006
XI-2	Allergology International, 56, 257, 2007
XI-3	Expert Opinion on Allergenicity Assessment of Polyglutamic Acid – Prof. Steve L. Taylor

GRAS EXMPTION CLAIM FOR POLYGLUTAMIC ACIDS

I. GRAS EXMPTION CLAIM

I.A Claim of Exemption from the Requirement for Premarket Approval as per Proposed 21 CFR 170.326 (c) (1) [62 FR 18938 (17 April, 1997)]

Ajinomoto Corporate Services, LLC (hereafter Ajinomoto) hereby notifies the U.S. Food and Drug Administration that polyglutamic acid has been determined to be Generally Recognized As Safe (GRAS) consistent with the requirements of the Federal Food, Drug and Cosmetic Act. The determination of GRAS for the use of polyglutamic acid defined in this dossier was made through scientific procedures described herein by a panel of experts qualified by their scientific training and experience to do so. The use of polyglutamic acid defined below is therefore exempt from the requirement of premarket approval.

Signed,

(b) (6)

2/19/10

Robert G. Bursey, Ph.D.
President
Ajinomoto Corporate Services, LLC

Date

I.B Name and Address of Notifier

Robert G. Bursey, Ph.D.
President

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I.C Common Name of Notified Substance

The common name of the substance which is the subject of this GRAS Notice is polyglutamic acid (PGA). PGA exists naturally in food and has a long history of consumption as a component of the traditional Japanese food 'natto', soybeans fermented with the microorganisum *Bacillus subtilis*.

I.D Intended Use and Consumer Exposure

Ajinomoto intends to market polyglutamic acid as a food ingredient in the U. S. for use as a 'debittering' agent in salt substitute products containing potassium chloride. PGA will be used in these salt substitutes at an inclusion rate of 0.15 – 0.4 % by weight (of the salt product). The basis of these use levels in shown in Section VIII, Table VIIIA on page 17 of this dossier. Use levels of PGA for the function which is the subject of this GRAS Notice is somewhat self-limiting, in that concentrations of the substance at 0.4 % or greater of a salt substitute create a slight sour taste in the salt substitute product.

The estimated mean consumption of total 'added salt' to the diet of Americans is 7.06 g/day per user and 7.01 g/day per capita, while the 90th percentile of 'added salt' intake is 11.6 g/day per user and 11.5 g/day per capita. At these levels of 'added salt' intake and a PGA use level of 0.4 % in all 'added salt' products, the mean and 90th percentile of PGA consumption would be 28.0 mg/day and 46.0 mg/day respectively (see VIII2 for details).

I.E Basis for GRAS Determination

As per the eligibility criteria described in 21 CFR 170.30 (and 21 CFR 170.35), polyglutamic acid (PGA) has been determined by Ajinomoto to be Generally Recognized AS Safe (GRAS) through the use of scientific procedures. This determination is supported by the opinions of highly reputed experts who are qualified by their scientific training and professional experience to assess the safety of PGA for the food use defined in this Notice. A copy of the fully endorsed 'Statement of the Expert Panel' is included on page 32 of this dossier.

This Expert Panel of independent scientists, at the request of Ajinomoto, after having reviewed and evaluated a dossier of data and information similar to that in this Notice, convened on April 7, 2009 to perform a collective critical assessment of whether the intended use of PGA as a food

ingredient was safe. The Panelists individually and collectively unanimously opined that PGA would be Generally Recognized As Safe (GRAS) based of scientific procedures. They subsequently endorsed a written statement to that effect (see attached statement of the Expert Panel).

The scientific experts on the Panel were Joseph F. Borzelleca, Ph.D. (Emeritus Professor – Virginia Commonwealth University), Michael W. Pariza, Ph.D. (Professor - University of Wisconsin), Steve L. Taylor, Ph.D. (Professor – University of Nebraska) and John A. Thomas, Ph.D. (Professor – University of Indiana Medical School). It is also the opinion of the Expert Panel that other competent scientist with knowledge and expertise in relevant scientific disciplines evaluating the same safety and toxicology data and information would arrive a the same conclusion.

Through the use of scientific procedures polyglutamic acid has been demonstrated to be a GRAS food ingredient for its intended uses. It therefore may be marketed and sold for its intended uses in the U.S. without the promulgation of a food additive regulation.

I.F Availability of Information

The detailed data and information that serve as a basis for this GRAS determination will be provided to the U.S Food and Drug Administration (FDA) upon request, or are available for review and copying during business hours a the office of:

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I.G Background Information

Salt a chemical consisting of sodium and chloride, is essential for the maintenance of a health, and appropriate amounts of salt enhance the taste of many foods and dishes as well. But too much sodium can be harmful, just like many other dietary components.

Excessive intake of sodium is not only a primary cause of hypertension in some individuals, which leads to cardiovascular disease such as strokes, heart attacks, heart failure etc., but also increased renal disease and risk of bone demineralization. A reduction in the intake of sodium by Americans is, and has been a major public goal in the U S.

The World Health Organization, has set a worldwide target of reducing salt intake to 5 g/day or less for all adults (WHO 2003), while actual salt intake in most developed countries remains 9- 12 g/day Efforts to reduce salt intake are made by individuals as well as food manufacturers, by using smaller amounts of salt and/or by using salt substitutes. In either way, taste is usually an issue and it appears difficult to continue a low sodium diet for a long time.

Potassium chloride is commonly used in salt substitute and is used in the form of a mixture with sodium chloride or by itself. Potassium chloride has a salty taste itself, but its metallic bitter aftertaste is a problem.

Polyglutamic acid offers a solution for making palatable low-sodium salt substitutes with potassium chloride by mitigating the bitter taste of potassium chloride in such products, whether for table top, food preparation or food production applications.

PGA is a polymer of many molecules of D- and L-Glutamic acid which are combined by γ-linkages PGA naturally exists in the traditional Japanese food natto (fermented soybeans) fermented with *Bacillus natto*. *Bacillus natto* is an idiomatically used name, and is officially classified as *Bacillus subtilis*.

Bacillus subtilis is an aerobic endospore-forming bacterium commonly found in nature and generally not considered to have a pathogenic or toxigenic potential. There is a history of safe use of this bacterium in large-scale fermentation production of specialty chemicals, of enzymes used in food production process,

and of several traditional relationships to food. It is used traditionally in some East Asian countries for the fermentive production of natto, a product also obtainable in western countries It is thus an organism with a tradition in food use (See Appendix 1-1)

PGA is a main component of natto mucilage (viscous substance) produced by *Bacillus natto*. PGA is registered as a food additive ("*Bacillus natto Gum*" ("納豆菌ガム")) in Japan. Ajinomoto PGA is produced with liquid fermentation by *Bacillus subtilis*.

II. DESCRIPTION OF SUBSTANCE

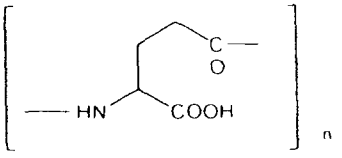
II.A Common or Usual Name

Polyglutamate, Polyglutamic acid, Poly-γ-Glutamate, Poly-γ-Glutamic acid

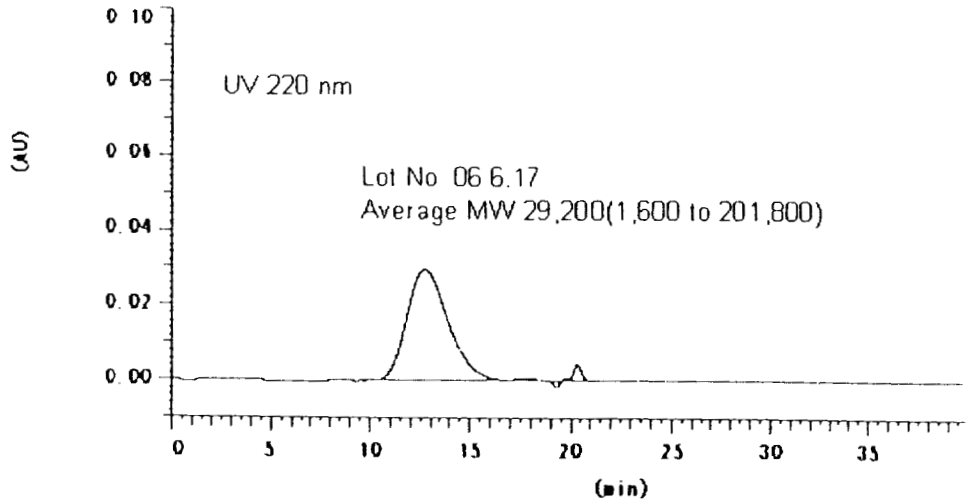
II.B Empirical Formula

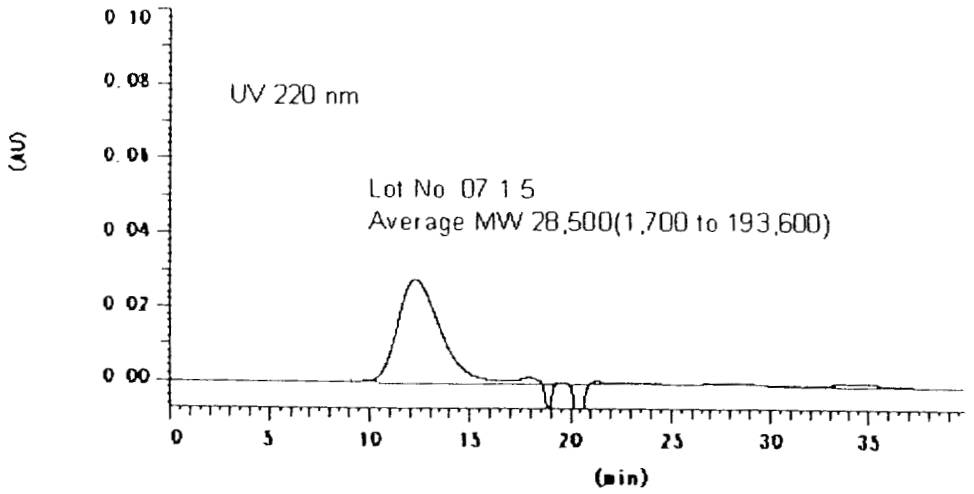
$[C_5H_7NO_3]_n$

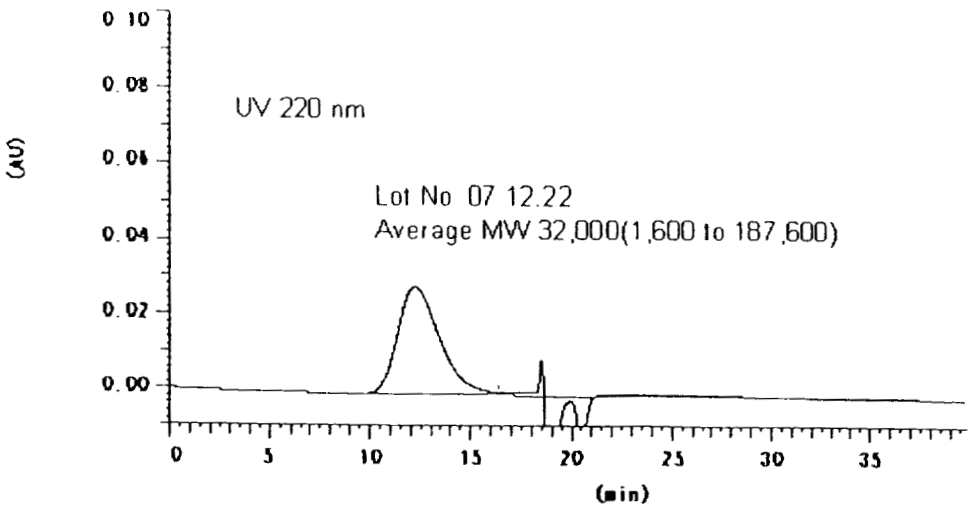
II.C Structural Formula



II.D Molecular Weight Distribution of Three Different Lots of Polyglutamic Acid







II.E-1 Product Specification and Analytical Methods for Polyglutamic Acid

Parameters	Acceptance criteria	Analytical method (See Appendix II-1)
Description (appearance)	White to yellowish fine powder, odorless or slightly characteristic	Observation
Identification Ninhydrin	Passed test	Ninhydrin Reaction
Assay (as Glu)	NLT 75.0 % w/w (dry)	Amino Acid Analysis, equivalent to glutamic acid test, FCC VI
Arsenic (as As_2O_3)	NMT 4.0 μg/g	Arsenic Limit Test, JSFA VIII Method 1, Apparatus B, equivalent to FCC VI
Lead (as Pb)	NMT 1 μg/g	Atomic Absorption Spectrophotometry
Loss on Drying	NMT 15.0 % w/w	Loss on Drying Test, JSFA VIII, equivalent to FCC VI
Residue on Ignition	NMT 43.0 % w/w	Residue on Ignition Test, JP XV, equal to USP XXXI
Microbiological Parameters		Microbial Limit Test, Standard Methods of Analysis in Food Safety Regulation (Biology)
Standard Plate Count (cfu/g)	NMT 10,000 cfu/g	Equivalent to FDA-BAM
Coliforms	Negative	FDA-BAM

FCC VI= Food Chemical Codex, 6th Edition
JP XV= The Japanese Pharmacopeia, 15th Edition
USP XXI= The United States Pharmacopeia, 31st Edition
FDA-BAM= FDA's Bacteriological Analytical Methods
JSFA VIII = The Japan's Specifications and Standards for Food Additives
NLT = Not Less Than
NMT = Not More Than

Storage Conditions: Store in well-closed, light-resistant containers and keep at ambient temperature between 15°C and 30°C

Shelf Life: 72 months after manufacturing date

000016

Table II E Analytical Results of Actual Production Batches (5 Lots) vs Specifications

Test item	Acceptance Criteria	Lot No.				
		0.7.4.28	07.5.10	08.5.24	08.6.8	09.3.29
Description (appearance)	White to yellowish fine powder, odorless or slightly characteristic	C	C	C	C	C
Identification Ninhydring	passed test	C	C	C	C	C
Assay (as Glu) (%)	NLT 75.0 % w/w(dry)	80 0	79.2	80.3	81.6	81.6
Arsenic (as As2O3) (µg/g)	NMT 4.0 µg/g	< 4.0	< 4.0	< 4.0	< 4 0	< 4.0
Lead (as Pb) (µg/g)	NMT 1 µg/g	0 09	0 08	0.07	0 06	0.07
Loss on Drying (%)	NMT 15.0 % w/w	2 8	3.2	3.1	2 9	2.2
Residue on Ignition (%)	NMT 43.0 % w/w	22.0	21.6	22.6	21 4	21.4
Microbiological Standard Plate Count (cfu/g)	NMT 10,000 cfu/g	0	0	0	0	0
Coliforms	negative	negative	negative	negative	negative	negative

C: conforms

II.F Stability of Food Grade Polyglutamic Acid Under Various Storage Temperatures

Samples of manufactured Polyglutamic acid were stored at 5°, 24°, 34°, 44° centigrade for up to 12 months. The samples were evaluated for their description (appearance), moisture content (%) and molecular weight (MW) at 0, 1, 2, 3, 6, 9 and 12 months. No changes indicative of product degradation

were seen in any of the evaluative criteria under any of the storage conditions at any time period up to 12 months.

Table II F Stability Test of Food Grade Polyglutamic Acid

Parameter	Time (months)						
	0	1	2	3	6	9	12
Storage Temperature 5°C							
Description*	C	C	C	C	C	C	C
Moisture %	2.5	2.2	2 3	2.6	2.5	2.5	2.4
Molecular weight	29,000	30,000	31,000	29,000	30,000	30,000	30,000
Storage Temperature 24°C							
Description*	C	C	C	C	C	C	C
Moisture %	2.5	2.2	1 9	2.5	2.3	2.5	2.2
Molecular weight	29,000	29,000	31,000	29,000	31,000	30,000	30,000
Storage Temperature 34°C							
Description*	C	C	C	C	C	C	C
Moisture %	2.5	2.5	2.3	2.5	2.3	2.6	2.5
Molecular weight	29,000	29,000	29,000	29,000	30,000	30,000	29,000
Storage Temperature 44°C							
Description*	C	C	C	C	C	C	C
Moisture %	2.5	2.4	1.9	2.7	2.6	2.4	2.8
Molecular weight	29,000	28,000	30,000	28,000	30,000	29,000	27,000

* Description= appearance

C = conforms

III METHOD OF MANUFACTURE

III.A Overview

Polyglutamic acid is produced by fermentation employing the microorganism *Bacillus subtilis* No foreign DNA has been intentionally introduced into the production strain of the microorganism Confirmation of the identity of the producer strain (*Bacillus subtilis*) was obtained by DNA-DNA hybridization analysis (See Section III.E and Appendix IX-13). The safety of the producer organism was affirmed by literature citation (Appendix I-1), by expert assessment (Appendix IX-14) and by laboratory evaluation (Appendix IX-12).

The fermentation process is characterized in Figure III-A Frozen aliquots of the producer organism are acclimatized to the production fermentation media and conditions for some 15 – 30 hours before being introduced to the main production fermented The PGA production fermentation runs for approximately 72 – 130 hours before the PGA – containing broth is then acid hydrolyzed, decolorized, and filtered multiple times (See Figure III-B). The liquid containing the purified PGA is then concentrated and heat sterilized. After further filtration, the concentrated, purified PGA is again sterilized and dried to a powder by spray drying.

All raw materials used in of the fermentation were food grade and widely used in food processing, as were substances used in the purification process (See Table III A). The list of substances and relative amounts of raw materials used in the fermentation media are shown in Table III B.

Table III A **Ingredients used in Fermentation Broth and Purification Process in the Production of Polyglutamic Acid**

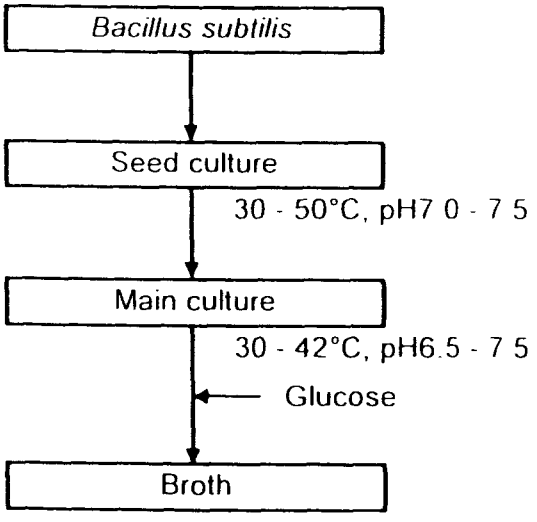
Production process	Raw material	CAS No.	Status	21CFR No.
Fermentation process	Dextrose (Glucose)	50-99-7	GRAS	184.1857
	L-Glutamic acid	56-86-0	Food Additive	172.320
	Hydrolyzed Soy Protein (HSP)	-	-	-
	Phosphoric acid (H_3PO4)	7664-38-2	GRAS	182 1073
	Potassium hydroxide (KOH)	1310-58-3	GRAS	184 1631
	Sodium hydroxide (NaOH)	1310-73-2	GRAS	184 1763
	Ammonia (NH_3)	7664-41-7	GRAS*1	184 1139
	Manganese sulfate ($MnSO_4$)	10034-96-5	GRAS	184 1461
	Magnesium sulfate ($MgSO_4$)	10034-99-8	GRAS	184 1443
	Ferrous sulfate ($FeSO_4$)	7782-63-0	GRAS	184.1315
	Ammonium sulfate ($(NH_4)_2SO_4$)	7783-20-2	GRAS	184 1143
	Antifoam. Polyoxyethylene-polyoxypropylene-glycerylether	9082-00-2	-*2	
Purification process	Hydrochloric acid (HCl)	7647-01-0	GRAS	182 1057
	Sodium chloride (NaCl)	7647-14-5	GRAS	182.1
	Activated carbon	64365-11-3	Food Additive	177.1210
	Diatomaceous earth	61790-53-2	Food Additive	178.3297

*1: as Ammonium hydroxide
*2: Safety assessment of the antifoam is described in Appendix III-1.
See Appendix III-2 for report on safety use of the antifoam by Dr. T. Adams

Table III B **Components/Composition of Fermentation Media/Broth Used to Produce Polyglutamic Acid**

Raw material	Seed culture		Main culture	
Glucose	2.0-4.0	g/dl	3.0-5.0	g/dl
L-Glutamic acid	0.0-1.0	g/dl	4 0-6.0	g/dl
HSP (Total-N	0.0 -0.2	g/dl	0.10-0.15	g/dl
H_3PO4	0.20-0.30	g/dl	0.20-0.30	g/dl
KOH	0.10-0.20	g/dl	0.10-0.20	g/dl
NaOH	0.30-0.40	g/dl	0 20-0.30	g/dl
$MnSO_4$	0.00-0 02	g/dl	0.00-0 02	g/dl
$MgSO_4$	0.03-0.04	g/dl	0.03-0.04	g/dl
$FeSO_4$	0.001-.01	g/dl	0.001-0.01	g/dl
$(NH_4)_2SO_4$	0.3-0.5	g/dl	0 3-0.5	g/dl
Antifoam	0.02-0.03	ml/dl	0.004-.007	ml/dl

Figure III A **Fermentation Process Used to Produce Polyglutamic Acid**



III.C Purification Process

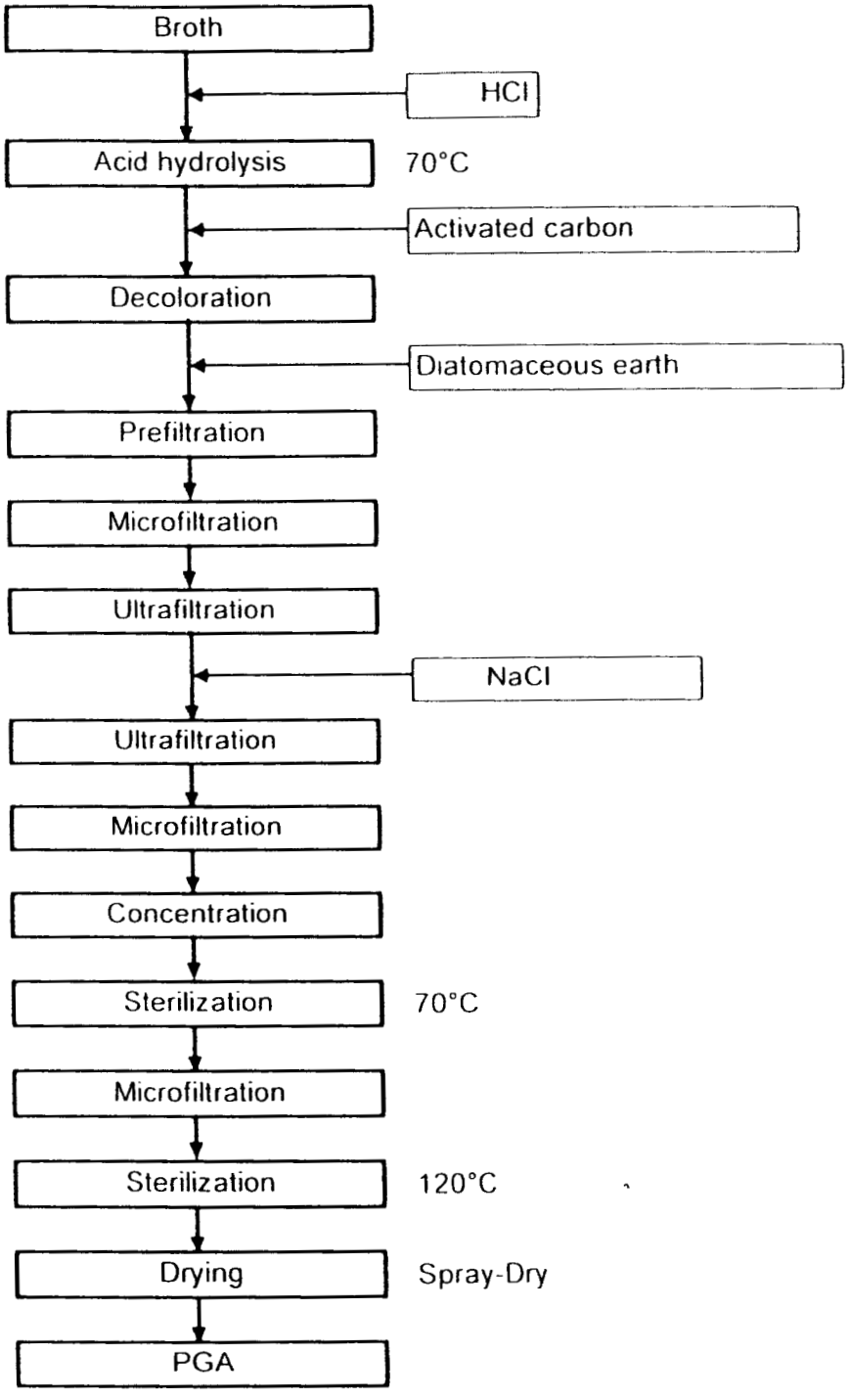


Figure III B The Fermentation, Purification, Sterilization and Drying Processes in the Production of Polyglutamic Acid

IV COMPOSITION OF PGA FINISHED PRODUCT

Table IV-A presents some analytical data on the composition of five different production batches of PGA product. The small batch to batch variance in the quantities of the different parameters measured indicates the production process is under control and capable of yield a consistent product.

Table IV-A Composition of Five Lots of Actual PGA Product

Test item	Lot No.				
	07 4 28	07.5.10	08.5.24	08.6 8	09.3 29
PGA as Glu (%)	76.3	76.1	76.8	77 0	76.1
Moisture (%)	4.7	4.5	4.1	4.5	4.2
Ash (%) *1	16 9	16.9	17.0	17.0	17.0
Total Sugars (%)	0.3	0.3	0.3	0.2	0.2
Acetic Acid (%)	0.02	0.02	0.02	0.02	0.03
Ammonium (%)	2.87	2.75	2.75	2.76	2.68
Lipid (%)*2	N D.	N.D.	N.D.	N.D	N.D.
SUM (%)	101.1	100.6	101.0	101.5	100.2

*1: Ash contains sodium 6.22%, chloride 1.94%, sulfate 0.55%, calcium 0.30%, and potassium 0.25%, and probably contains other minerals, oxide and carbonate, etc.

*2: Limit of detection is 0 1%

N.D. = not detected

See Appendix IV-1 for the analytical methods.

V DISCOVERY AND HISTORY OF USE

V. A Discovery

PGA is a main component of mucilage (viscous substances) of the traditional Japanese food, natto. Natto is made by fermenting soybeans with the microorganism *Bacillus subtilis*. The amount of PGA in natto is about 300 mg/100 g

V. B History of Use

PGA has long history of safe use (over 1,000 years) in Japan through the consumption of natto Natto production (consumption) in Japan is approximately 250 Kt per year Thus, about 750 t of PGA is consumed in Japan each year.

Natto has been also produced and sold in the US for some time Natto is listed in the 1963 Revision of "USDA Handbook No. 8: Composition of Foods". A natto manufacturing company "Yonai Natto Mfg Co." was listed in 1941 edition of "The Japanese American Directory".

Soybeans fermented with *Bacillus subtilis* is traditionally eaten in several areas of Asia as well, eg. Kinema in Nepal, Thua Nao in Thailand, and Chongkukjang in Korea. They also contain PGA made with *Bacillus subtilis*.

PGA is currently registered as a food additive (*Bacillus natto gum*) in Japan. Ajinomoto has been producing and selling PGA made by liquid fermentation employing *Bacillus subilis* since 2001. This product is used as an ingredient in calcium supplements, low-sodium table salt, protein powders, barbecue sauces and several other products. Cumulative sales of Ajinomoto produced PGA were approximately 29 tons during the seven year period from 2001 – 2007

Table V B Ajinomoto PGA sales (t)

fiscal year	2001	2002	2003	2004	2005	2006	2007
t (per year)	1	3	2	2	9	6	6
t (Cumulative)	1	4	6	8	17	23	29

VI CURRENT REGULATORY STATUS

PGA is currently registered as food additive ("*Bacillus natto gum* " 納豆菌ガム")) in Japan.

PGA is being used as an ingredient in calcium supplements, low-sodium table salt, protein powders, barbecue sauces, and several food products.

VII PRODUCTION AND USE

Ajinomoto PGA is produced with liquid fermentation by *Bacillus subtilis*. PGA functions to mask the bitter taste of potassium chloride, thus low-sodium salt preparations with potassium chloride can be made palatable by using PGA

VIII PROPOSED CONDITIONS OF USE

VIII.A Food Categories and Intended Usage Levels

A-1. Food Categories

Ajinomoto intends to market PGA in the United States in the food category characterized as "Added Salt". This category includes salt used for table top salt as well as in food preparation for both commercial and home use.

A-2. Use Levels

Potassium chloride is a common constituent of salt replacers (i.e. low sodium salt products). Due to its undesirable bitter and metallic taste, a typical salt substitute containing potassium chloride is a mixture of sodium chloride in a ratio of up to one to one. As this common type of salt substitute has saltiness similar to that of sodium chloride (See Appendix VIII-1), the mixture may be used in lieu of regular salt. In order to mask the undesirable bitter metallic taste of potassium chloride, an inclusion rate of 0.15-0.4 % PGA by weight is used. If the ratio of potassium chloride to sodium chloride is changed from 50 %, the recommended inclusion rate of PGA is 0.3-0.88 % of the potassium chloride.

PGA not only functions to mask the bitter, metallic and aftertaste of potassium chloride, it also may contribute other taste attributes such as sourness, etc. These other taste attributes of PGA act to self-limit the levels of use in 'added salt' applications. Therefore, it is necessary to examine the suitable inclusion rate sufficient to mask the undesirable bitterness but not produce other taste attributes which regular salt does not have.

A-3. Study to Determine PGA Use Levels

Studies were undertaken to determine the range and optimal use level of PGA in salt substitutes containing sodium chloride and potassium chloride. A salt mixture containing sodium chloride and potassium chloride in a ratio of 1:1 (by weight) was prepared. This mixture was felt to contain the highest concentration of potassium chloride (hence, the greatest amount of PGA) that would be used in a salt substitute PGA was added to the sodium chloride; potassium chloride mixture (ratio 1:1) at levels ranging from 0 % to 0.4% in increments of 0.05 %. Thus, PGA was tested at concentrations of 0 %, 0.05%, 0.10 %, 0.15 %, 0.20 %, 0.25 %, 0.30 %, 0.35 % and 0.40 %. A 1.0 % aqueous solution of each of the nine sodium chloride: potassium chloride. PGA mixtures was prepared and evaluated for salty and bitter (metallic) taste by a panel of tasters. The salty taste was compard to that provided by a 1.0% sodium chloride solution.

Table VIII A **Saltiness and Bitterness of Sodium Chloride:Potassium Chloride (1:1) Solutions Containing 0 – 0.4 % PGA**

PGA inclusion rate**										
	Cont.	0.05 %	0.10 %	0.15%	0.20 %	0.25 %	0.30 %	0.35 %	0.40%	Target
Inclusion rate in 1 % Aqueous Solution (%)***										
PGA	0	0.0005	0.0010	0.0015	0.0020	0.0025	0.0030	0.0035	0.0040	-
NaCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.0
KCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0
Saltiness	++	++	++	++	++	++	++	++	++	+++
Bitterness	+++	+++	++	++	++	++	++	++	++	
Others									1	

*1) Slight sour taste was perceived.
** PGA addition to sodium chloride : potassium chloride mixture (1 : 1)
*** Concentration of PGA in 1 % solution of sodium chloride : potassium chloride (1 : 1) mixture
++ Degree of saltiness or bitterness

The results of the taste test to evaluate the range and optimal use level of PGA in potassium-containing salt substitutes indicated that masking of the bitter metallic taste occurred at concentrations as low as 0.15 % (of PGA). At levels of PGA addition to the potassium-containing salt substitute of 0.40 % a slight, but detectable, sour (and other 'off' tastes) taste was reported. Earlier studies had indicated that inclusion

rates of PGA of more than 0.40 % produced culture broth flavor and other 'off' flavors. It was concluded that the appropriate inclusion rate of PGA in potassium-containing salt substitutes is between 0.15 % and 0.35 %. The data also indicate that use levels of PGA in potassium-containing salt substitutes are somewhat self-limiting with concentrations exceeding 0 35% producing undesirable 'off" flavors.

VIII B Estimated Consumption of PGA

The estimated consumption of PGA was determined using the assumption that all added salt (low-sodium salt + regular salt) is replaced with a low-sodium salt with 50 % potassium chloride concentration and with the maximum PGA inclusion rate of 0.4 % was used.

The estimated mean consumption for the US population of total "Added Salt" (total of low-sodium salt and regular salt) is **7.06 g/day per user** and **7.01 g/day per capita** and **11.6 g/day per user** and **11.5 g/day per capita** for the 90th percentile of consumption (See Appendix VIII-2, Table 9A). Assuming a 0.4% use level of PGA in 'Added Salt' the estimated consumption of PGA (salt intake x 0.4 %) for total "Added Salt" (mg/day) is then **28.24 mg/day per user (7.06 g/day x 0.4 %)** for the mean and **46.4 mg/day per user (11.6 g/day x 0.4 %)** for the 90th percentile. On the per capita basis, the estimated consumption of PGA (mg/day) is **28.04 mg/day per capita (7.01 g/day x 0.4%)** for the mean and **46.0 mg/day per capita (11.5 g/day x 0.4%)** for the 90th percentile of "Added Salt" users.

The above consumption figures are converted to the kg body-weight (bw) basis by assuming an average weight for the US population is 60 kg per person. The estimated consumption of total "Added Salt" (total of low-sodium salt and regular salt) by the US population is **0.12 g/kg bw/day per user** and **0.12 g/kg bw/day per capita** for the mean, and **0.23 g/kg bw/day per user** and **0.23 g/kg bw/day per capita** for the 90th percentile uses (See Appendix VIII-2, Table 9B). The estimated potential total consumption of PGA (salt x 0.4 %) from total "Added Salt" (mg/kg bw/day) is then **0.48 mg/kg bw/day per user (0.12 g/kg bw/day x 0.4 %)** for the mean and **0.92 mg/kg bw/day per user (0.23 g/kg bw/day x 0.4%)** for the 90th percentile. On the per capita basis, the estimated maximum potential consumption of PGA (mg/kg bw/day) for uses indicated in this Notification would be **0.48 mg/kg bw/day per capita (0.12 g/kg bw/day x 0.4%)** for the mean and **0.92 mg/kg bw/day per capita (0.23 g/kg bw/day x 0.4 %)** for the

90th percentile (See Appendix VIII-2, report from the Exponent, Inc. for the basis for regular and low sodium salt consumption)

IX SAFETY AND TOXICOLOGY STUDIES

IX A Introduction

PGA has a long history of consumption as a food ingredient in the fermented soybean product, natto To reconfirm the safety of PGA as a food additive, which had been approved in Japan, several key toxicology studies (mutagenicity and 90 day subchronic) were undertaken in 1996 as a national research project by the Japanese Ministry of Health, Labor and Welfare. The Subcommittee on Food Additives of the Food Sanitation Committee of the Ministry of Health, Labor and Welfare in June 2004 concluded that no additional toxicology studies of PGA were needed

The determination that PGA is GRAS is based upon scientific procedures. PGA is produced by fermentation employing the *Bacillus subtilis* organism that has a long history of safe use in foods. All of the raw materials used in the fermentation and purification of PGA are food grade and/or have been determined to be safe. The identification of the producing organism, *Bacillus subtilis,* was confirmed by analysis by DNA-DNA hybridization (Appendix IX-13) and its safety affirmed by reference from the scientific literature (Appendix I-1), by laboratory testing (Appendix IX-12) and expert assessment (Appendix IX-14).

The safety of PGA is clearly suggested by a long history (hundreds of years) of human consumption as a component of natto (a soybean product fermented with *Bacillus subtilis*) and a decade of ingestion as a government approved food ingredient in Japan. In addition, its mutagenicity was evaluated in two independent reverse mutation tests, a chromosome aberration test and a micronucleus test, all of which demonstrated the absence of mutagenicity of PGA. One of these reverse mutation assays and the chromosome aberration and micronucleus tests were conducted by the Japanese government's Ministry of Health, Labor and Welfare.

In addition, an acute toxicity study and a 90 day subchronic study (conducted and published by the Japanese National Institute of Health Sciences) were conducted with doses of PGA as high as 5.0 % of the diet showing no apparent biochemical, macroscopic or microscopic signs of toxicity. Pharmacokinetic-type studies were

performed in rats to access the digestibility of PGA. Orally administered solutions of PGA delivering 0.5 g/kg bw or 0.8 g/kg bw were only 30 % or less digestible. The digestibility of PGA fed as 2 % of the diet for 18 days proved to be substantially more digestible (45 % - 90 %) although the variability between individual animals was markedly higher. However, the difference in digestibility (about 50 %) between animals was almost non-existent in germ-free rats fed 1 % PGA.

Finally, the potential allergenicity of PGA was evaluated by comparing its amino acid sequence homology to that of all renown allergens. Although there was 38 % homology to beta-conglycinin, a soy protein, over an 80 amino acid window, a RAST inhibition (serum screening) test demonstrated that PGA was unable to inhibit soy-specific antibodies from binding to soy protein. The failure to inhibit binding indicates the soy-specific antibodies did not recognize any epitopes on PGA and hence there would be none of the binding required to trigger an allergic reaction.

Thus, and overwhelming weight of scientific evidence reinforces the safety of PGA for the proposed used in food and supports the conclusions noted in the statement of an Expert Panel convened to evaluate the GRAS status of PGA for those uses.

IX B Mutagenicity Studies

1) Reverse Mutation Assay (Ames Test)

The mutagenicity of PGA was investigated by means of reverse mutation test conducted by Ajinomoto Company, Inc. in five bacterial strains (*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 *uvr*A). The results of this assay were no significant increase of revertant was observed at dose levels from 156 to 5,000 μg/plate in the absence and presence of mammalian metabolic activation system (See Appendix IX-2). The investigators concluded that PGA was negative to reverse mutation induction regardless of whether or not there was metabolic activation.

Three additional mutagenicity studies were conducted under a national research project managed by the Japanese Ministry of Health, Labor and Welfare. PGA produced by Ajinomoto was used in these studies as the test material.

2) Reverse Mutation Assay (#2)

A second Ames Test was performed by the Nagoya City Public Health Research Institute for the Japanese Ministry of Health, Labor and Welfare. This reverse mutation test of Bacillus natto gum that used *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1538) examined levels of PGA of up to 10 0 mg/plate. The results were negative regardless of the presence of metabolic activation (S9 mix) (See Appendix IX-3, 4). The original research report (in Japanese) and an English translation are attached

3) Chromosome Aberration Test in Cultured Mammalian Cells

A chromosome aberration test in cultured mammalian cells (CHL/IU) was performed on PGA by the Japanese National Institute of Health Sciences for the Japanese Ministry of Health, Labor and Welfare Abnormality was observed with a short treatment in 5.0 mg/ml of PGA of the maximum limit dosage with metabolic activation in the 6 % cells. There was no other chromosomal abnormality in the study. Since the abnormality was not as frequent in the high concentration tested and no dose-response relationship was seen and there was no mutagenicity in the following micronucleus test, it was concluded that there was no toxic influence on the living body (See Appendix IX-5, 6). The original research report (in Japanese) and an English translation are attached.

4) Micronucleus Test with rodents

A study of micronucleus inducibility in mouse bone marrow was performed at the Akita University Faculty of Medicine with funding from the Japanese National Institute of Health Sciences Six male ddy-mice per group were administered PGA (twice with 24 hour intervals) at doses of 0.75, 1 5 and 3.0 g/kg BW. The frequency of micronucleated immature erythrocytes and the percentage of immature erythrocytes among total erythrocytes in mice administered PGA were not statistical different from the control (See Appendix IX-7, 8). The original research report (in Japanese) and an English translation are attached.

IX C Acute Toxicity Study in Rats

A single oral dose of PGA was administered by gavage to five SD rats [Crj:C (SD) IGS] per sex at level of 0 (control), 2500 and 5000 mg/kg and the rats observed for 15 days. No deaths were observed at any dose in either sex. Watery diarrhea was observed at 3 or 6 hours after treatment in more than half of the animals in the 2500 and 5000 mg/kg groups but disappeared by day 2. Necropsy revealed no treatment related changes in either sex in any group The lethal dose of PGA was demonstrated to be greater than 5000 mg/kg in either male or female rats (Appendix IX-1).

IX D Subchronic Toxicity Study in Rats

A 90 day subchronic toxicity study of PGA was conducted by the Japanese National Institute of Health Sciences for the Japanese Ministry of Health, Labor and Welfare to evaluate the safety of PGA as food ingredient. PGA was administered to groups of five male and five female F344 rats per treatment group by feeding CRF-1 pellet diet containing 0 %, 0.18 %, 0.55 %, 1.66 %, or 5.0 % of PGA.

Table IX-D-1 Food Consumption and Intake of PGA during Subchronic Study

PGA Dose Level	Food consumption (g/animal/day)		Daily intake (mg/kg bw/day)		Total intake (g/kg bw)	
(%)	Male	Female	Male	Female	Male	Female
5	13.4	8.5	2,616.4	2,727.6	238.1	248.2
1 66	13.7	8.7	875.9	931.7	79.7	84.8
0.55	13.5	8.5	288.0	302.8	26.2	27.6
0 18	13.9	8.7	97.6	102.3	8.9	9.3
0	14.0	8.9	-	-	-	-

There was no mortality in any of the animals in any treatment group and there were no differences in feed intake or body weight between control animals and those in either sex in any of the treatment groups. Sporadic differences from control values in hematological and serum biochemical parameters were seen in different dose or sex groups but the changes observed were not dose-dependent.

Slightly increased kidney weights, compared to the controls, were observed in the higher dose groups of both males and females but there were no abnormal findings in the histopathological examination of the kidneys. The authors concluded that there were no treatment related serious indications of toxicity by feeding PGA in the diet for 90 days even at the highest dose studied (5 % of the diet) (See Appendix IX-9, 10, 11).

The results of this suchronic toxicity study of PGA were published in the Japanese Journal, Bull. Natl. Health Sci., 117, 119-122 (1999) (Appendix IX-11). A copy of the English translation of the paper is also attached (Appendix IX-9).

IX E Identification of the PGA-producing Strain

To confirm the identity of the strain of microorganism producing PGA a preliminary study 16S rRNA gene phylogenic analysis was conducted. The result of this analysis indicated the PGA-producing strain was most closely related to *Bacillus subtilis* and *Bacillus amyloliquefaciens* (homology 98.9 % and 98.6 %, respectively). A definitive identification could not be done by this analysis.

DNA-DNA hybridization analysis was then conducted. The result of this analysis conducted by Higeta Shoyu Co., Ltd., confirmed the PGA-producing strain was identified as *Bacillus subtilis* (See Appendix IX-13).

IX F Safety Assessment of the PGA-Producing Strain

Bacillus subtilis is an aerobic endospore-forming bacterium commonly found in nature and generally not considered to have either pathogenic or toxigenic potential (See Appendix I-1). The organism has a

history of use in the production of food ingredients. The PGA-producing strain was non-producer of toxin (See Appendix IX-12).

Dr Michael W. Pariza was asked to evaluate all available data and information about the safety (toxicity and pathogenicity) of the PGA-producing organism. In his expert opinion (Appendix IX-14) Dr. Pariza concludes that the PGA-producing organism employed by Ajinomoto "is safe for use in the manufacture of food-grade poly gamma glutamic acid (PGA)".

IX G Safety Study of the PGA-Producing Strain

A study of the potential virulence of the *Bacillus subtilis* used by Ajinomoto to produce PGA was conducted at the Kitasato Institute. The pathogenicity and toxin productivity of the PGA-producing strain were compared with that of *Bacillus cereus* as a positive control strain.

The living microorganisms and supernatants produced by centrifugation were administrated intraperitoneally to ddY mice. LD_{50} values of the PGA-producing strain and *Bacillus cereus* were estimated to be more than 10^8 and $10^{6\,9}$ cfu/ml, respectively. No death was observed in mice treated by the PGA-producing strain and both of the supernatants. There were no special findings in the organs in all surviving mice at necropsy.

Both of the supernatants sterilized by filtration were assayed by CRET-RPLA test measuring enterotoxic activity (diagnosed by reverse passive latex agglutination). Agglutination was detected in a 2-fold diluted supernatant as compared to a 3,200-fold dilution of *Bacillu cereus* supernatant Since the 2-fold titer was non-specific agglutination, the PGA-producing strain was determined to be non-producer of toxin (See Appendix IX-12).

IX H Safety Assessment of DISFOAM GD Anti-foaming Agent

A safety assessment and toxicological evaluation of the anti-forming agent DISFOAM GD was performed to support its use in the fermentation process used to PGA. Dr. Timothy Adams reviewed all of the available safety data on the substance and evaluated a worst case potential exposure from the proposed use of PGA and determined the experimental no effect level was 10,000,000 times greater than any likely intake (Appendices III-1 and III-2).

The acute oral LD_{50} of DISFOAM GD in male and female Sprague-Dawley CD rats was greater than 2000 mg/kg bw (OECD Guidelines No. 401) In a 28-day repeated dosing study (OECD Guidelines No. 407) in male and female Sprague Dawley rats, the No Observed Adverse Effect Level was 94 mg/kg bw/day, the highest dose tested. The concentrations tested were 0, 100, 300, 1000 ppm, equivalent to dose of 0, 9.9, 28 or 94 mg/kg bw/day. DISFOAM GD showed no evidence of mutagenicity in a bacterial reverse mutation assay in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538, TA 100, TA 98 and TA 102 and *Escherichia coli* strain WP2 uvr A with and without metabolic activation (OECD Nos. 471, 472)

X PHARMACOKINETIC INFORMATION

Since PGA is an ingredient in fermented soybean, natto, a food that has been eaten for long time by the Japanese, and there appears to be little doubt about its safety, there is a little information about the pharmacokinetics of PGA.

Fecal elimination of PGA was investigated in rats when it is orally administered. The PGA used in the experiments had a MW 26,000 (average), D/L=85/15.

Three experimental protocols were used in the studies:

1) A PGA solution was orally ingested by rats.
2) A diet containing PGA was fed to rats.
3) A diet containing PGA was fed to germ free rats.

In all experiments, rat feces were collected during the period when PGA was given and when PGA was not given. After the feces were acid hydrolyzed, glutamic acid (Glu) was measured by amino acid analysis. By subtracting fecal Glu content during the period when PGA was being ingested from fecal Glu content during the term when PGA was not given, fecal Glu derived from PGA was calculated [Glu (F)]. Oral Glu administered via PGA solution (Exp. 1) or in diets containing PGA (Exp. 2, 3) was calculated by amino acid analysis after acid hydrolysis of PGA [Glu (O)]. Fecal excretion of PGA was estimated by the following equation:

Fecal excretion of PGA = Glu (F) / Glu (O) x 100 (%).

PGA in the feces was also directly analyzed by HPLC (gel permeation chromatography, PGA was detected at UV220nm) after extracting PGA from the feces [fecal PGA]. Oral PGA was the amount of PGA in the PGA solution (Exp. 1) or the amount of PGA added into the diet (Exp.2).
Fecal excretion of PGA was also estimated by the following equation:

Fecal excretion of PGA = fecal PGA / oral PGA x 100 (%).

Fecal excretion of PGA form both estimations resulted in similar results, suggesting that the estimation of fecal excretion of PGA was reliable.

1) PGA solution orally ingested by rats.

Five-week-old Sprague Dawley rats were administered PGA solutions orally containing either 0.5 g/kg bw (n=4) or 0.8 g/kg bw (n=4). Feces were collected before and after PGA administration. Fecal excretion of PGA was 70 % or more of that ingested with small individual differences. The average MW of PGA in the feces was about 5,000 – 8,000.

Table X-1 Percent and Molecular Weight of Ingested PGA in Feces

Rat	Dose	Fecal excretion of PGA (%)	MW of PGA in Feces
1	0.5g/kg BW	68	5,100
2	0.5g/kg BW	78	5,300
3	0.5g/kg BW	69	5,500
4	0.5g/kg BW	69	6,900
5	0.8g/kg BW	78	5,100
6	0.8g/kg BW	74	4,900
7	0.8g/kg BW	99	8.100
8	0.8g/kg BW	85	6.700

2) Diets containing PGA fed to rats.

Five-week-old Sprague Dawley rats were fed diets containing 2 % PGA for 18 days (n=7). The results indicate that fecal excretion of PGA differed markedly between individual rats (7 % to 54 % of PGA found in feces). The average MW of PGA in the feces was about 5,000 – 10,000. There was a negative correlation between average MW of PGA in the feces and fecal excretion of PGA. The level of residual PGA found in the feces was substantially lower than when PGA was administered orally in a solution. Hence it appears that PGA is only partially digested when ingested as part of the diet.

Table X-2 Percent and Molecular Weight of Dietary PGA in Feces

Rat #		Day of Collection								Mean
		5	6, 7	8	9	10	11	12, 13	14, 15	
1	% in Feces*	12	16	17	7	16	18	22	21	17
	MW	10,900	9,200	11,200	11,300	10,100	10,300	7,800	9,300	-
2	% in Feces*	25	15	10	11	13	9	10	11	13
	MW	16,400	7,500	8,100	8,500	8,400	8,000	8,000	7,700	-
3	% in Feces*	45	47	32	12	25	54	24	14	30
	MW	4,900	5,100	7,600	10,200	7,100	6,200	8,000	12,000	-
4	% in Feces*	44	56	43	56	43	59	58	41	54
	MW	5,400	5,400	5,600	5,600	5,600	5,800	5,700	5,600	-
5	% in Feces*	46	47	33	49	40	35	50	43	44
	MW	5,200	5,000	4,800	4,900	5,000	4,900	4,900	5,000	-
6	% in Feces*	13	11	9	7	8	5	6	4	7
	MW	10,200	9,800	9,700	10,500	9,100	8,200	9,000	7,400	-
7	% in Feces*	12	9	9	11	7	5	7	5	8
	MW	7,800	8,500	8,900	9,200	8,600	9,300	9,400	8,900	-

* % Feces=% of ingested PGA in feces

The difference in fecal excretion of PGA and wideness of individual difference between when it is ingested in solution or as part of the diet was thought to depend on the difference in intestinal transit time, susceptibility to intestinal enzymes, and especially on individual difference of function of intestinal bacteria. A study was undertaken using germ free rats to assess the role of the intestinal microflora in the breakdown of PGA.

3) Diets containing PGA fed to germ free rats.

Five-week-old Fisher germ free rats were fed diets containing 1 % PGA for 25 days (n=5). Fecal excretion of PGA in germ free rats was about 50 % in all animals with small individual differences Average MW of PGA in the feces was about 6,000.

Hence, there appears to be substantial amount of breakdown of PGA by the intestinal microflora although the degree of degradation varies markedly between individual animals.

Table X-3 Percent and Molecular Weight of PGA in Feces of Germ-Free Rats Fed PGA In the Diet

Rat #		Day of Collection								Mean
		4	7	10	13	16	19	22	24	
1	% in Feces*	31	33	69	51	57	52	49	61	58
	MW	6,000	6,400	6,300	6,100	6,000	6,000	6,100	6,500	-
2	% in Feces*	25	32	62	42	58	32	87	32	53
	MW	6,600	6,200	6,100	6,200	6,000	6,000	5,800	6,300	-
3	% in Feces*	38	31	45	44	60	65	79	29	51
	MW	9,600	6,700	6,400	6,200	6,200	5,900	6,100	5,800	-
4	% in Feces*	38	32	74	32	40	26	53	24	49
	MW	6,900	6,100	6,500	6,200	6,100	5,800	6,500	6,400	-
5	% in Feces*	44	51	22	63	50	57	35	26	50
	MW	6,400	6,200	6,600	5,900	5,800	5,900	6,400	6,600	-

* % Feces=% of ingested PGA in feces

It might be hypothesized from the above results that, half the amount of PGA ingested moves to the large intestine, where PGA is utilized by intestinal bacteria, the function of which depends on the dietary and physiological situation. PGA is degraded into the lower molecular size of about average MW 5,000 – 8,000 within the gastrointestinal tract

XI ALLERGENICITY

The fermentation products of *Bacillus subtilis* are generally thought of as non-allergenic; e.g. natto is know as non-allergenic even among patients with soybean allergy. The absence of any allergenicity potential is due to the lack of exact homology to the amino acid sequence of the portion of the beta-conglycinin (soy allergen) molecule that binds with IgE antibodies and proteolytic digestion of soybean allergen which occurs during fermentation.

There is no report on food allergy caused directly by PGA in the result of PubMed search for (allergy + allergenicity) X (poly-γ-glutamic acid + poly-γ-glutamate) One report, Arerugi (in Japanese), 55, 832-836, 2006 (See Appendix XI-1) of an allergic reaction to ingesting fermented soy beans (natto) is found by the above search, in which involvement of PGA to natto allergy was speculated. This late-onset anaphylaxis caused by fermented soybeans was also reported in Allergology International, 56, 257-261, 2007 (See Appendix XI-2), in which the authors hypothesized that unknown allergens produced by *Bacillus subtilis* caused the reaction and their slow release resulted in late-onset anaphylaxis. However, these cases are very rare, only 8 patients have been reported among over 1,000 years food use of natto.

Three approaches were employed to evaluate the potential allergenicity of PGA, comparison of amino acid sequence homology to that of known allergens, serum screening using a RAST inhibition test and the expert opinion of Dr. Steve Taylor, Professor, University of Nebraska. All three approaches are described in Appendix XI-3.

The amino acid sequence (chains of glutamic acids) of PGA was compared with that of all known allergens (food and other allergens) in the University of Nebraska's Allergen Online data base. PGA was seen to have 38 % homology to beta-conglycinin (soybean allergen) over an 80-amino acid window.

However, the gamma linkages in the PGA molecule were seen to be distinct from the alpha linkages found in known allergens and further reduced the potential allergenicity of PGA. Further the PGA amino acid sequence homology was in a low complexity region of the beta-conglycinin molecule unlikely to be involved in binding to soy-specific IgE antibodies.

Serum screening of PGA was undertaken to assess its potential for binding to soy-specific IgE antibodies using a RAST inhibition test. The serum screening (employing antibodies of soy allergic individuals) was performed at the University of Nebraska. PGA was incapable inhibiting the binding of soy-specific IgE antibodies to soy protein, indicating the soy-specific antibody did not recognize any epitope on PGA (Appendix XI-3)

An earlier soy ELISA assay had been performed and demonstrated that there was no intact soy proteins in PGA. These results corroborated the above described RAST inhibition test result (Appendix XI-3).

Professor Steve L. Taylor prepared an Expert Opinion on the allergenicity of PGA (Appendix XI-3) Professor Taylor addressed three issues:

"(1) Since soybean protein is incorporated into the fermentation medium and since PGA is found naturally in a popular Japanese fermented soy product called natto, does PGA pose any risk to soybean-allergic individuals?
(2) PGA is a novel polypeptide as outlined above. Would any likelihood exist that PGA might induce allergic sensitization in susceptible consumers?
(3) Would any carryover of soy protein or soy protein fragments occur as a result of the use of soy protein in the fermentation medium?"

With respect to soybean allergic individuals, Professor Taylor concludes:

"In my expert opinion, PGA will not pose any risk to soy-allergic consumers. It is not homologous to known soy allergents and does not bind to soy-specific IgE antibodies present in the sera of soy-allergic subjects. Furthermore, no evidence exists that any soy protein or fragments of soy protein are carried over from the fermentation medium to the PGA ingredient.

However, some possibility exists that susceptible individuals might become sensitized to PGA through oral exposure. Based on the low level of anticipated exposure, this risk seems small. If some small number of consumers develop PGA allergy, any risk can be easily managed by product avoidance through labeling of the ingredient."

Recognizing that PGA is a unique polypeptide, Professor Taylor notes that

"As previously noted, PGA is a rather unique polypeptide especially with the respect to the gamma linkages for the glutamic acid residues and the presence of preponderance of D-glutamic acid isomers The gamma linkages in PGA tend to render this polypeptide resistant to digestion. Digestive stability, especially pepsin resistance, is thought to correlate to some extent with the potential allergenicity of food proteins (Astwood et al., 1996). Because of its digestive stability, PGA might be able to reach the intestinal tract of susceptible consumers in a form that is sufficiently large to be immunoreactive. PGA feeding studies conducted by Ajinomoto confirm that 70-80 % of PGA appears in the feces of rats after ingestion. The molecular weight of the PGA fragments found in the rat feces was approximately 5300 daltons corresponding to 35-40 glutamic acid residues. While such short fragments are unlikely to elicit allergic sensitization, the possibility cannot be totally excluded "

XII. STATEMENT OF THE EXPERT PANEL

Statement of the Expert Panel

The Ajinomoto Company conducted a search of the scientific literature on Polyglutamate (PGA) through December 2008, assembled available information of PGA including manufacturing, specifications, and exposure, and prepared this dossier. Ajinomoto then convened a panel of Experts (Expert Panel) to determine the safety of the intended use of PGA as a taste modifier for potassium chloride. We, the members of that Expert Panel, independently and collectively critically evaluated the information assembled by Ajinomoto and other materials deemed appropriate and then conferred by telephone with members of the Ajinomoto Company.

We conclude that the intended use of Polyglutamate, meeting appropriate food grade specifications presented in the dossier and manufactured and used consistent with current Good Manufacturing Practice (cGMP), as a taste modifier of potassium chloride, is Generally Recognized As Safe (GRAS) based on scientific procedures.

It is our opinion that other experts, also qualified to evaluate the safety of foods and food ingredients, would concur with this conclusion.

(b) (6)

07 April 2009
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REPORT OF THE EXPERT PANEL ON THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE INTENDED USES OF POLY-GAMMA-GLUTAMIC ACID (PGA)

19 April 2010

INTRODUCTION

Ajinomoto Co. Inc. convened a panel of independent biomedical scientists (hereinafter, the Expert Panel) qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients and food, to conduct an independent, critical and comprehensive evaluation of the available data and information on Poly-gamma-glutamic acid (PGA) and to determine whether the intended uses of Poly-gamma-glutamic acid are safe and suitable and are Generally Recognized as Safe (GRAS) based on scientific procedures. The Panel consisted of the following qualified experts: Professors Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Michael W. Pariza, Ph.D. (University of Wisconsin-Madison), John Thomas (University of Indiana School of Medicine) and Steve Taylor, Ph.D. (University of Nebraska).

Ajinomoto conducted a comprehensive search of the scientific literature on Poly-gamma-glutamic acid (PGA) through December 2009, summarized the pertinent literature and prepared a dossier, "Polyglutamate (PGA) Dossier for GRAS Panel Review," and made this available to the Panel. Ajinomoto provided information on the identity, manufacturing, specifications, batch analyses, potentially toxic natural contaminants, functionality and intended uses and levels, estimated daily intakes, nutrition and absorption and digestibility, safety/toxicity data and potential adverse effects, including potential allergenicity. The Expert Panel, independently and collectively, critically evaluated the dossier and other information deemed appropriate.

Members of the Panel conferred by telephone with technical experts from Ajinomoto. Following a critical review and discussion of the materials evaluated and information provided by Ajinomoto, the Panel unanimously concluded that the intended uses of Ajinomoto's Poly-gamma-glutamic acid (PGA) protein, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications presented in the dossier, are safe and suitable and are GRAS based on scientific procedures.

A summary of the information evaluated and the basis for the determination that the intended uses of Ajinomoto's Poly-gamma-glutamic acid (PGA) are safe and suitable and GRAS based on scientific procedures is presented below.

BASIS FOR GRAS DETERMINATION

Background

Salt, NaCl, is essential for maintenance of health. However, excessive intakes of salt may result in hypertension. Public health officials recommend reducing daily salt intake for adults to 5 grams or less (WHO, 2003). Potassium chloride is a widely used salt substitute used singly or in combination with sodium chloride to reduce salt intake. However, potassium chloride has a better aftertaste. Ajinomoto research discovered that poly-gamma-glutamic acid (PGA) could effectively block this bitter aftertaste, thereby enhancing the use of potassium chloride.

Poly-gamma-glutamic acid (PGA) is a polymer of gamma-linked D- and L- glutamic acid that exists naturally in the traditional Japanese food, Natto (soybeans fermented with Bacillus subtilis). Natto contains about 300 mg PGA/100 grams. Natto has been consumed in Japan for at least 1000 years and has been produced in the USA since 1963. PGA is registered as a food additive in Japan. PGA is used in a number of products in Japan including as a Ca supplement, low sodium table salt, protein powder, and in barbecue sauces.

The mean molecular weight of poly-gamma-glutamic acid (PGA) is 28, 000 to 32, 000 Daltons (range: 1, 600 to 201, 800).

Manufacturing Process

Ajinomoto's poly-gamma-glutamic acid (PGA) is produced by liquid fermentation of glucose (dextrose) and other ingredients (e.g. vitamins, minerals) providing all the nutrients required by Bacillus subtilis. All components of the fermentation medium are approved food grade ingredients After fermentation, the broth is acid (HCl) hydrolyzed, decolored, filtered, purified, sterilized, and dried to a powder. Ajinomoto produces poly-gamma-glutamic acid (PGA) consistent with cGMP.

The results of five, non-sequential batch analyses demonstrate that the manufacturing process consistently yields a product that meets the food-grade specifications presented in the dossier.

Product Specifications

Ajinomoto's poly-gamma-glutamic acid (PGA) meets appropriate food grade specifications (presented in the dossier)

Functionality, Intended Uses and Use-Levels and Exposure

Poly-gamma-glutamic acid (PGA) is proposed for use in "Added salt" including table-use and salt used in food preparation to mask the bitter after taste of potassium chloride. A common salt replacer, the potassium chloride is often mixed with sodium chloride up to 50% (that is, equal

parts of potassium chloride and sodium chloride). To further mask the bitter after taste of this mixture (to modify taste perception), it is recommended that PGA be used at a level of 0.15-0.4 % by weight. If the ratio of potassium chloride is changed from 50%, the recommended inclusion rate of PGA is 0.3- 0.8% of potassium chloride. It is noted that in addition to masking the bitter after taste of potassium chloride, PGA also enhances spiciness, enhances absorption of calcium, and improves dispensability in dehydrated beverages.

Estimated consumption of PGA is calculated by assuming that all added salt (low sodium salt + regular salt) is replaced by 50% low-sodium salt with potassium chloride and that the maximum PGA inclusion rate is 0.4 % for the 50 % low-sodium salt with potassium.

The estimated consumption for the US population of total "Added Salt" (total of low-sodium salt and regular salt) is 7 06 g/day per user and 7.01 g/day per capita for the mean, and 11.6 g/day per user and 11.5 g/day per capita for the 90th percentile (See Appendix VIII-1, Table 9A). The estimated consumption for the US population of PGA (= salt x 0.4 %) for total "Added Salt" (mg/day) is then 28.24 mg/day per user (= 7.06 g/day x 0.4 %) for the mean and 46.4 mg/day per user (= 11.6 g/day x 0.4 %) for the 90th percentile. On the per capita basis, the estimated consumption of PGA (mg/day) is 28.04 mg/day per capita (= 7.01 g/day x 0.4 %) for the mean and 46.0 mg/day per capita (= 11.5 g/day x 0.4 %) for the 90th percentile.

The above consumption figures may be expressed as g PGA/ kg body-weight (bw) by taking the above figures and dividing by the average weight for the US population, 60 kg. The new figures became as follows. The estimated consumption for the US population of total "Added Salt" (total of low-sodium salt and regular salt) is 0.12 g/kg bw/day per user and 0.12 g/kg bw/day per capita for the mean, and 0.23 g/kg bw/day per user and 0.23 g/kg bw/day per capita for the 90th percentile (See Appendix VIII-1, Table 9B). The estimated consumption for the US population of PGA (= salt x 0.4 %) for total "Added Salt" (mg/kg bw/day) is then 0.48 mg/kg bw/day per user (= 0.12 g/kg bw/day x 0.4 %) for the mean and 0.92 mg/kg bw/day per user (= 0.23 g/kg bw/day x 0.4 %) for the 90th percentile. On the per capita basis, the estimated consumption of PGA (mg/kg bw/day) is 0.48 mg/kg bw/day per capita (= 0.12 g/kg bw/day x 0.4 %) for the mean and 0.92 mg/kg bw/day per capita (= 0.23 g/kg bw/day x 0.4 %) for the 90th percentile.

Safety Assessment

History of Consumption(Common Use in Foods and Feeds)

PGA is found in the mucilage of Natto, a traditional Japanese food from soybeans fermented with Bacillus subtilis. It has been consumed for at least 1000 years. Natto has also been produced and used in the USA since 1963. PGA is a registered food additive in Japan. PGA is also used as a Ca supplement, in low-sodium table salt, protein powder, and barbecue sauces.

PGA-producing Strain (Bacillus subtilis)

B.subtilis, an aerobic endospore-forming bacterium commonly found in nature, is neither pathogenic nor toxigenic. There is a long history of safe use of B. subtilis in food production.

Animal Studies

Absorption, Distribution, Biotransformation, Excretion

Digestibility

Five-week old male Sprague-Dawley rats received PGA (MW: 26,000) in solution by gavage at doses

of 0.5 or 0.8 g PGA/kg bw (4 rats/dose). Fecal analysis demonstrated 71 % PGA with a MW of 5, 700 (in rats that received the 0.5 g dose) and 84 % PGA with a MW of 6,200 (in rats that received the 0.8 g dose).

Five-week old male Sprague-Dawley rats received PGA (MW: 26,000) as a dietary admixture at a level of 2% for 18 days. 25% PGA (MW: 8,000) was found in the feces.

Five-week old male Fisher rats received PGA (MW: 26,000) as a dietary admixture at a level of 1% for 25 days. 52% PGA (MW: 6,300) was found in the feces.

These findings support the digestibility of PGA following oral administration.

Acute Toxicity

The acute oral LD_{50} of PGA (MW: 26,000) in male and female SD rats [Crj:CD(SD)IGS]was greater than 5,000 mg/kg bw, the limit dose. Watery diarrhea was observed in more than half the rats that receive 2,500 or 5,000 mg/kg bw at 3 and 6 hours post-treatment. There were no other reported signs of toxicity.

Sub-chronic Toxicity

Male and female F344 rats (5/sex/group) received PGA (MW: 26,000) as a dietary admixture at levels of 0, 0.18, 0.55, 1.66, and 5% for 90 consecutive days. This study was conducted consistent with GLP and currently accepted testing guidelines. There were no statistically significant, consistent, treatment and dose-related adverse effects reported in any of the parameters evaluated. The only statistically significant finding was increased relative kidney weights in both sexes at 1.66 and 5% levels but there were no abnormal histopathological renal findings. The significance of these renal findings is unclear.

Genotoxicity Studies.

The following studies were conducted with PGA (MW: 26,000):

- Ames Reverse Mutation Tests (Strains TA98, TA100, TA 1535, TA 1537, TA1538 and E. coli WP2 uvrA. PGA was not mutagenic with or without metabolic activation.
- Mouse Micronucleus Test (male ddy mice). PGA was administered by gavage at doses of 0, 750, 1500, and 3000 mg/kg bw twice with 24 hour intervals. No evidence of mutagenicity.
- Chromosome Aberration Test in Cultured Mammalian Cells (CHL/IU). Cells were treated with PGA (up to 5.0 mg/ml) for 6, 24, or 48 hours. Only 6% of cells at maximum level showed aberration with metabolic activation at 6 hours. No abnormalities were seen at 24 and 48 hours. No evidence of clastogenicity.

Allergenicity

Fermentation products by B. subtilis are generally considered non-allergenic. Natto is reported to be non-allergenic among subjects with soybean allergy. Nevertheless, Ajinomoto requested Professor Steve Taylor to address the potential allergenicity of PGA.

Professor Taylor compared the amino acid sequence homology of PGA with known allergens and reported a 38% homology with beta-conglycinin, a soy allergen; there was no IgE antibody binding in a RAST assay using antibodies from soy allergic individuals; and no soy proteins were detected in an ELISA test for soy proteins. Professor Taylor concluded:

"In my expert opinion, PGA will not pose any risk to soy-allergic consumers. It is not homologous to known soy allergens and does not bind to soy-specific IgE antibodies present in the sera of soy-allergic subjects. Furthermore, no evidence exists that any soy protein or fragments of soy protein are carried over from the fermentation medium to the PGA ingredient." It is concluded that the allergenic potential of PGA is exceedingly low, if existent.

Human Studies

There are no reported human studies conducted with PGA.

Summary

The safety and suitability and the GRAS status of the intended uses of Ajinomoto's Poly-gamma-glutamic acid (PGA) are based on:

- The history of safe consumption of Natto, a food produced from soybeans fermented with B.subtilis Natto contains 300 mg PGA/100 grams.
- The recognized safety of the PGA-producing strain of B. subtilis, non-pathogenic and non-toxigenic (non-toxin producer).
- The absorption and digestibility of PGA; at least 50% absorption in rats. PGA is degraded to lower molecular species and excreted in the feces.
- The low acute oral toxicity of PGA; the acute oral LD50 is > 5000 mg/kg bw.
- A NOAEL of 5% in a 90-day sub chronic study, the highest level fed, equivalent to 2,616 mg PGA/kg bw/day in male rats and 2,727 mg PGA/kg bw in female rats.
- The estimated consumption of PGA is 0.48 mg/kg bw/day for the mean and 0.92 mg/kg bw/day for the 90th percentile.

CONCLUSION

The Ajinomoto Company conducted a search of the scientific literature on Polyglutamate (PGA) through December 2008, assembled available information of PGA including manufacturing, specifications, and exposure, and prepared this dossier. Ajinomoto then convened a panel of Experts (Expert Panel) to determine the safety of the intended use of PGA as a taste modifier for potassium chloride. We, the members of that Expert Panel, independently and collectively critically evaluated the information assembled by Ajinomoto and other materials deemed appropriate ,conferred by telephone with members of the Ajinomoto Company, and then, in executive session, unanimously concluded that the intended use as a taste modifier of potassium chloride of Ajinomoto's Poly-gamma-glutamic acid (PGA), manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food grade specifications presented in the dossier, is safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures and supported by a history of safe consumption of Natto, a Japanese food containing PGA.

It is our opinion that other experts, also qualified to evaluate the safety of foods and food ingredients, would concur with this conclusion.

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CONCLUSION

The Ajinomoto Company conducted a search of the scientific literature on Polyglutamate (PGA) through December 2008, assembled available information of PGA including manufacturing, specifications, and exposure, and prepared this dossier. Ajinomoto then convened a panel of Experts (Expert Panel) to determine the safety of the intended use of PGA as a taste modifier for potassium chloride. We, the members of that Expert Panel, independently and collectively critically evaluated the information assembled by Ajinomoto and other materials deemed appropriate ,conferred by telephone with members of the Ajinomoto Company, and then, in executive session, unanimously concluded that the intended use as a taste modifier of potassium chloride of Ajinomoto's Poly-gamma-glutamic acid (PGA), manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food grade specifications presented in the dossier, is safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures and supported by a history of safe consumption of Natto, a Japanese food containing PGA.

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Appendices

Opinion on Riboflavin as a colouring matter authorized for use in foodstuffs produced by fermentation using genetically modified *bacillus subtilis* (expressed on 10 december 1998)

Terms of Reference

To advise on the safety in use of riboflavin, produced by fermentation using genetically modified *Bacillus subtilis*, as a colouring matter authorized for use in foodstuffs intended for human consumption.

Introduction

The Committee has previously reported on the safety in use of riboflavin and its metabolite riboflavin-5'-phosphate as a colouring matter for foodstuffs for human consumption (1). It has now been requested to review the microbiological and toxicological safety of riboflavin produced by fermentation as a colouring matter for foodstuffs generally.

Background

Riboflavin (vitamin B_2) is a water-soluble vitamin, that is synthesised by plants and many microorganisms but is not produced by higher animals. It is an essential micronutrient in the human diet, where it serves as precursor to coenzymes e.g. flavine adenine dinucleotide and flavin mononucleotide, which function as hydrogen carriers in biological redox processes.

Riboflavin occurs naturally in peas, beans, grains, yeast, milk, egg yolk and liver. Hitherto riboflavin has been chemically synthesised for use in food fortification and in small amounts as a colouring agent in foods e.g. ice cream, processed meat, fish products, sauces and soups. Riboflavin is also used in the fortification of animal feedstuffs.

Although it has been known for many years that riboflavin could be produced by bacteria using fermentation technology, it was not until recently that a very pure product could be produced using a genetically modified strain of *Bacillus subtilis*. The pathway of biosynthesis of riboflavin in *B. subtilis* involves 6 steps controlled by enzymes and the precursors guanosine triphosphate and ribulose-5-phosphate.

To evaluate the possible microbiological risks for consumer health the microbiological status of the production process requires consideration with regard to the safety of the producer organism as well as the fermentation process and product purification procedure. Although riboflavin is not a Novel

Food per se the safety assessment can be performed following the strategy proposed by the SCF in its Guide to the Assessment of Novel Foods (2), particularly with reference to the section on Novel Foods derived from genetically modified microorganisms.

Producer organism

B. subtilis is an aerobic endospore-forming bacterium commonly found in nature and generally not considered to have a pathogenic or toxigenic potential. There is a history of safe use of this bacterium in large-scale fermentation production of specialty chemicals, of enzymes used in food production processes, and of several traditional relationships to food. It is used traditionally in East Asia for the fermentative production of Natto from wheat starch, a product also obtainable in western countries. It is thus an organism with a tradition in food use although the actual food product is little known in the European Union. The organism is also involved in many types of food spoilage.

The strain of *B. subtilis* used in riboflavin production is RB50::[pRF69] $_n$[pRF93] $_m$Ade+ (3), a non-sporing derivative of *B.subtilis* 168, which had been produced by classical mutation and into which the two specific plasmids pRF69 and pRF93 had been chromosomally, and thus stably, integrated into the bacterial genome and amplified 20-25-fold and 10-15-fold respectively. The strain was identified by the Deutsche Sammlung von Mikroorganismen (DSM Braunschweig, Germany) as *Bacillus subtilis*. The genetic modification includes the chromosomal integration of the following elements:

- pUC 19, a derivative of the common *E. coli* cloning vector pBR 322, harbouring a DNA fragment carrying the *rib* operon and the *bla* gene encoding ampicillin resistance, which latter is not expressed in *B. subtilis*.

- SPO1-15, the constitutive promoter derived from a *B. subtilis* bacteriophage which enables efficient expression of the *rib* operon.

- the marker gene *tet*, encoding tetracycline resistance, originally derived from *Bacillus cereus*.

- the marker gene *cat*, encoding chloramphenicol resistance, originally derived from *Staphylococcus aureus*.

The *tet* as well as the *cat* gene can be considered to be constituents of the natural gene pool of a wide range of Gram-positive organisms including *B. subtilis*. The chromosomal organisation of the gene inserts has been demonstrated by their functionality as well as by southern blot analysis. These data were supplemented by sequencing of specific fragments.

The fermentation process

The producer organism is grown by controlled submerged growth in well-defined media containing accepted components such as carbohydrates, nitrogen sources, mineral salts, an antifoam agent and the antibiotics chloramphenicol and/or tetracycline in the inoculation media.

The production process uses inoculum build-up followed by a feed-batch production fermentation. It is carried out in contained vessels using axenic large-scale microbiological techniques. The fermentation is routinely tested to ensure cultural purity, the quality control as described can be considered to be of the highest standard to exclude contamination. The final fermentation process will be performed in practice in compliance with Good Industrial Large-Scale practice.

Down-stream processing

After fermentation the whole broth is pasteurised to ensure that no viable cells of the production organism are present in the final product. The further

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processing is designed to remove cell debris, DNA and contaminants. Hydrochloric acid is added as the only purification agent The concentration of acid and the temperature conditions chosen were shown to degrade any DNA still present, so that it can no longer serve as a substrate in transformation or as a template in a polymerase chain reaction. After removal of any residual biomass the purity of the final product is 96% and this material after washing and drying can then be used as such in animal feed. Further processing with hot hydrochloric acid produces a material of 98% purity (food grade). This final purification step is identical with that currently used for producing a chemically synthesised material of the same 98% purity. Comparison of the HPLC chromatograms of the chemically synthesised and the fermentation product confirmed that no further impurities had resulted from the fermentation process, the main degradation product resulting from the acid treatment being 8-hydroxymethylriboflavin (4). The final product meets the specification for riboflavin used as colouring matter for foodstuffs for human consumption listed in Commission Directive 95/45 EC (3).

Intake

The riboflavin produced by fermentation is intended to be used for the same purposes as the chemically synthesised product. No increased consumption is expected as no new applications exclusive to the fermentation-derived product are anticipated.

Nutritional aspects

As the synthetic product and the fermentation product have identical specifications and quality criteria and the same application levels, it is unlikely that there will be any difference in bioavailability, when one product is substituted for the other. Hence a nutritional assessment is unnecessary.

Toxicological aspects

The 96% fermentation product (feed grade), the 98% fermentation product (food grade) and the 98% chemically synthesised product were examined in a 13-week oral feeding study in Wistar rats at doses of 20, 50 or 200 mg/kg b.w. There were no significant differences between test and control groups regarding clinical signs, feed consumption and water intake. There was a 6% retardation of growth of females given 200 mg/kg b.w. of the 98% fermentation product. No consistent or dose-related changes in haematological parameters, urinalysis or clinical chemistry were noted. Females given 200 mg/kg b.w. of the 98% fermentation product showed a slight increase in relative liver weight and males given 50 and 200 mg/kg b.w. fermentation product showed a slight increase in relative spleen weight, neither of these weight increases being dose-related nor associated with any histopathological findings. Gross and histopathology showed no significant treatment-related lesions in any test group. The observed changes were considered to be of no toxicological concern (5)

A bacterial microsomal reverse mutation test in *S. typhimurium* was negative with and without S9 mix over a dose range of 50-5000 µg/plate for the 96% and 98% fermentation product (6,7)

Evaluation

The only genetic modifications of the producer organism *B .subtilis* concerned the introduction of the 2 genetic constructs containing the *rib* operons, the *tet* and *cat* genes and the selected mutational changes for deregulating the purine and riboflavin pathways and also for preventing sporulation. PCR analysis, southern blot analysis and sequencing of specific fragments showed that the expected composition of the modified genome remained stable under production conditions and that biologically active DNA of the production strain was no longer present in the fermentation-derived purified material. A hypothetical genetic transfer to the human consumer or the gut microorganisms of the novel genes in GM *B. subtilis* following consumption of fermentation-derived riboflavin could not occur since the purification process destroyed any DNA that may have been present.

There are no indications that the impurities present in the fermentation-produced riboflavin were different from those occurring in the chemically synthesised riboflavin and were also shown with existing analytical methods to be present at similar levels.

The toxicity study and the mutagenicity assay showed no toxicologically relevant adverse effects either in the 98% fermentation product or the 98% synthetic material. On microbiological-hygienic grounds there are no objections to the acceptance of the fermentation product.In conclusion the Committee considered that riboflavin produced by fermentation using genetically modified *Bacillus subtilis* is acceptable as a food colour.

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Appendix II-1

Analytical Methods of Specification

1. Description

(1) Appearance and odor of the sample are tested using Standard PGA, previously tested and released

(2) Appearance

Weigh about 1 g of the sample, put in a 50-ml beaker, place on a piece of white paper, and observe from above Determine whether the sample is a white to yellowish fine powder

(3) Odor

Weigh about 1 g of the sample, put in a 50-ml beaker, smell, and determine whether the sample is odorless or slightly characteristic

2. Identification (Ninhydrin)

(1) To 5 ml of the sample solution (1→200), add 5 ml of concentrated hydrochloric acid, and heat at 110°C for 24 hours, and then adjust the pH of the sample solution to between 4 0 and 6.0 using 6 N sodium hydroxide solution (short-range pH indicator paper as an external indicator)

(2) To 5 ml of this solution, add 1 ml of ninhydrin solution, heat (about 80°C) for 5 minutes in the water bath, and then, a blue-purple color develops

3. Assay (as Glu)

(1) Weigh accurately about 0.1 g of the dried sample (dried under the same way described in Loss on Drying), and dissolve in 10 ml of water.

(2) To 5 ml of this solution, add 5 ml of concentrated hydrochloric acid, and heat at 110°C for 24 hours, and then analyze with amino acid analyzer

(3) Assay (Glu) is given in the following formula

$$\textbf{Assay (Glu) \% W/W} = \frac{A \times B}{C \times D} \times 100$$

A: analyzed concentration of glutamic acid

B: 129 (molecular weight of glutamic acid residue in PGA)

C: concentration of the sample solution

D: 147 (molecular weight of glutamic acid)

4. Arsenic (as As_2O_3)

(1) Test Solution

Weigh 0 50 g of the sample, add 5 ml of water or 5 ml of diluted hydrochloric acid, dissolve by heating if necessary, and use the solution as the test solution.

(2) Transfer the test solution into the generator bottle, add 1 drop of bromophenol blue TS, neutralize with aqueous ammonia, ammonia TS, or diluted hydrochloric acid (1→4), add 5 ml of diluted hydrochloric acid (1→2) and 5 ml of potassium iodide TS, and allow to stand for 2 to 3 minutes. Add 5 ml of acidic stannous chloride TS, and allow to stand for 10 minutes. Then, add water to make 40 ml, add 2 g of arsenic-free zinc, and immediately connect the rubber stopper, fitted with exit tube and glass tube, with generator bottle Insert the tip of glass tube to the bottom of absorber tube containing 5 ml of the absorbing solution for arsenic hydride, then immerse the generator bottle up to the shoulder in water maintained at 25°C, and allow to stand for 1 hour. Disconnect the absorber tube, add pyridine to make 5 ml, if necessary, and observe the color of the absorbing solution The color produced is not deeper than the standard color

(3) Preparation of Standard Color is carried out at the same time as the test solution Measure 2 0 ml of Arsenic Standard Solution (1 μg/ml as As_2O_3), transfer into a generator bottle, add 5 ml of potassium iodide TS, allow to stand for 2 to 3 minutes Add 5 ml of acidic stannous chloride TS, allow to stand at room temperature for 10 minutes Proceed in the same manner as the test solution, use the color produced by the absorbing solution as the standard color

(4) Bromophenol blue TS Weigh 0 1 g of bromophenol blue, dissolve in 100 ml of 50% ethanol, and filter if necessary.

Ammonia TS. Measure 40 ml of aqueous ammonia, and add water to make 100 ml

Potassium iodide TS Weigh 16 5 g of potassium iodide, and dissolve in water to make 100 ml Store, protecting from light. Prepare freshly before use.

Acidic stannous chloride TS: Weigh 4 g of stannous chloride, dissolve in 125 of arsenic-free hydrochloric acid, and add water to make 250 ml. Store in a bottle with a ground-glass stopper, tightly stoppered Use within 1 month after preparation.

Absorbing solution for arsenic hydride: Weigh 0.50 g of Ag-DDTC (silver diethyldithiocarbamate) and dissolve in pyridine to make 100 ml. Store in a light-resistant bottle with a ground-glass stopper in a cool place.

5. Lead (as Pb)

(1) Weigh the sample (6 to 9 g) into a Kjeldahl flask, and add 20 ml of nitric acid and 7 ml of sulfuric acid

(2) Heat until white fumes of sulfur trioxide appear, and then cool

(3) Add 10 ml of 20 % hydrochloric acid and heat

(4) Transfer the digests to a separatory funnel, and add 10 ml of 50 % ammonium citrate solution

(5) Neutralize with ammonium hydroxide using thymol blue as an indicator, and cool

(6) Make up the volume to about 100 ml, add 5 ml of 3 % ammonium pyrrolidine dithiocarbamate solution (APDC), and stand for 5 minutes

(7) Add 5 ml of butyl acetate and shake for 5 minutes

(8) After allowing the sample to separate into water and butyl acetate layer, collect the butyl acetate layer and measure with atomic absorption spectrophotometer using the following condition:

Wave Length: 217.0 nm
Flame: Acetylene - Air

6. Loss on Drying

(1) Weigh 2 g of the sample into the weighing bottle (dried and weighed accurately), spread the sample so that the layer is not thicker than 5 mm, and weigh it accurately.

(2) Place the bottle in the desiccator with silica gel as desiccant and dry under the pressure at 1 3 kpa or less at 40°C for 24 hours

(3) Weigh the sample again, and calculate the loss on drying as follows

$$\textbf{Loss on Drying \% W/W} = \frac{\textbf{Weight Loss (g)}}{\textbf{Weight of the sample (g)}} \times 100$$

7. Residue on Ignition

(1) Ignite a crucible of porcelain in electric furnace at 600°C for 30 minutes, allow to cool it in a desiccator, and weigh it accurately Place 1 g of the sample into the crucible and weigh it accurately

(2) Moisten the sample with a small amount (usually 1 ml) of sulfuric acid, then heat gently at a temperature as low as practicable until the sample is thoroughly charred After cooling, moisten the residue with a small amount (usually 1 ml) of sulfuric acid, heat gently until white fumes are no longer evolved, and ignite at 600°C for 1 hour in electric furnace

(3) Cool the crucible in a desiccator and weigh accurately

(4) Calculate the residue on ignition as follows

$$\textbf{Residue on Ignition \% W/W} = \frac{\textbf{Weight of ash (g)}}{\textbf{Weight of the sample (g)}} \times 100$$

8. Standard Plate Counts

(1) Test solution

Weigh 10 g of the sample and dissolve with sterile phosphate buffer to make 100 ml PH of the solution is adjusted between 6 8 and 7 2 by adding of dilute hydrochloric acid solution.

(2) Collect 1 ml of the test solution, inoculate on the dried surface of Standard Agar Medium plate, and spread thoroughly over the surface Allow the plate to stand level surface

(3) Cultures are incubated at 35±1°C for 48±3 hours.

(4) After incubation count the number of colonies grown on the surface of the plate.

(5) <u>Standard Agar Medium (1,000 ml, pH 7 1±0 1)</u>

Yeast extract . . . . 2 5 g

Peptone. . .. .5.0 g

Glucose. . . . .1 0 g

Agar ... . .20 g

9. Coliforms

(1) Test solution

Weigh 10 g of the sample, add 90 ml of sterile phosphate buffer, and blend for 2 minutes Prepare decimal dilutions of 10^{-1} and 10^{-2} with sterile phosphate buffered physiological saline

(2) Transfer 1 ml portions to 3 LST tubes for each 3 consecutive dilutions (undiluted test solution, 10^{-1} and 10^{-2} dilutions) Incubate LST tubes at 35°C Examine tubes and record reactions at 24±2 hours for gas Re-incubate gas-negative tubes for an additional 24 hours and examine and record reactions again at 48±2 hours

(3) From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth Incubate BGLB tubes at 35°C and examine for gas production at 48±2 hours Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

(4) <u>Lauryl Tryptose (LST) Broth (1,000 ml, pH 6.8±0 2)</u>

Tryptose or trypticase	20 g
Lactose	5 g
K_2HPO4	2 75 g
KH_2PO4	2 75 g
NaCl	5 g
Sodium lauryl sulfate	0 1 g

<u>Brilliant Green Lactose Bile (BGLB) Broth (1,000 ml, pH 7 2±0.1)</u>

Peptone	10 g
Lactose	10 g
Oxgall	20 g
Brilliant green	0 0133 g

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Toxicological Evaluation of DISFOAM GD Antifoaming Agent

Prepared by Timothy B. Adams, Ph.D.

3/10/09

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Appendix IV -1

Analytical Methods of Purities

1. PGA as Glu (%)

(1) Dissolve the sample with 20 % hydrochloric acid containing β-mercaptoethanol, and heat at 110°C for 24 hours.

(2) Dilute with sodium citrate buffer (pH 2.2), and then analyze with amino acid analyzer.

PGA as Glu (%) is given in the following formula:

$$\textbf{PGA as Glu (\%)} = \frac{A \times B}{C \times D} \times 100$$

A: analyzed concentration of glutamic acid

B: 129 (molecular weight of glutamic acid residue in PGA)

C: concentration of the sample solution

D: 147 (molecular weight of glutamic acid)

2. Moisture (%)

(1) Weigh the dish (constant weight).

(2) Place the sample into the dish and weigh the dish containing the sample.

(3) Place the dish in the vacuum drying oven and dry at 70°C to constant weight.

(4) Cool in the desiccator and weigh the dish containing the sample.

Moisture (%) is given in the following formula:

$$\textbf{Moisture (\%)} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

W_1: Constant weight of the dish

W_2: Weight of the dish containing the sample

W_3: Weight of the dish containing the sample after drying

3. Ash (%)

(1) Weigh the porcelain crucible (constant weight).

(2) Place the sample into the porcelain crucible and weigh the porcelain crucible containing the sample.

(3) Place the porcelain crucible in the electric furnace and make preliminary ashing at low temperature until smoking ceases.
(4) Ignite it at 550°C to constant weight.
(5) Cool in the desiccator and weigh the porcelain crucible containing the sample.

Ash (%) is given in the following formula:

$$\text{Ash (\%)} = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

W_1: Constant weight of the porcelain crucible
W_2: Weight of the porcelain crucible containing the sample
W_3: Weight of the porcelain crucible containing the sample after igniting

4. Total Sugars (%)

(1) Dissolve the sample with water and fill up with water to constant volume.
(2) Measure the sample solution using phenol-sulfuric acid colorimetric method with glucose standard (calculated as glucose).

5. Acetic Acid (%)

(1) Dissolve the sample with 5% perchloric acid, add water into the sample solution, and shake it well.
(2) Fill up with water to constant volume, filtered the sample solution, and then apply to HPLC using the following condition:

Detector: UV-VIS Spectrophtometric Detector
Column: Shodex RSpak KC-811 X 2, Φ 8 mm X 300 mm
Temperature: 40°C
Mobile Phase: 3 mmol/l perchloric acid
Reaction Reagent: 15 mmol/l disodium hydrogen phosphate solution containing 0.2 mmol/l bromothymol blue
Flow Rate: Mobile Phase1.0 ml/min, Reaction Reagent 1.4 ml/min
Wave Length: 445 nm

6. Ammonium (%)

(1) Dissolve the sample with 20 % hydrochloric acid containing β-mercaptoethanol, and heat at 110°C for 24 hours.

(2) Dilute with sodium citrate buffer (pH 2.2), and then analyze with amino acid analyzer.

7. Lipid (%)

(1) Weigh the Soxhlet flask (constant weight) and weigh the sample into the paper extraction thimble.

(2) Dry the sample with the forced convection oven at 105°C for 2 hours.

(3) Perform Soxhlet extraction with diethyl ether for more than 16 hours into the Soxhlet flask.

(4) Evaporate the Soxhlet flask and dry it with the forced convection oven at 105°C for 1 hour.

(5) Cool in the desiccator and weigh the Soxhlet flask.

Lipid (%) is given in the following formula:

$$\textbf{Lipid (\%)} = (W_2 - W_1) \times 100 / S$$

W_1: Constant weight of the Soxhlet flask

W_2: Weight of the Soxhlet flask containing the sample extract after evaporation

S: Weight of the sample

8. Sodium (%)

(1) Weigh the sample and ignite at 550°C for about 12 hours.

(2) Extract and fill up with hydrochloric acid to constant volume.

(3) Measure with atomic absorption spectrophotometer using the following condition:

Wave Length: 589.6 nm

Flame: Acetylene - Air

9. Chloride (%)

(1) Weigh the sample and extract with ion-exchange water.

(2) Add diluted nitric acid and measure with potentiometric titrator using 0.1 mol/L silver nitrate standard solution.

10. Sulfate (%)

(1) Weigh the sample, add 10 ml of diluted hydrochloric acid (1→2), and heat on the hot plate.

(2) Filter through No. 5C filter paper and add water to about 100 ml.

(3) Neutralize and finally slightly acidify with diluted hydrochloric acid (1→2) and 50% sodium hydroxide solution using methyl orange as an indicator.

(4) Add 1 ml of diluted hydrochloric acid (1→2) and boil.

(5) Add 10 to 15 ml of 10% barium chloride solution and stand overnight.

(6) Filter through No. 5C filter paper and wash the precipitate using about 200 ml of water until the filtrate is chloride-free.

(7) Transfer the filter paper containing the precipitate into the porcelain crucible (previously weighed constant weight) and ignite at 500°C for 3 hours.

(8) Dry to constant weight at 750°C for 20 minutes.

(9) Cool in the desiccator and weigh the porcelain crucible.

Sulfate (%) is given in the following formula:

$$\textbf{Sulfate (\%)} = (W_1 - W_2) / W \times 0.4116 \times 100$$

W: Weight of the sample

W_1: Weight of the porcelain crucible containing the sample after igniting

W_2: Constant weight of the porcelain crucible

11. Calcium (%)

(1) Weigh the sample and ignite at 550°C for 5 to 6 hours.

(2) Dissolve with 20 % hydrochloric acid and evaporate to dryness on the hot plate.

(3) Dissolve with 20 % hydrochloric acid again and heat on the hot plate.

(4) Filter through No. 5A filter paper and fill up to constant volume.

(5) Measure with inductively coupled plasma-atomic emission spectrometer using the following wave length: **315.887 nm**.

12. Potassium (%)

(1) Weigh the sample and ignite at 550°C for about 12 hours.

(2) Extract and fill up with hydrochloric acid to constant volume.

(3) Measure with atomic absorption spectrophotometer using the following condition:

Wave Length: 766.5 nm

Flame: Acetylene - Air

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Chemical Regulation and Food safety

Exponent®

Estimated Intakes of Regular and Low Sodium Salt for The US Population

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VIII-2-1

Estimated Intakes of Regular and Low Sodium Salt for The US Population

Prepared for

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Acronyms and Abbreviations

CDC	Centers for Disease Control
FARE™	Foods and Residue Evaluation Program
NHANES	National Health and Nutrition Examination Survey
NCHS	National Center for Health Statistics
USDA	United States Department of Agriculture

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Executive Summary

At the request of Ajinomoto Corporate Services, LLC (Ajinomoto) Exponent has estimated the intake of salt from "low sodium" or "reduced sodium", regular salt, and total salt for the US population. Intakes of "optional" salt (used at table and in food preparation) as well as "non-optional" (e.g., used in food processing) were estimated. To estimate "low sodium" salt intakes from processed foods, Exponent derived the amount of salt intakes from processed foods identified as "low sodium" or "reduced sodium". To estimate "low sodium" table salt intakes, Exponent assumed that optional table salt intake for subjects who rarely or never use regular salt in food preparation or at the table is low sodium salt. Therefore, since "low sodium" and "reduced sodium" foods could include foods prepared with low sodium salt as well as foods prepared with less salt, and since subjects who rarely or never user regular salt in food preparation or at the table may not be using any salt at all, the estimates derived in this report are conservative estimates of the consumption of low sodium salt. More representative estimates may be obtained by conducting a targeted survey focusing on the frequency of use and amounts usually consumed of these salts during food preparation and at the table.

Introduction

At the request of Ajinomoto Corporate Services, LLC (Ajinomoto) Exponent has conducted an intake assessment to estimate the consumption of table top "regular" and "low sodium" salt using data from CDC's 2003-2004 National Health and Nutrition Examination Survey (NHANES), the most recent publicly available food consumption database. The purpose of the assessment is to derive data for estimation of intakes of a food additive that is used in a low sodium salt that Ajinomoto has developed.

Intakes estimates were derived for the total US population and five subpopulations: Infants and toddlers1-3 years old, Children 4-8 years old, Children 9-13 years old, Teenagers 14-18 years old, and Adults 19 years old or older.

Data and Methods

Dietary consumption of salt was estimated using Exponent's Foods and Residue Evaluation Program (FARE™ 8.13) software, and data from NHANES 2003-2004. The NHANES 2003-2004 (NCHS, 2007) is a complex multistage probability sample designed to be representative of the civilian U.S. population. The survey collects two days of food intake data, in addition to nutrition, demographic, and health information. The NHANES survey over-samples minorities, low-income groups, adolescents (12-19 years), and adults 60 years of age and older, and statistical weights are provided by the National Center for Health Statistics (NCHS) to adjust for the differential probabilities of selection. Participants included 10,122 subjects in 2003-2004. Only individuals with complete and reliable 2-day dietary records were included in the analysis (N=8,354).

Exponent estimated the daily intake on a per "user" basis. In this analysis, a "user" is anyone who reported consuming a food on either of the survey days (USDA's "user" definition). Specifically, each individual who reported consuming a food on either of the survey days is identified, and that individual's responses for both survey days are used in the assessment. Zero consumption days are included in calculating that individual's average daily intake. Provided the ingredient/contaminant of interest is not an acute toxicant or teratogen, it is appropriate to average exposures over a longer period than one day. Therefore, Exponent® used each respondent's food consumption averaged over the two days of the NHANES 2003-2004 survey. For example, if someone reported consuming 100 grams of bread on day 1 and 150 grams of bread on day 2, his/her 2-day average bread consumption would be 125 grams ([100+150]/2). A 2-day average typically overestimates long-term (chronic) daily intake; however, only two nonconsecutive days' worth of food consumption data are available in the NHANES 2003-2004 survey database.

Exponent used the statistically weighted values from the survey in its analyses. The statistical weights compensate for variable probabilities of selection, adjust for non-response, and provide intake estimates that are representative of the U.S. population and the selected age-gender subgroups.

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Low sodium or lite salt can be used by food processors in the manufacturing of "low sodium" or "reduced sodium" foods. In addition, consumers can use low sodium or lite table salt at home either during food preparation or at the table. Consumption data in the NHANES survey are reported on an "as consumed basis". That is, if a survey participant consumed an apple pie, the consumption amount reported in the survey for that subject would be for the amount of pie consumed, and not for the ingredients (flour, butter, apples, sugar, etc...) used to make that pie. Exponent has developed recipes that allow for the translation of foods as consumed into their various ingredients. These recipes build on "intermediate" recipes that have been developed by USDA and CDC and are used in Exponent's FARE software. "Table salt" is an ingredient that is captured in these recipes, however, the recipes do not identify the type of salt, as "reduced sodium/lite" or "regular" salt. NHANES has included questions about the frequency of use of regular salt during cooking and at the table and has generated two sets of estimates for sodium content of foods, one that refers to the total sodium content of foods and one that excludes the contribution of "optional salt". Exponent obtained these databases and estimated the optional salt content of all foods reported in the NHANES survey and estimated the intakes of low sodium and regular salt as follows:

- Low sodium salt from processed foods: intakes were derived using the salt content of "reduced sodium" and "low sodium" foods (see Appendix 1 for the list of foods included).

- Table top low sodium salt: intakes were derived using the optional salt content for those survey participants who reported that they rarely add **regular** salt at the table and never or rarely add **regular** salt during food preparation. Specifically, the sodium content of foods due to optional salt use was derived as the difference between the total sodium content and the sodium content excluding optional salt. The optional salt content was then estimated by back-calculation from the sodium content, assuming 0.393 g of sodium to 0.607 g of chloride in 1g of salt. Optional salt intake was then derived for all subjects. For subjects who reported they rarely add **regular** salt at the table and never or rarely add **regular** salt during food preparation, optional salt intake was assumed to be "low sodium" table salt intake. For all other subjects, the optional salt intake was assumed to be regular table salt intake.

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- Regular salt: All other salt intakes, including salt in processed foods and optional table salt were used to represent "regular" (i.e., non low-sodium') salt intakes.

Figure 1 summarizes the approach used in estimating salt intakes for each subject.

The intake of salt is based on 2-day average intakes among subjects with 2 days of valid intake data in NHANES 2003-2004. Each individual's intake of salt, presented in g/day was divided by his/her bodyweight to provide the *per capita* and *per user* intakes on a bodyweight basis. The results are presented in g/day and g/kg-bw/day on a *per capita* and *per user* basis. Analyses were conducted using Exponent's proprietary software program FARE™ version 8.13 and SPSS® Version 7.5.3. The subgroups selected for this consumption analysis include the U.S. population, infants and toddlers 1-3 years of age, children 4-8 and children 9-13 years of age, teenagers 14-18 years of age, and adults 19 years and over.

Results and Discussion

Tables 1A and 1B summarize the estimated intake of "low sodium" salt, based on the salt content of "reduced sodium" or "low sodium" processed foods listed in Appendix 1. Table 1A provides estimates g/day, while Table 1B provides the estimates in g/kg bw/day. The proportion of the subjects reporting consumption of "reduced" or "low" sodium processed foods in the NHANES 2003-2004 survey is 27%. Per user mean and 90th percentile estimates are 0.30 g/day and 0.68 g/day, respectively.

Tables 2A and 2B summarize the estimated intake of low sodium table salt. The intakes are estimated by assuming that the optional salt intake of subjects who reported that they never or rarely use regular salt in food preparation or at the table represents low sodium table salt intake. The estimated proportion of subjects consuming low sodium table salt is 7%. Per user mean and 90th percentile estimates are 2.23 g/day and 4.08 g/day, respectively.

Tables 3A and 3B summarize the total low sodium intake for NHANES survey participants. The intake estimates are derived by adding for each survey participants their estimated intakes of low sodium table salt and low sodium salt from low or reduced sodium processed foods. Per user mean and 90th percentile estimates are 0.76 g/day and 2.16 g/day, respectively.

Table 4A and 4B summarize the regular table (optional) salt intake for NHANES survey participants. The intake estimates are derived as the total optional salt intake that is not assumed to be low sodium salt (that is, excluding the salt intake included in Table 2 above). Per user mean and 90th percentile estimates are 2.11 g/day and 4.00 g/day, respectively.

Table 5A and 5B summarize the regular non-optional salt intake for NHANES survey participants. The intake estimates are derived as the total regular salt intake that is not assumed to be optional (that is, excluding the salt intake estimated in Table 4 above). Per user mean and 90th percentile estimates are 4.90 g/day and 8.80 g/day, respectively.

Table 6A and 6B summarize the total regular salt intake for NHANES survey participants. The intake estimates are derived as the total salt intake that is not assumed to be low sodium salt (that is, excluding the salt intake included in Table 3 above). Per user mean and 90th percentile estimates are 6.81 g/day and 11.22 g/day, respectively.

Tables 7A and 7B summarize the estimated daily intake of total table top (optional) salt for NHANES 2003-2004 participants. The intakes are derived by adding intakes of low sodium table salt and regular table top (optional) salt. Per user mean and 90th percentile estimates for the U.S. population are 2.12 g/day and 4.01 g/day, respectively.

Tables 8A and 8B summarize the estimated daily intake of total (both regular and low sodium) table (optional) salt and low sodium salt from processed foods for NHANES 2003-2004 participants. The intakes are derived by adding intakes of low sodium table salt, low sodium salt from low or reduced sodium processed foods, and regular table top (optional) salt. Per user mean and 90th percentile estimates for the U.S. population are 2.19 g/day and 4.14 g/day, respectively.

Tables 9A and 9B summarize the estimated daily intake of total (table top or optional and non-optional) salt (low sodium and regular) for NHANES 2003-2004 participants. Per user mean and 90th percentile estimates for the U.S. population are 7.06 g/day and 11.6 g/day, respectively.

The FDA Guidance on Sodium Claims[1] defines a reduced or low sodium food as a food that has that at least 25 percent less sodium per serving than the reference food. The intake estimates for low sodium salt derived in this report for processed foods are based on the assumption that low sodium processed foods are made with low sodium salt. However, while low sodium foods can be prepared with low sodium salt, some may simply include a lower amount of regular salt.

Further, the estimates of low sodium table salt intakes provided in this report are based on the assumption that subjects who reported that they rarely or never use regular salt in food preparation or at the table use low sodium or lite salt instead, which may not be true, as these subjects may be not using any type of salt. Thus the estimates provided in this reported are

[1] http://www.fda.gov/fdac/foodlabel/sodium.html

likely conservative estimates (overestimates) of the low sodium salt intakes in the US. Unfortunately, NHANES did not collect quantitative information on the frequency and amount of use of lite salt or salt substitute at the table or during food preparation, hence it is not possible to confirm our assumption that those subjects who rarely or never use regular salt at the table or in food preparation are using alternative types of salt. However, NHANES included a question on the first dietary interview day to assess the type of salt usually added at the table. Table 10 summarizes the reported answers to this question. Sixty six percent of the respondents said that they usually add regular salt at the table, while 3.8% said that usually add lite salt or salt substitute at the table. This estimate (3.8%) is slightly lower than the estimate derived above for the fraction of the subjects who use low sodium table salt during cooking or at the table (7%). The difference could either be due to the added fraction of subjects who use reduced sodium salt during food preparation or could reflect the potential overestimation in our estimates of low sodium table salt intakes.

Conclusion

Exponent has estimated the intake of salt from "low sodium" or "reduced sodium" foods using data from CDC's 2003-2004 NHANES. These estimates were used to represent the consumption of "low sodium" salt in processed foods. In addition estimates of low sodium table salt were derived using information on sodium intake from optional salt, and frequency of use of regular salt at the table and in food preparation. Since "low sodium" and "reduced sodium" foods could include foods prepared with low sodium salt as well as foods prepared with less salt, the estimates derived in this report are conservative estimates of the consumption of low sodium salt from these foods. The estimates of low sodium table salt intakes were derived using information on the frequency of use of regular salt in food preparation or at the table. Since subjects who rarely or never user regular salt in food preparation or at the table may not be using any salt at all, the estimates derived in this report are conservative estimates of the consumption of low sodium table salt. A more representative estimate may be obtained by conducting a targeted survey focusing on the frequency and amount of consumption of these salts and of processed foods made with these salts by consumers.

Figure 1. Approach used estimating salt intakes for each NHANES survey participant

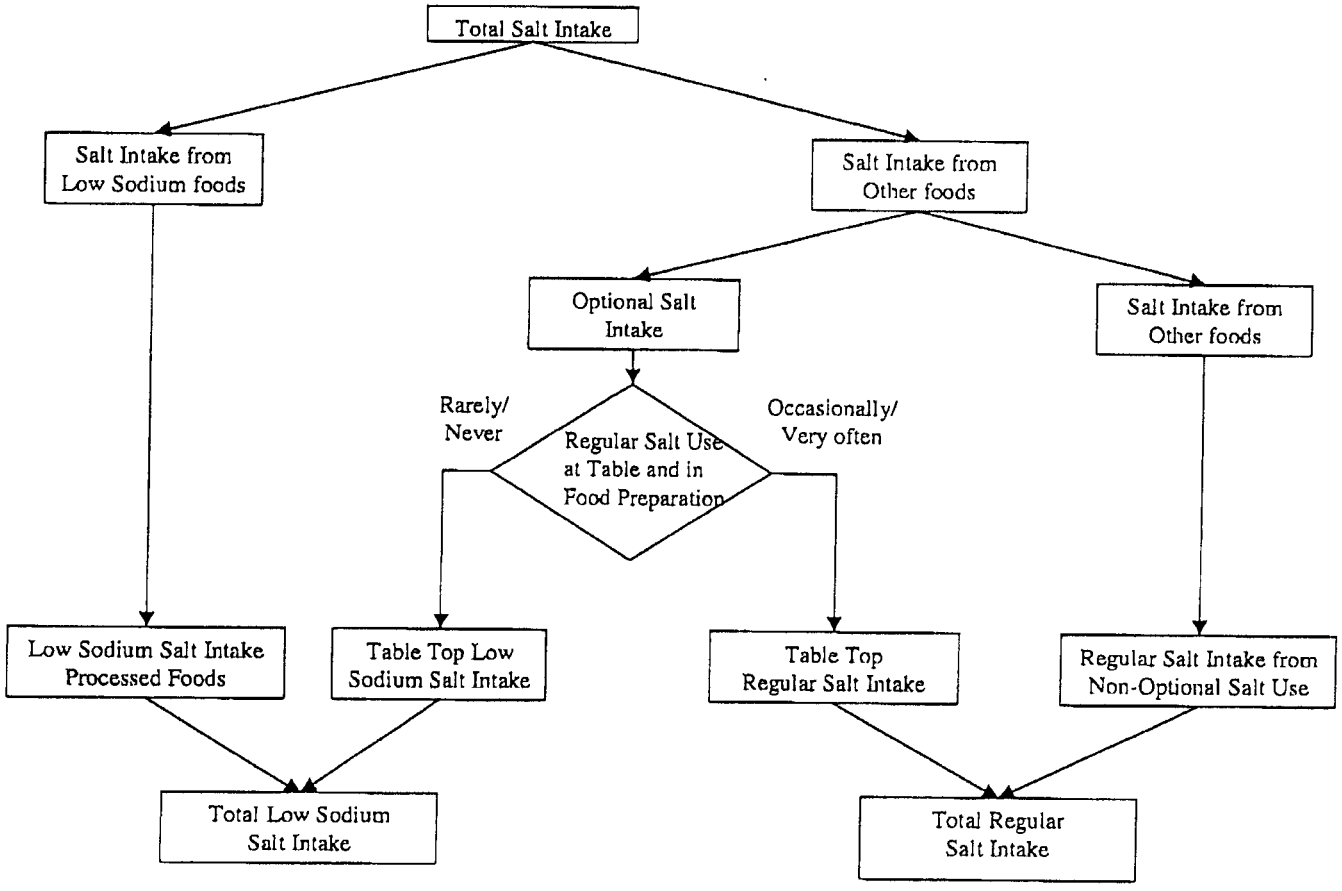

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Table 1A. Estimated Daily Intake of Salt from "Low or Reduced" Sodium Processed Foods (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	2467	27	0.08	0.25	0.30	0.68
Infants 1-3	215	31	0.05	0.15	0.15	0.30
Children 4-8	333	42	0.12	0.30	0.28	0.60
Children 9-13	369	39	0.11	0.31	0.28	0.60
Teenagers 14-18	456	35	0.11	0.30	0.30	0.76
Adults 19+	1077	24	0.08	0.20	0.32	0.75

Table 1B. Estimated Daily Intake of Salt from "Low or Reduced" Sodium Processed Foods (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	2467	27	0.002	0.004	0.006	0.013
Infants 1-3	215	31	0.003	0.009	0.011	0.023
Children 4-8	333	42	0.005	0.014	0.012	0.024
Children 9-13	369	39	0.002	0.007	0.006	0.013
Teenagers 14-18	456	35	0.002	0.005	0.005	0.012
Adults 19+	1077	24	0.001	0.003	0.004	0.009

Table 2A. Estimated Daily Intake of Low Sodium Table Salt (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	571	7	0.16	0.00	2.22	4.08
Infants 1-3	20	3	0.03	0.00	1.01	2.15
Children 4-8	40	6	0.06	0.00	1.02	2.08
Children 9-13	77	6	0.10	0.00	1.57	4.78
Teenagers 14-18	98	10	0.20	0.00	2.08	3.49
Adults 19+	334	8	0.19	0.00	2.41	4.32

Table 2B. Estimated Daily Intake of Low Sodium Table Salt (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	571	7	0.002	0.000	0.033	0.062
Infants 1-3	20	3	0.002	0.000	0.076	0.152
Children 4-8	40	6	0.003	0.000	0.045	0.102
Children 9-13	77	6	0.002	0.000	0.033	0.080
Teenagers 14-18	98	10	0.003	0.000	0.034	0.061
Adults 19+	334	8	0.002	0.000	0.031	0.055

Table 3A. Estimated Total Daily Intake of Low Sodium Salt (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	2852	32	0.25	0.60	0.76	2.16
Infants 1-3	229	32	0.07	0.15	0.23	0.60
Children 4-8	353	45	0.18	0.60	0.40	0.96
Children 9-13	413	42	0.20	0.45	0.48	1.46
Teenagers 14-18	515	40	0.31	0.83	0.76	2.21
Adults 19+	1323	30	0.26	0.60	0.89	2.68

Table 3B. Estimated Total Daily Intake of Low Sodium Salt (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	2852	32	0.004	0.009	0.012	0.035
Infants 1-3	229	32	0.005	0.012	0.017	0.045
Children 4-8	353	45	0.008	0.018	0.017	0.042
Children 9-13	413	42	0.004	0.011	0.011	0.030
Teenagers 14-18	515	40	0.005	0.014	0.012	0.031
Adults 19+	1323	30	0.003	0.008	0.011	0.035

Table 4A. Estimated Daily Intake of Regular Table (Optional) Salt (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	7332	90	1.90	3.87	2.11	4.00
Infants 1-3	610	93	0.87	1.87	0.93	1.89
Children 4-8	662	90	1.22	2.55	1.36	2.64
Children 9-13	828	90	1.44	2.94	1.59	2.98
Teenagers 14-18	1124	87	1.76	3.97	2.03	4.19
Adults 19+	3956	91	2.11	4.09	2.33	4.21

Table 4B. Estimated Daily Intake of Regular Table (Optional) Salt (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	7332	90	0.031	0.064	0.034	0.067
Infants 1-3	610	93	0.065	0.134	0.069	0.137
Children 4-8	662	90	0.052	0.114	0.058	0.114
Children 9-13	828	90	0.033	0.071	0.037	0.075
Teenagers 14-18	1124	87	0.027	0.060	0.031	0.063
Adults 19+	3956	91	0.027	0.053	0.030	0.056

000106

Table 5A. Estimated Daily Intake Non-Optional Regular Salt (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	8148	99	4.87	8.78	4.90	8.80
Infants 1-3	656	100	3.43	5.67	3.43	5.67
Children 4-8	726	100	4.96	7.89	4.96	7.89
Children 9-13	930	100	5.23	8.64	5.23	8.64
Teenagers 14-18	1258	100	5.34	9.10	5.34	9.10
Adults 19+	4359	100	4.94	9.00	4.94	9.00

Table 5B. Estimated Daily Intake of Non-Optional Regular Salt (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	8148	99	0.088	0.176	0.088	0.176
Infants 1-3	656	100	0.256	0.421	0.257	0.421
Children 4-8	726	100	0.209	0.335	0.209	0.335
Children 9-13	930	100	0.123	0.206	0.123	0.206
Teenagers 14-18	1258	100	0.084	0.145	0.084	0.145
Adults 19+	4359	100	0.064	0.118	0.064	0.118

Table 6A. Estimated Daily Intake of Total Regular Salt (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	8148	99	6.77	11.22	6.81	11.22
Infants 1-3	656	100	4.30	6.85	4.30	6.85
Children 4-8	726	100	6.18	9.92	6.18	9.92
Children 9-13	930	100	6.67	10.83	6.67	10.83
Teenagers 14-18	1258	100	7.11	11.44	7.11	11.44
Adults 19+	4359	100	7.05	11.66	7.05	11.66

Table 6B. Estimated Daily Intake of Total Regular Salt (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	8148	99	0.118	0.222	0.119	0.223
Infants 1-3	656	100	0.321	0.516	0.321	0.516
Children 4-8	726	100	0.262	0.410	0.262	0.410
Children 9-13	930	100	0.157	0.272	0.157	0.272
Teenagers 14-18	1258	100	0.112	0.184	0.112	0.184
Adults 19+	4359	100	0.091	0.156	0.091	0.156

000108

Table 7A. Estimated Daily Intake of Total Optional Salt (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	7903	97	2.06	3.97	2.12	4.01
Infants 1-3	630	96	0.90	1.88	0.94	1.89
Children 4-8	702	95	1.28	2.57	1.34	2.62
Children 9-13	905	96	1.53	2.98	1.59	3.04
Teenagers 14-18	1222	97	1.97	4.15	2.03	4.19
Adults 19+	4290	99	2.30	4.20	2.33	4.24

Table 7B. Estimated Daily Intake of Total Optional Salt (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	7903	97	0.033	0.066	0.034	0.067
Infants 1-3	630	96	0.067	0.135	0.070	0.139
Children 4-8	702	95	0.055	0.114	0.058	0.114
Children 9-13	905	96	0.035	0.075	0.037	0.075
Teenagers 14-18	1222	97	0.030	0.061	0.032	0.061
Adults 19+	4290	99	0.030	0.055	0.030	0.056

000109

Table 8A. Estimated Daily Intake of Total Optional Salt and Low Sodium Salt from Processed Foods (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	7958	98	2.14	4.11	2.19	4.14
Infants 1-3	636	97	0.94	1.92	0.97	1.93
Children 4-8	712	97	1.40	2.77	1.44	2.80
Children 9-13	915	99	1.64	3.16	1.66	3.16
Teenagers 14-18	1230	97	2.07	4.19	2.13	4.26
Adults 19+	4307	99	2.38	4.35	2.40	4.35

Table 8B. Estimated Daily Intake of Total Optional Salt and Low Sodium Salt from Processed Foods (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	7958	98	0.035	0.069	0.036	0.070
Infants 1-3	636	97	0.070	0.144	0.072	0.146
Children 4-8	712	97	0.060	0.118	0.062	0.119
Children 9-13	915	99	0.038	0.079	0.038	0.079
Teenagers 14-18	1230	97	0.032	0.063	0.033	0.064
Adults 19+	4307	99	0.031	0.057	0.031	0.057

Table 9A. Estimated Daily Intake of Total Salt (g/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/day) Mean	Per Capita (g/day) 90th Percentile	Per User (g/day) Mean	Per User (g/day) 90th Percentile
US population	8148	99	7.01	11.5	7.06	11.6
Infants 1-3	656	100	4.37	6.92	4.38	6.92
Children 4-8	726	100	6.36	10.5	6.36	10.5
Children 9-13	930	100	6.87	11.2	6.87	11.2
Teenagers 14-18	1258	100	7.41	12.0	7.41	12.0
Adults 19+	4359	100	7.32	12.0	7.32	12.0

Table 9B. Estimated Daily Intake of Total Salt (g/kg bw/day), NHANES 2003-2004

Subpopulations	Unweighted Number of Users	% Users	Per Capita (g/kg bw/day) Mean	Per Capita (g/kg bw/day) 90th Percentile	Per User (g/kg bw/day) Mean	Per User (g/kg bw/day) 90th Percentile
US population	8148	99	0.12	0.23	0.12	0.23
Infants 1-3	656	100	0.33	0.52	0.33	0.52
Children 4-8	726	100	0.27	0.43	0.27	0.43
Children 9-13	930	100	0.16	0.27	0.16	0.27
Teenagers 14-18	1258	100	0.12	0.19	0.12	0.19
Adults 19+	4359	100	0.09	0.16	0.09	0.16

Table 10. Type of salt usually added at the table (NHANES 2003-2004)

Type of salt added at table	Percent
Ordinary salt	65.8
Lite salt	2.7
Salt substitute	1.1
Does not add salt at table	29.7
Other	0.3
Don't know	0.4

Appendix 1: Low or Reduced Sodium Foods Reported Consumed in the 2003-2004 NHANES Survey

14107210	Cheese, muenster, low sodium
14108050	Cheese, Parmesan, low sodium
14108800	Cheese, semi-soft, low sodium
14109020	Cheese, Swiss, low sodium
14110010	Cheese, Cheddar or Colby, low sodium
14410210	Cheese, processed, American or Cheddar type, low sodium
22602010	Pork bacon, smoked or cured, lower sodium
25220470	Bologna, beef, lower sodium
25230220	Ham, sliced, low salt, prepackaged or deli, luncheon meat
25230550	Ham, pork, and chicken, luncheon meat, chopped, minced, pressed, spiced, canned, reduced sodium
28141300	Chicken with rice and vegetable, reduced fat and sodium (diet frozen meal)
28143220	Chicken in barbecue sauce, with rice, vegetable and dessert, reduced fat and sodium (diet frozen meal)
28316020	Beef and mushroom soup, canned, low sodium
28345030	Chicken or turkey soup, cream of, canned, reduced sodium, made with water
28345040	Chicken or turkey soup, cream of, canned, reduced sodium, undiluted
28355140	Clam chowder, New England, canned, reduced sodium, ready-to-serve
41221010	Baked beans, low sodium
41420350	Soy sauce, reduced sodium
41420410	Teriyaki sauce, reduced sodium
41602070	Split pea soup, canned, reduced sodium, prepared with water or ready-to-serve
42202010	Peanut butter, low sodium
42202100	Peanut butter, reduced sodium
51130520	BREAD, WHITE, LOW SODIUM/NO SALT, TOASTED
51184100	BREAD STICK, HARD, LOW SODIUM
51300610	BREAD, WHOLE WHEAT, NS AS TO 100%, LOW SODIUM
51300620	BREAD, WHOLE WHEAT, NS 100%, LOW SODIUM, TOAST
54201010	CRACKERS, MATZO, LOW SODIUM
54202010	CRACKERS, SALTINE, LOW SODIUM
54202050	CRACKERS, SALTINE, FAT FREE, LOW SODIUM
54203010	CRACKERS, TOAST THINS (RYE/WHEAT/WHITE), LOW SODIUM
54204010	CRACKER, 100% WHOLE WHEAT,LO SODIUM
54205010	CRACKER, SNACK, LOW SODIUM
54205030	CRACKER, CHEESE, LOW SODIUM
54205100	CRACKER, SNACK, LOWFAT, LOW SODIUM
54403060	Popcorn, popped in oil, lowfat, low sodium
58301080	Lasagna with cheese and meat sauce, reduced fat and sodium (diet frozen meal)
58302060	Spaghetti or noodles with beef in tomato-based sauce, lowfat, reduced sodium (diet frozen meal)
58403030	Chicken noodle soup, canned, low sodium, ready-to-serve

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58403060	Chicken noodle soup, canned, reduced sodium, ready-to-serve
58404040	Chicken rice soup, canned, reduced sodium, prepared with water or ready-to-serve
71103210	White potato, boiled, without peel, canned, low sodium, fat not added in cooking
71201200	White potato, chips, restructured, reduced fat and reduced sodium
72128510	Turnip greens, canned, low sodium, cooked, fat not added in cooking
73103010	Carrots, canned, low sodium, fat not added in cooking
73103020	Carrots, canned, low sodium, fat added in cooking
73111270	Peas and carrots, canned, low sodium, fat not added in cooking
74204500	Tomatoes, canned, low sodium
74301150	Tomato juice, low sodium
74303100	Tomato and vegetable juice, mostly tomato, low sodium
74401010	Tomato catsup
74402010	Tomato chili sauce (catsup-type)
74404050	Spaghetti sauce, meatless, low sodium
74406050	Barbecue sauce, low sodium
74602100	Tomato soup, canned, low sodium, ready-to-serve
74602200	Tomato soup, canned, reduced sodium, prepared with water
74602300	Tomato soup, canned, reduced sodium, prepared with milk
75204110	Beans, lima, immature, canned, low sodium, fat not added in cooking
75205110	Beans, string, green, canned, low sodium, NS as to fat added in cooking
75205120	Beans, string, green, canned, low sodium, fat not added in cooking
75205130	Beans, string, green, canned, low sodium, fat added in cooking
75208110	Beets, canned, low sodium, fat not added in cooking
75208120	Beets, canned, low sodium, fat added in cooking
75216310	Corn, yellow, canned, low sodium, fat not added in cooking
75216320	Corn, yellow, canned, low sodium, fat added in cooking
75224120	Peas, green, canned, low sodium, fat not added in cooking
75224130	Peas, green, canned, low sodium, fat added in cooking
75311110	Mixed vegetables (corn, lima beans, peas, green beans, and carrots), canned, low sodium, fat not added in cooking
75311120	Mixed vegetables (corn, lima beans, peas, green beans, and carrots), canned, low sodium, fat added in cooking
75503110	Cucumber pickles, dill, reduced salt
75503140	Cucumber pickles, sweet, reduced salt
75607050	Mushroom soup, cream of, low sodium, prepared with water
75607140	Mushroom soup, cream of, canned, reduced sodium, prepared with water
75607150	Mushroom soup, cream of, canned, reduced sodium, undiluted
75609050	Pea soup, canned, low sodium, prepared with water
75649030	Vegetable soup, canned, low sodium, prepared with water or ready-to-serve
75650990	Minestrone soup, canned, reduced sodium, ready-to-serve
75651090	Vegetable chicken soup, canned, low sodium, prepared with water
75651150	Vegetable noodle soup, canned, reduced sodium, prepared with water or ready-to-serve

Final Report

A Single Oral Dose Toxicity Study of Poly-γ-glutamic Acid (γ-PGA) in Rats

Project No. H-01074

May 28, 2001

Nippon Experimental Medical Research Institute Co., Ltd.

STATEMENT OF COMPLIANCE

Study title : A Single Oral Dose Toxicity Study of Poly-γ-glutamic Acid(γ-PGA) in Rats

Project No. H-01074

I, the undersigned, hereby declare that this report is the correct translated English version of the original report that written in Japanese language.

(b) (6)

Date: Jan. 22, 2002

Masaaki Shirai

Study Director (translator)

Nippon Experimental Medical Research Institute Co. Ltd.

Approval of the Final Report

Study title: A Single Oral Dose Toxicity Study of Poly-γ-glutamic Acid (γ-PGA) in Rats
Project No.H-01074

I, the undersigned, hereby declare that this study was conducted in non-GLP, while this final report precisely reflects the raw data in the study.

Study Director: Masaaki Shirai
Date: May 28, 2001
Nippon Experimental Medical Research Institute Co., Ltd.

Study title: A Single Oral Dose Toxicity Study of Poly-γ-glutamic Acid (γ-PGA) in Rats
Project No.H-01074

1. Study purpose

To test for toxic signs following exposure of poly-γ-glutamic acid to rats and assess the approximate lethal dose

2. Sponsor

Name: Food Research & Development Laboratories, Ajinomoto Co., Inc.
Address: 1-1 Suzuki-machi, Kawasaki-ku, Kawasaki-shi

5. Contract laboratory

Name: Nippon Experimental Medical Research Institute Co., Ltd.
Address: 3303-58 Ohdo, Agatsuma-machi, Agatsuma-gun, Gunma-ken

6. Testing facility

Name: Haruna Laboratory, Nippon Experimental Medical Research Institute Co., Ltd.
Address: 3303-58 Ohdo, Agatsuma-machi, Agatsuma-gun, Gunma-ken
Managing Director: Masaaki Kashima

5. Retention of records and data

(1) Retention period: for 5 years after completion of the study

(2) Storage items and location

The following items will be stored in the specified areas of Haruna Laboratory, Nippon Experimental Medical Research Institute Co., Ltd.

1) Study protocol, documents relating to the study, final report (original), raw data obtained in the study: Raw data storehouse
2) Data, etc.: Data storeroom

Study title: A Single Oral Dose Toxicity Study of Poly-γ-glutamic Acid (γ-PGA) in Rats
Project No.H-01074

6. Study Schedule

Study initiation:	April 20, 2001
Animal delivery:	April 20, 2001
Quarantine end:	April 26, 2001
Acclimatization end:	April 30, 2001
Grouping:	April 30, 2001
Experimental start:	May 1, 2001
Administration:	May 1, 2001
Necropsy:	May 15, 2001
Experimental end:	May 15, 2001
Preparation of a draft final report:	May 16, 2001
Preparation of the final report:	May 28, 2001
Study completion:	May 28, 2001

7. Work assignment and responsible personnel

Study director, protocol preparation, final report preparation, work instruction/management and test substance preparation/management:

	Masaaki Shirai*)
Animal health assessment:	Michiko Takahashi
Test substance administration:	Masaaki Shirai, Tasaburo Hashizume
Clinical observation:	Masaaki Shirai, Junji Katagai, Tasaburo Hashizume, Kishio Hashizume, Masahiko Ota, Mitsuo Hashizume, Akira Fukutome
Body weight measurement:	Masaaki Shirai, Tasaburo Hashizume, Junji Katakai, Mitsuo Hashizume
Necropsy:	Masaaki Shirai
Statistical analysis:	Yukio Tanaka, Masahiro Kasumi, Wataru Koike, Yukiko Takebuchi

*) Post: Safety Research No.1 Dept., Nippon Experimental Medical Research Institute Co., Ltd.

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I. Summary

Single oral doses of poly-γ-glutamic acid (γ-PGA) at 0 (vehicle control), 2500 and 5000 mg/kg was administered by gavage to 5 SD [Crj:CD(SD)IGS] rats per sex per group. The rats were observed for clinical signs for the following 15 days to determine the approximate lethal dose of the test substance. As a result, no death was observed for either sex at any doses tested during the observation period.

For clinical signs, watery diarrhea was observed in more than half the animals of the 2500 and 5000 mg/kg groups at 3 or 6 hours after treatment, accompanied by soiled peritoneal region. These signs disappeared or tended to disappear by Day 2 of administration, and there was no special findings observed on and after Day 3.

The male and female body weights of each treatment group did not significantly differ from those of the vehicle control group, indicating a robust body weight gain.

Necropsy revealed no treatment related changes in either male or female rats in any groups including vehicle controls.

Based on the above findings, we conclude that the approximate lethal dose of γ-PGA is 5000 mg/kg or more either in the male or female rat.

II. Study purpose

To test for toxic signs following exposure of poly-γ-glutamic acid to rats and assess the approximate lethal dose

III. Materials and methods

1. Test substance

(1) Name:	Poly-γ-glutamic acid (γ-PGA)
(2) Lot No.:	001GP01
(3) Appearance:	White to light brown powder
(4) Constituent:	γ-PGA
(5) Molecular weight:	28000
(6) Manufacture date:	December 27, 2000
(7) Expiration date:	December 26, 2001 (for 1 year after manufacture)
(8) Storage conditions:	At room temperature away from the light
(9) Supplier:	
Name:	Healthy & Nutritional Food Development Section, Food Research & Development Laboratories, Ajinomoto Co., Inc.
Address:	1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi
(10) Disposition of the remaining test substance:	The remaining test substance was returned to the sponsor after the completion of the study.

2. Test substance preparations

An amount of the test substance was weighed with an electronic balance (Sartrius Co., Ltd.) and suspended in water for injection to yield concentrations of 125 and 250 mg/mL. The stability and homogeneity of the test substance preparations were checked by visual inspection, and no heat, foam, discoloration, etc. were found to be produced. The preparation was carried out upon use.

3. Test animals and environmental conditions

(1) Test animals

Thirty-five SD[Crj:CD(SD)IGS] rats of each sex, 5 weeks of age (body weights at delivery: males: 128-139 g; females: 87-103 g), were purchased from Atsugi Breeding Center, Charles River Japan Inc. (795, Shimofurusawa, Atsugi-shi, Kanagawa, Japan). After the following 7 days of quarantine period, the animals were assessed for the state of health, and healthy animals were acclimatized for the following 4 days. At the end of the acclimatization period, the animals were distributed to groups by random sampling

with stratified body weights. The animals were 6 weeks old at the start of administration. The remaining animals were discarded as pool animals

(2) Environmental conditions

The animals were housed individually in stainless bracket cages for rats (260W×380D×180H mm) in a barrier system animal room (Room No.2 of Building E) under the following set conditions: temperature: 22±3°C (actual values: 20-23 C); relative humidity: 50±20% (actual values: 53-69%); ventilation frequency: 10 times/hr, all-fresh-air system; lighting: 12 hr/day (6:00 a.m. to 6:00 p.m., 150-300 lux). The animals were allowed free access to pellet chow for experimental animals CE-2 (Lot No.E2021, Clea Japan Inc, 2-20-14 Aobadai, Meguro-ku, Tokyo, Japan) in feeders, and household tap water in polycarbonate watering bottles (500 mL).

Cages, feeders, trays and watering bottles were autoclaved (121°C, 30 min) before use, and the trays and watering bottles were changed at least twice a week. The animal room was cleaned after work every day, and the floor was sterilized by wiping with a 400-fold dilution of benzethonium chloride (Hyamine, Sankyo Co., Ltd.).

(3) Identification of cages and animals

Animals were identified with abbreviated codes of the animal numbers marked on the root of the tail with an oil-marker pen. Cages were identified with affixed colored cards showing Project No., group names, administration route, animal species, name of test substance, date of administration, animal numbers, etc.

(4) Impurity and contaminant analysis of feed and water

Feed was analyzed by the lot for impurity and contamination by Tokyo Kenbikyo-in (44-1, Hakozaki-cho, Nihonbashi, Chuo-ku, Tokyo, Japan) at the request of the manufacturer. A copy of the results was obtained and checked for integrity before serving the feed to animals. Drinking water was periodically sampled (August 3, 1999, August 8, 2000 and April 3, 2001) by our institute at a specified location, and analyzed for impurity and contamination by Environmental Hygiene Laboratory Center of Gunma Pharmaceutical Association (5-18-36 Nishikatagai-cho, Maebashi-shi, Gunma, Japan). The results conformed to the standards specified by this institute or water quality standards (1992) specified in the Water Works Law of the Ministry of Health and Welfare, Japan.

4. Group designs and dose levels

We set up the following 3 groups consisted of 5 animals per sex per group.

(1) Dose levels, dose volume and number of animals used

Group	Test substance	Dose (mg/kg)	Volume (mL/kg)	No. of animals used Male	Female
00	Water for injection (vehicle control)	0	20	5	5
01	γ-PGA	2500	20	5	5
02	γ-PGA	5000	20	5	5
Total				15	15

(2) Animal numbers

Group	Test substance	Animals numbers Male	Female
00	Water for injection (vehicle control)	00M01-00M05	00F01-00F05
01	γ-PGA	01M01-01M05	01F01-01F05
02	γ-PGA	02M01-02M05	02F01-02F05

(3) Rationale for the dose selection

According to the instructions from the sponsor, dose levels of 2500 and 5000 mg/kg and vehicle control were selected.

5. Route, procedures and frequency of administration

The treatment was administered orally by gavage. The animals were fasted for about 18 hours before administration. The dose volume (20 mL/kg) was determined based on the body weights taken just before administration (body weight range: males: 194-206 g; females: 128-138 g), and the treatment was given once in the morning by gavage using a disposable syringe (2.5 mL capacity) with a gastric probe for rats. Feed was served at least 3 hours after administration.

6. Observation, measurement and examination

The day of administration was referred to as Day 1 of administration, and the following observations, measurements and examinations were performed on all animals in all groups up to Day 15.

(1) Clinical signs and mortality

Animals were observed for clinical signs and mortality at approximately 15 min, then 30 min, 1, 3 and 6 hr on Day 0, and thereafter once daily for 15 days.

(2) Body weight

Body weights were determined with an electronic balance (Sartrius Co., Ltd.) on Day 1 (before treatment) and on Days 2, 3, 4, 8, 11 and 15.

(3) Necropsy

The surviving animals were killed by exsanguination under ether anesthesia by cutting the abdominal aorta on Day 15. The body surface and intracranial, intrathoracic and intraabdominal organs were macroscopically examined.

7. Statistical analysis

The body weights taken in this study were represented as mean ± S.D., and F-test was performed for analysis of variance. Then the vehicle control group was compared with the treatment groups using Student *t*-test in the case of homogeneous variance; or using Aspin-Welch *t*-test in the case of inhomogeneous variance. As a result, there was no significant difference between the vehicle control group and each test substance group.

IV. Results

1. Mortality and approximate lethal dose (Tables 1, 2)

No deaths occurred during the observation period in either male or female rats in any of the treatment groups. The approximate lethal dose of the test substance was estimated to be 5000 mg/kg or more for either the male or female rat.

2. Clinical signs (Tables 3, 4, Appendices 1, 2)

In the 2500 mg/kg group, watery diarrhea and soiled peritoneal region were observed in 3/5 males and 4/5 females at 6 hours after administration.

In the 5000 mg/kg group, watery diarrhea was observed in all male and female rats, and soiled peritoneal region in all male rats from 3 to 6 hours after treatment, and then soiled peritoneal region was observed in 1 male on Day 2 of administration.

Most of the above findings disappeared or tended to disappear by Day 2, and there was no special findings observed on and after Day 3.

No special findings were observed in the control group throughout the observation period.

3. Body weight changes (Figs. 1, 2, Tables 5, 6, Appendices 3, 4)

The body weight changes of the male and female rats in each treatment group did not significantly differ from those of the vehicle controls, indicating a robust body weight gain.

4. Necropsy (Tables 7, 8, Appendices 5, 6)

No treatment-related changes were observed either in the male or female rats in any groups including vehicle controls.

V. Discussion and conclusion

Single oral doses of poly-γ-glutamic acid (γ-PGA) at 0 (vehicle control), 2500 and 5000 mg/kg was administered by gavage to 5 SD [Crj:CD(SD)IGS] rats per sex per group. The rats were observed for clinical signs for the following 15 days to determine the approximate lethal dose of the test substance. As a result, no death was observed for either sex at any doses tested during the observation period.

For clinical signs, watery diarrhea was observed in more than half the animals of the 2500 and 5000 mg/kg groups at 3 or 6 hours after treatment, accompanied by soiled peritoneal region. These changes are attributable to the fact that a substance with low absorbability was administered in a large amount. The signs disappeared or tended to disappear by Day 2 of administration, and there was no special findings observed on and after Day 3.

The male and female body weights of each treatment group did not significantly differ from those of the vehicle control group, indicating a robust body weight gain.

Necropsy revealed no treatment related changes in either male or female rats in any groups including vehicle controls.

Based on the above findings, we conclude that the approximate lethal dose of γ-PGA is 5000 mg/kg or more either in the male or female rat.

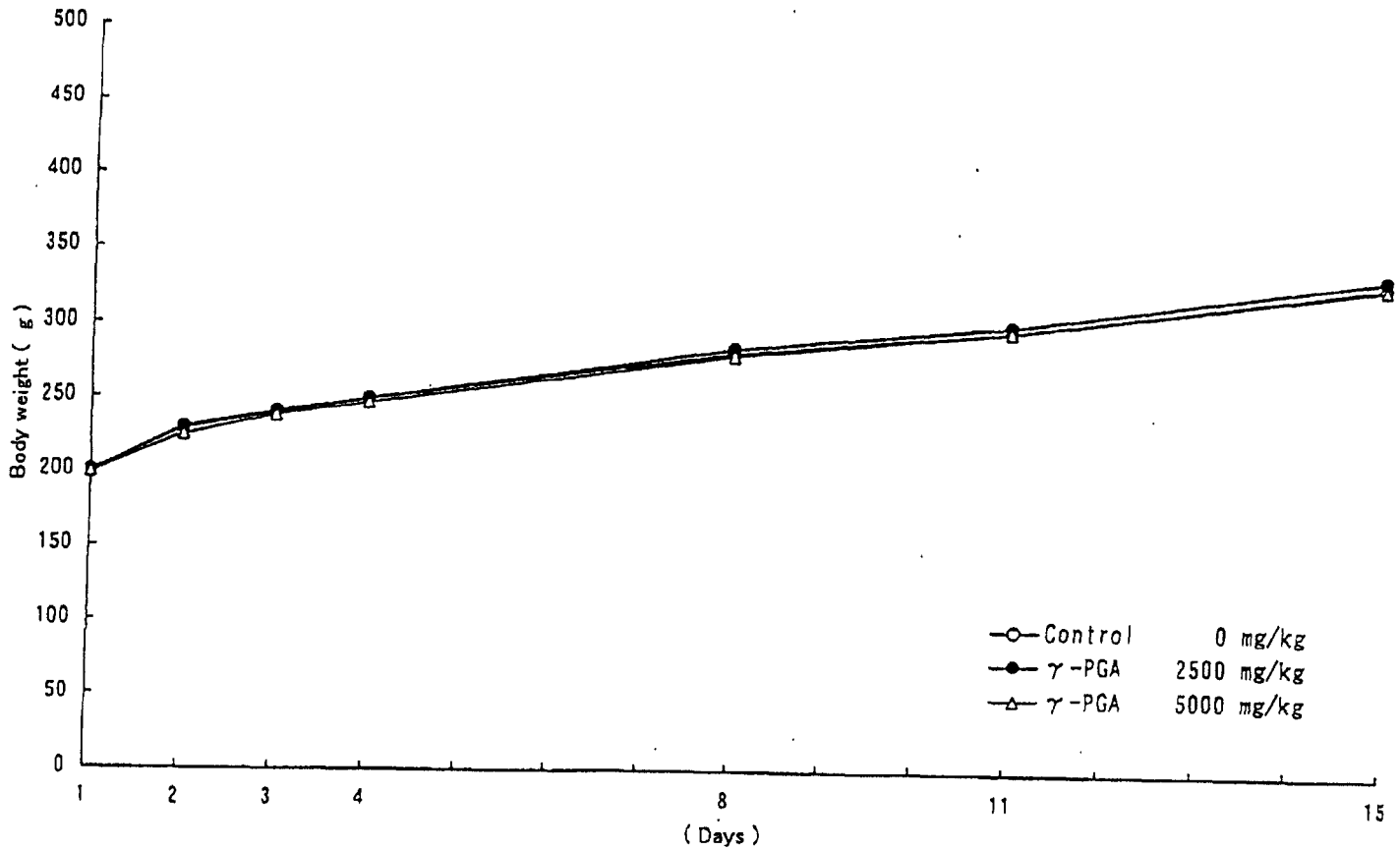

Fig.1 Body weight changes of male rats treated orally with γ-PGA

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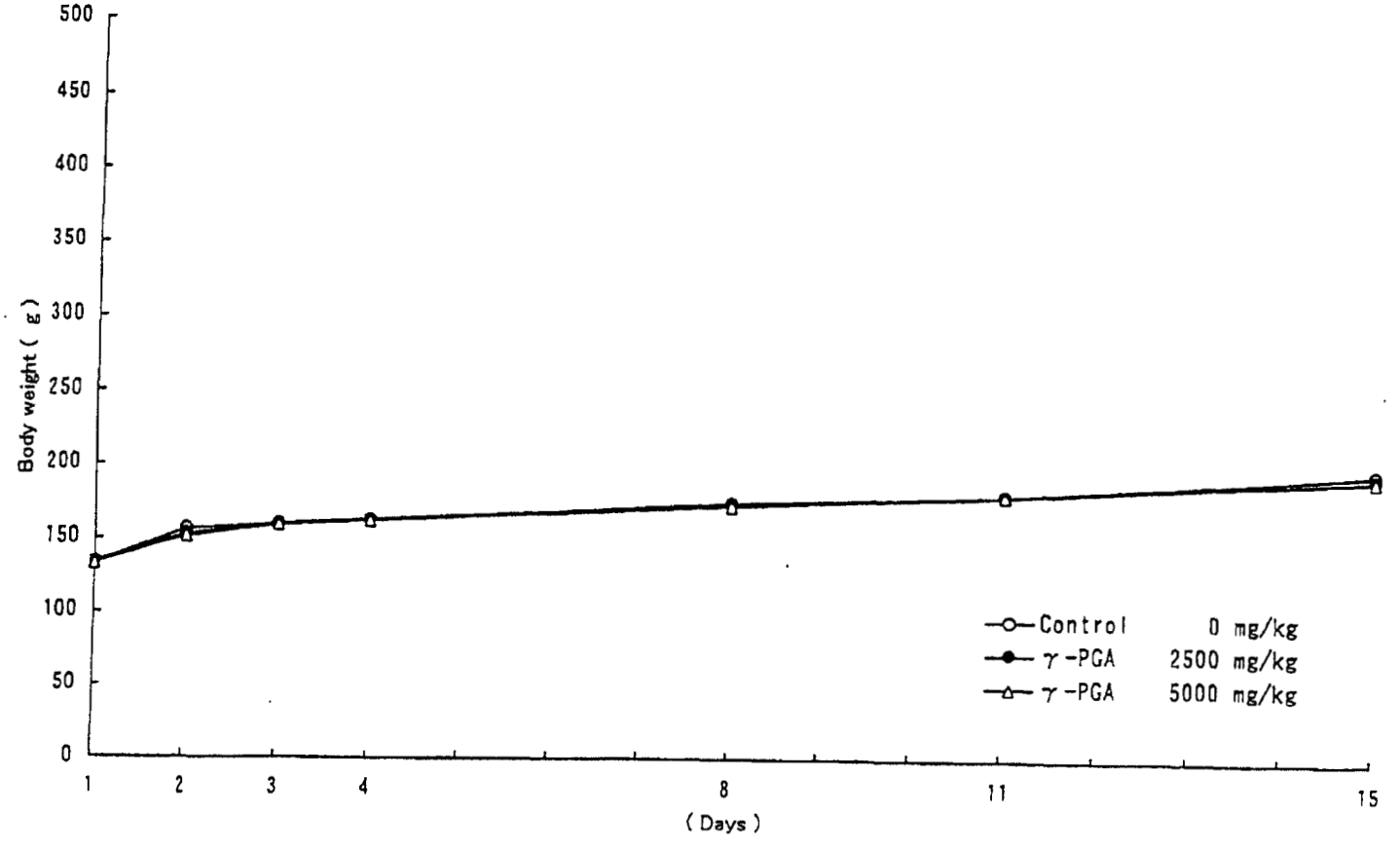

Fig.2 Body weight changes of female rats treated orally with γ-PGA

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Table 1 Mortality of male rats treated orally with γ-PGA

Group-No.		Dose (mg/kg)		1 min 15	1 min 30	1 h 1	1 h 3	1 h 6	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Mortality
												< Days >											
00	Control	0	No. of death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
01	γ-PGA	2500	No. of death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
02	γ-PGA	5000	No. of death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5

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Table 2 Mortality of female rats treated orally with γ-PGA

Group-No.	Dose (mg/kg)		1 min 15	1 min 30	1 h 1	1 h 3	1 h 6	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Mortality
										< Days >												
00	Control 0	No. of death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
01	γ-PGA 2500	No. of death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
02	γ-PGA 5000	No. of death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5

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Table 3 Clinical signs of male rats treated orally with γ-PGA

Group-No. Dose (mg/kg)	Findings	1 min 15	1 min 30	1 h 1	1 h 3	1 h 6	2	3	4	5	6	7	8	9	10	11	12	13	14	15
										< Days >										
00 Control	No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	no abnormality	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
01 γ-PGA	No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
2500	no abnormality	5	5	5	5	2	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	watery diarrhea					3														
	soiled perineal region					3														
02 γ-PGA	No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5000	no abnormality	5	5	5	3	0	4	5	5	5	5	5	5	5	5	5	5	5	5	5
	watery diarrhea				2	5														
	soiled perineal region				1	5	1													

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000132

Table 4 Clinical signs of female rats treated orally with γ-PGA

Group-No. Dose (mg/kg)	Findings	1 min 15	1 min 30	1 h 1	1 h 3	1 h 6	2	3	4	5	6	7	8	9	10	11	12	13	14	15
							< Days >													
00 Control	No.of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	no abnormality	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
01 γ-PGA	No.of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
2500	no abnormality	5	5	5	5	1	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	watery diarrhea					4														
	soiled perineal region					4														
02 γ-PGA	No.of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5000	no abnormality	5	5	5	3	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	watery diarrhea				2	5														

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Table 5 Body weights of male rats treated orally with γ-PGA

Group-No. Dose (mg/kg)		Days 1	2	3	4	8	11	15
00	N	5	5	5	5	5	5	5
Control	Mean	200	229	240	250	282	298	332
0	S.D.	4	8	6	6	6	9	10
01	N	5	5	5	5	5	5	5
γ-PGA	Mean	200	230	241	250	285	302	337
2500	S.D.	3	6	5	4	6	5	9
02	N	5	5	5	5	5	5	5
γ-PGA	Mean	199	225	238	247	280	298	331
5000	S.D.	3	2	1	4	5	8	13

Unit : g N : No.of animals

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Table 6 Body weights of female rats treated orally with γ-PGA

Group-No. Dose (mg/kg)		< Days > 1	2	3	4	8	11	15
00	N	5	5	5	5	5	5	5
Control	Mean	134	156	160	163	175	181	198
0	S.D.	3	6	7	6	6	6	7
01	N	5	5	5	5	5	5	5
γ-PGA	Mean	134	153	159	163	175	181	195
2500	S.D.	2	4	2	5	4	4	5
02	N	5	5	5	5	5	5	5
γ-PGA	Mean	133	151	159	162	173	181	194
5000	S.D.	4	3	5	5	8	7	9

Unit : g N : No.of animals

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000135

Table 7 Necropsy of male rats treated orally with γ-PGA

Organs / Findings	Group No. Dose (mg/kg)	00 Control 0	01 γ-PGA 2500	02 γ-PGA 5000
	No. of animals	5	5	5
abnormality		0	0	0

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000136

Table 8 Necropsy of female rats treated orally with γ-PGA

Organs / Findings	Group No. Dose (mg/kg)	00 Control 0	01 γ-PGA 2500	02 γ-PGA 5000
	No. of animals	5	5	5
abnormality		0	0	0

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000137

Appendix 1 Clinical signs of male rats treated orally with γ-PGA

Group-No. Dose (mg/kg)	Animal No.	1 min 15	1 min 30	1 h 1	1 h 3	1 h 6	2	3	4	5	6	7	8	9	10	11	12	13	14	15
							< Days >													
00	00M01	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Control	00M02	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	00M03	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	00M04	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	00M05	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
01	01M01	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
γ-PGA	01M02	—	—	—	—	ab	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2500	01M03	—	—	—	—	ab	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	01M04	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	01M05	—	—	—	—	ab	—	—	—	—	—	—	—	—	—	—	—	—	—	—
02	02M01	—	—	—	—	ab	—	—	—	—	—	—	—	—	—	—	—	—	—	—
γ-PGA	02M02	—	—	—	ab	ab	b	—	—	—	—	—	—	—	—	—	—	—	—	—
5000	02M03	—	—	—	—	ab	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	02M04	—	—	—	a	ab	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	02M05	—	—	—	—	ab	—	—	—	—	—	—	—	—	—	—	—	—	—	—

- : no abnormality a : watery diarrhea b : soiled perineal region

PROJECT No. H-01074

Appendix 2 Clinical signs of female rats treated orally with γ-PGA

Group-No. Dose (mg/kg)	Animal No.	1 min 15	1 min 30	1 h 1	1 h 3	1 h 6	2	3	4	5	6	7	8	9	10	11	12	13	14	15
							< Days >													
00	00F01	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Control	00F02	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0	00F03	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	00F04	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	00F05	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
01	01F01	–	–	–	–	ab	–	–	–	–	–	–	–	–	–	–	–	–	–	–
γ-PGA	01F02	–	–	–	–	ab	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2500	01F03	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	01F04	–	–	–	–	ab	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	01F05	–	–	–	–	ab	–	–	–	–	–	–	–	–	–	–	–	–	–	–
02	02F01	–	–	–	–	a	–	–	–	–	–	–	–	–	–	–	–	–	–	–
γ-PGA	02F02	–	–	–	a	a	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5000	02F03	–	–	–	–	a	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	02F04	–	–	–	–	a	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	02F05	–	–	–	a	a	–	–	–	–	–	–	–	–	–	–	–	–	–	–

– : no abnormality a : watery diarrhea b : soiled perineal region

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Appendix 3 Body weights of male rats treated orally with γ-PGA

Group-No. Dose (mg/kg)	Animal No.	1	2	3	4	8	11	15
		< Days >						
00	00M01	195	218	231	243	274	292	329
Control	00M02	197	226	238	246	278	291	323
0	00M03	201	232	245	256	283	292	332
	00M04	203	238	246	255	291	311	349
	00M05	206	232	242	251	284	305	328
	N	5	5	5	5	5	5	5
	Mean	200	229	240	250	282	298	332
	S.D.	4	8	6	6	6	9	10
01	01M01	201	231	246	254	292	309	349
γ-PGA	01M02	203	231	242	250	281	299	326
2500	01M03	196	224	235	244	279	299	333
	01M04	202	225	237	248	285	299	341
	01M05	199	239	245	253	290	305	335
	N	5	5	5	5	5	5	5
	Mean	200	230	241	250	285	302	337
	S.D.	3	6	5	4	6	5	9
02	02M01	203	226	240	250	284	302	334
γ-PGA	02M02	197	226	238	245	273	285	311
5000	02M03	194	223	236	245	277	295	328
	02M04	199	228	238	252	286	307	347
	02M05	201	224	238	244	280	299	333
	N	5	5	5	5	5	5	5
	Mean	199	225	238	247	280	298	331
	S.D.	3	2	1	4	5	8	13

Unit : g N : No.of animals

PROJECT No.H-01074

Appendix 4 Body weights of female rats treated orally with γ-PGA

Group-No. Dose (mg/kg)	Animal No.	Days 1	2	3	4	8	11	15
00 Control 0	00F01	136	157	158	161	174	183	204
	00F02	129	151	153	153	165	171	187
	00F03	135	152	155	164	176	185	205
	00F04	137	165	170	166	178	185	198
	00F05	134	155	164	169	180	181	196
	N	5	5	5	5	5	5	5
	Mean	134	156	160	163	175	181	198
	S.D.	3	6	7	6	6	6	7
01 γ-PGA 2500	01F01	136	158	162	168	176	185	201
	01F02	132	153	157	156	170	174	191
	01F03	134	149	158	162	174	181	189
	01F04	133	150	157	161	174	183	196
	01F05	136	154	161	169	180	183	198
	N	5	5	5	5	5	5	5
	Mean	134	153	159	163	175	181	195
	S.D.	2	4	2	5	4	4	5
02 γ-PGA 5000	02F01	132	150	157	163	170	176	186
	02F02	135	151	164	168	182	186	202
	02F03	138	155	165	163	175	190	205
	02F04	128	151	157	153	161	172	188
	02F05	132	148	153	161	175	181	189
	N	5	5	5	5	5	5	5
	Mean	133	151	159	162	173	181	194
	S.D.	4	3	5	5	8	7	9

Unit : g N : No.of animals

PROJECT No.H-01074

000141
IX-1-27

Appendix 5 Necropsy of male rats treated orally with γ-PGA

Group No.	00					01					02				
Dose (mg/kg)	Control 0					γ-PGA 2000					γ-PGA 5000				
Animal No.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	1	1	1	1	1	2	2	2	2	2
	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Organs / Findings															
abnormality	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

— : Negative

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IX-1-28

Appendix 6 Necropsy of female rats treated orally with γ-PGA

Group No.	00					01					02				
Dose (mg/kg)	Control 0					γ-PGA 2000					γ-PGA 5000				
Animal No.	0001F01	0002F02	0003F03	0004F04	0005F05	0011F01	0012F02	0013F03	0014F04	0015F05	0021F01	0022F02	0023F03	0024F04	0025F05
Organs / Findings															
abnormality	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

— : Negative

PROJECT No.H-01074

Study Report

Title:

Mutagenicity study using γ-polyglutamic acid microbes

Development Laboratories
Pharmaceutical Laboratories
Ajinomoto Co., Inc.

000144

Study Controller

Orie Yokoi,
Development Laboratories, Pharmaceutical Laboratories
Ajinomoto Co., Inc.

Study Investigator

Atsuko Sano,
Development Laboratories, Pharmaceutical Laboratories
Ajinomoto Co., Inc.

Introduction

We conducted a mutagenicity study on γ-polyglutamic acid using *Escherichia coli* and four strains of *Salmonella*, so herein report our results.

Study Methods

1. Study Schedule

Study bacteria pre-culturing:	May 11, 1999
Date of study:	May 12, 1999
Plate culturing:	May 12-14, 1999
Mutated colony counting:	May 14, 1999

2. Test Substance

Name:	γ-polyglutamic acid (γ-PGA)
Lot No.:	KL9903-1
Character:	White powder
Preservation conditions:	Sealed refrigerated at 5°C
Supplier:	Food R & D Laboratories (FLX-1) Ajinomoto Co., Inc.
Commissioned dept.:	Food R & D Department Ajinomoto Co., Inc.

3. Control Substance

(1) Negative control substance

Name:	Injectable distilled water
Lot No.:	8F78
Manufacturer:	Otsuka Pharmaceutical Co., Ltd.

(2) Positive control substance

Name:	AF-2 (2-(Furyl)-3-(5-nitro-2-furyl)acrylamide
Lot No.:	LEN0571
Manufacturer:	Wako Pure Chemical Industries, Ltd.

Name:	ENNG (N-Ethyl-N'-nitro-N-nitrosoguanidine)
Lot No.:	V6B7626

Manufacturer:	Nacalai Tesque, Inc.
Name:	ICR-191 (6-Chloro-9-(3-(2-chloroethylamino) -propylamino)-2-methoxyacridine didrochloride
Lot No.:	432098
Manufacturer:	Polyscience, Inc.
Name:	2-aminoanthracene (2AA)
Lot No.:	M5G9888
Manufacturer:	Nacalai Tesque, Inc.

4. Study Materials

(1) Study strains

- Strains used: *Salmonella typhimurium* TA100, TA1535, TA98, TA1537
  Escherichia coli WP2uvrA

(2) S9Mix

- S9 (Lot No. 98112608, 99011409)

Manufacturer:	Oriental Yeast Co., Ltd.
Preservation method:	Refrigerated at –80°C or less

- Cofactor (Lot No. 999803)

Manufacturer:	Oriental Yeast Co., Ltd.
Preservation method:	Refrigerated at –20°C or less

(3) Culture media

- Minimum glucose agar plate medium (Lot No. AN830LN)

Name:	"TESUMEDIA" AN culture medium
Manufacturer:	Oriental Yeast Co., Ltd.
Preservation method:	Preserved at room temperature

- Pre culturing culture medium

Name:	Nutrient Broth No. 2 (Oxoid) (Lot No. 028 59365)
Preparation method:	Extra pure water was added to 2.5 g of Nutrient Broth No. 2 (Oxoid) to obtain 100 ml, and this was subjected to high pressure steam sterilization for 15 min at 121°C.

5. Study Methods

(1) Preparation of test solution

A solution of 50,000 μg/ml was prepared using injectable water and a test solution at the maximum concentration obtained.

(2) Setting of concentrations

Six concentrations were obtained, with the maximum concentration set at 5,000 μg/plate and then the concentration halved each time to a minimum concentration of 156 μg/plate.

(3) Pre-culturing of study strain

Ten milliliters of Nutrient Broth No. 2 (Oxoid) medium was placed in an L-shaped tube and inoculated with 20 μl of each study strain (10 μl for *E. coli*). These were set at 17:00 in a mixing incubator at a constant temperature maintained at 4-5°C. The temperature was increased (37°C) at 22:30 and at 23:00 shaking began. The timer was set so the next day at 9:00 the temperature/shaking switch would turn off.

(4) Study operation

To 0.1 ml of test substance, 0.5 ml of 0.1 M sodium/ phosphate buffer solution (pH7.4) was added when there was no metabolic activation and 0.5 ml of S9Mix was added when there was metabolic activation. Next, 0.1 ml of bacterial solution from the completed culture was added and the solution was subjected to pre-incubation for 20 min at 37°C.

At the end of pre-incubation, 2.0 ml of Top Agar was added, and the mixture was superpositioned on a minimum glucose agar plate culture medium and solidified. The substance was then cultured for 48 hr at 37°C and the revertant colonies were counted. The plate was also observed to see if growth had been inhibited or not.

(5) Viable cell count calculation

The cell count for the bacterial suspension used in the study was calculated by normal method.

(6) Number of plates

Two plates were used for the test substance and positive control substance, and three plates were used for the negative control substance.

(7) Evaluation of results

A positive evaluation was given when the revertant colony count per plate of test substance increased to at least twice that of the negative control substance, and dose dependency was evident.

Study results

1. Viable Cell Count

TA100:	3.65×10^9/ml
TA1535:	5.37×10^9/ml
WP2uvrA:	7.79×10^9/ml
TA98:	3.76×10^9/ml
TA1537:	1.94×10^9/ml

2. Revertant Colony Counts and Plate Observation

   We conducted a mutagenicity study on γ-polyglutamic acid using microbes. The revertant colony counts were calculated 48 hr after culturing, and the results are shown in the table (Table 1, Appendices 1, 2, 3, 4 and 5).

   There were no marked differences in the mutated colony counts compared to the colony counts from the injectable water, which was the negative control substance, in all the studies conducted regardless of whether or not there was metabolic activation. The growth of the study bacteria had not been inhibited, nor was sedimentation seen on the plates. The colony count clearly increased for the positive control substance that was used. Thus, the induction of reverse mutation of γ-polyglutamic acid was judged to be negative.

Conclusion

We conducted a mutagenicity study on γ-polyglutamic acid using five strains of microbes, *Salmonella* TA100, TA1535, TA98, and TA1537 and *E. coli* WP2uvrA.
From the results obtained, it was concluded that γ-polyglutamic acid tested negative to reverse mutation induction regardless of whether or not there was metabolic activation.

Table 1 Ames Test of γ-PGA

Compound	Dose	Number of Revertant Colonies / Plate									
		S-9 mix(-)					S-9 mix(+)				
	(μg/plate)	TA100	TA1535	WP2	TA98	TA1537	TA100	TA1535	WP2	TA98	TA1537
H_2O	0	120	20	41	29	16	133	23	45	33	37
γ-PGA	156	116	25	48	27	14	127	23	40	43	35
	313	117	21	41	38	17	120	18	38	40	27
	625	120	28	44	32	16	133	21	38	46	31
	1250	129	22	39	33	19	134	23	41	42	35
	2500	127	27	57	37	21	145	31	46	35	37
	5000	138	26	51	30	18	136	24	48	42	27
Positive Control		AF2	ENNG	AF2	AF2	(CR-191	2AA	2AA	2AA	2AA	2AA
μg/plate		[0.01]	[5]	[0.01]	[0.1]	[0.5]	[1]	[2]	[10]	[0.5]	[2]
Colonies / Plate		609	507	228	681	>2000	1186	460	1340	299	513

000150

000150

Appendix 1

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with TA100

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)			Mean	S-9 mix (+)			Mean
H_2O	0	116	126	119	120	144	115	139	133
r-PGA	156	116	115		116	127	127		127
	313	115	119		117	112	127		120
	625	124	115		120	144	121		133
	1250	139	118		129	136	131		134
	2500	127	127		127	141	148		145
	5000	141	135		138	146	126		136
AF2	0.01	598	619		609				
	0.02	1101	1137		1119				
2AA	0.5					447	524		486

000151

000151

Appendix 1

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with TA100

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)	Mean	S-9 mix (+)	Mean
2AA	1			1217 1155	1186

Toxicity : +

Precipitation of Test Article : +

000152

000152

Appendix 2

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with TA1535

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)			Mean	S-9 mix (+)			Mean
H_2O	0	19	19	21	20	19	24	26	23
r-PGA	156	23	26		25	23	23		23
	313	25	16		21	16	19		18
	625	28	27		28	17	24		21
	1250	17	26		22	24	22		23
	2500	24	29		27	24	37		31
	5000	26	26		26	22	26		24
2AA	1					208	215		212
	2					457	463		460
ENNG	5	473	541		507				

000153

IX-2-10

000153

Appendix 2

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with TA1535

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)		Mean	S-9 mix (+)	Mean
ENNG	10	2000	2000	2000		

Toxicity : +

Precipitation of Test Article : +

Appendix 3

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with WP2uvrA

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)			Mean	S-9 mix (+)			Mean
H_2O	0	32	45	47	41	40	47	48	45
r-PGA	156	49	46		48	30	49		40
	313	42	39		41	40	35		38
	625	32	55		44	34	42		38
	1250	41	37		39	44	37		41
	2500	53	60		57	40	52		46
	5000	51	51		51	45	51		48
AF2	0.01	217	238		228				
	0.02	352	319		336				
2AA	10					1361	1319		1340

000155

Appendix 3

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with WP2uvrA

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)	Mean	S-9 mix (+)	Mean
2AA	20		1322	1116	1219

Toxicity : +
Precipitation of Test Article : *

000156

Appendix 4

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with TA98

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)			Mean	S-9 mix (+)			Mean
H_2O	0	25	32	29	29	35	25	40	33
r-PGA	156	25	29		27	39	46		43
	313	38	37		38	36	43		40
	625	30	34		32	40	52		46
	1250	28	38		33	34	49		42
	2500	42	31		37	36	33		35
	5000	28	31		30	35	49		42
AF2	0.05	303	299		301				
	0.1	670	691		681				
2AA	0.5					309	288		299

000157

Appendix 4

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with TA98

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)		Mean	S-9 mix (+)		Mean
2AA	1			1095	1231		1163

Toxicity : +
Precipitation of Test Article : *

000158

Appendix 5

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with TA1537

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)			Mean	S-9 mix (+)			Mean
H_2O	0	14	15	18	16	36	28	46	37
r-PGA	156	11	16		14	45	25		35
	313	18	16		17	28	25		27
	625	15	16		16	29	32		31
	1250	21	17		19	36	34		35
	2500	28	14		21	36	37		37
	5000	20	16		18	23	31		27
2AA	1					99	80		90
	2					527	498		513
ICR-191	0.5	2000	2000		2000				

000159

Appendix 5

[AMES TEST OF r-POLYGLUTAMIC ACID]

Item : Number of Revertant Colonies with TA1537

Compound	Dose (mcg/plate)	Number of Revertant Colonies / Plate S-9 mix (-)		Mean	S-9 mix (+)	Mean
ICR-191	1	2000	2000	2000		

Toxicity : +

Precipitation of Test Article : +

000160

Appendix IX-3

English Translation of Mutagenicity Test of *Bacillus subtilis* Gum–(PGA)

(see Appendix IX-4)

Fiscal 1996

Test for reevaluating the safety of food additives:

Mutagenicity test (Ames test)

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1. Samples

Ten natural additives, namely, "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), soybean saponin, orange color, chitin, perilla extract, Fukuronori (*Gloiopeltis furacata*) extract, bitterwood (*Picrasma quassioides*) extract, horseradish (*Armoracia rusticana*) extract, Mousouchiku (*Phyllostachys pubescens*) extract and levan were examined (Table 1).

2. Test methods

Pursuant to the "New guidebook for mutagenicity tests with microorganisms" (edited by Chemical Substances Investigation Division of the Ministry of Labour), the samples were preincubated at 37℃ for 20 minutes. The maximum concentration of a sample solution was set at which cytotoxicity against the test strains was exhibited or at the technically feasible highest concentration. Each sample was examined at more than six concentrations and more than two plates were used for each concentration. However, more than six plates were used for the solvent control.

1) Test strains

Five strains, *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 were used for all samples.

2) S9

According to the routine procedure, S9 was prepared from the liver of Sprague-Dawley rats to which phenobarbital and 5, 6-benzoflavone were administered. Fifty microliters of S9 was used for each plate.

3) Preparation of sample solutions

Sample solutions were prepared by dissolving or suspending "Bacillus natto (*Bacillus subtilis*)

Gum"("納豆菌ガム"), chitin and levan in sterile distilled water; soybean saponin, perilla extract, fukuronori extract, bitterwood extract, horseradish extract and mousouchiku extract in dimethylsulfoxide (DMSO); and orange color in acetone. The samples were previously checked for contamination with microorganisms by adding soft agar (containing L-histidine 0.05 mM and D-biotin 0.05 mM) to the minimum glucose agar plates with the maximum concentrations of the samples. Since it was confirmed that "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム") and perilla extract were contaminated with microorganisms, they were examined after "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム") was suspended in distilled water and heat-treated for 5~10 seconds in a microwave (Toshiba ER-120) and the stock solution of perilla extract was filtered to remove the contaminating microorganisms (0.45 μm). Regarding orange color, after the sample was dispensed into test tubes to which 50~100 μl of DMSO was added and mixed, acetone was removed by applying nitrogen gas stream in 40℃ water bath for testing.

4) Evaluation criteria of test results

It was determined that the samples that induced His+ revertant colonies twofold or more as compared with the control and showed a concentration dependence in one or more test strains were considered to be positive, and those that induced His+ revertant colonies at 1.5-fold or more but less than twofold as compared with the control and showed a concentration dependence were suspected to be positive. The samples other than those stated above were determined to be negative.

3. Results and discussion

The test results for the ten natural additives are shown in Table 2.

Horseradish extract was examined in the concentration range from 0.005 μl to 0.5 μl for each plate. The results revealed that in the TA100 strain, horseradish extract induced His+ revertant colonies 1.7-fold as many colonies as the control without the S9 mix (hereafter referred to as -S9mix) and 1.5-fold in the case of with the S9 mix (hereafter referred to as +S9mix) and showed a concentration dependence (Table 3-5, Figure 1). Therefore, the mutagenicity of horseradish extract determined by the Ames test was suspected to be positive.

"Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム") and perilla extract induced 1.5-fold or more but less than twofold His+ revertant colonies as compared with the control at the concentrations of 2 mg/plate in the TA 1537 strain (+S9mix) and 100 μl in the TA100 strain (+S9mix) (Tables 4-1 and 5-1). In addition, because some plates of soybean saponin, orange

color, bitterwood extract and mousouchiku extract induced 1.5-fold or more His+ revertant colonies as compared with the control, they were reexamined. As a result, the mean level of induction of His+ revertant colonies in each sample was less than 1.5-fold as compared with the control (Tables 4-4, 5-4, 6, 7, 10 and 11). Furthermore, chitin, fukuronori extract and levan did not induce 1.5-fold or more His+ revertant colonies as compared with the control in the five test strains, regardless of whether metabolism was activated or not (Tables 8, 9 and 12). Therefore, the mutagenicity of the nine natural additives excluding the horseradish extract ("Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), soybean saponin, orange color, chitin, perilla extract, fukuronori extract, bitterwood extract, mousouchiku extract and levan) was determined to be negative by the Ames test.

Additionally, the nine natural additives excluding horseradish extract did not show any marked cytotoxicity in all the five test strains at the test concentration range, regardless of whether metabolism was activated or not.

平成８年度

食品添加物安全性再評価等の試験検査

変異原性試験（Ａｍｅｓ法）

名古屋市衛生研究所
宮部　正樹

000165

IX-4-1

1．試料

納豆菌ガム、ダイズサポニン、オレンジ色素、キチン、シソ抽出物、フクロノリ抽出物、ニガキ抽出物、セイヨウワサビ抽出物、モウソウチク抽出物およびレバンの10品目の天然添加物について試験した（表1）．

2．試験法

「新・微生物を用いる変異原性試験ガイドブック」（労働省化学物質調査課編）に準じ、37℃、20分間のプレインキュベーション法を用いて試験した。供試試料溶液の最高濃度は試験菌株に対し細胞毒性を示すか、または技術的に可能な上限の濃度を最高濃度とした。全試料に対し6段階以上の濃度で試験し、各濃度とも2プレート以上を使用した。ただし溶媒対照は6プレート以上を使用した。

1）試験菌株

全試料に対し、*Salmonella typhimurium* TA98、TA100、TA1535、TA1537 および TA1538 の5種類の菌株を用いて試験した。

2）S9

S9は常法により、フェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawleyラット肝臓より調製し、1プレート当たり 50 μl を使用した。

3）試料溶液の調製

納豆菌ガム、キチンおよびレバンは滅菌蒸留水に、ダイズサポニン、シソ抽出物、フクロノリ抽出物、ニガキ抽出物、セイヨウワサビ抽出物およびモウソウチク抽出物はジメチルスルホキシド（以下DMSO）に、オレンジ抽出物はアセトンにそれぞれ溶解または懸濁し、試料溶液とした。ただし試料はあらかじめ、試験に供した最高濃度を軟寒天（L－ヒスチジン 0.05 mM、D－ビオチン 0.05 mM添加）とともに最少グルコース寒天平板に重層し、試料が微生物に汚染されていないことを確認した。納豆菌ガムおよびシソ抽出物は微生物汚染が確認されたため、納豆菌ガムは蒸留水に懸濁後、電子レンジ（東芝 ER-120）で5～10秒間加熱処理し、シソ抽出物は原液をろ過除菌（0.45μm）して試験した。オレンジ抽出物は試験管に供試量を分注し、DMSOを50～100μl加え混和後、40℃の温浴中で窒素気流によりアセトンを除去して試験した。

4）試験結果の判定法

1種類以上の試験菌株に対し、対照の2倍以上のHis^+復帰変異コロニーを誘発し、かつ濃度依存性を示したものを陽性、1.5倍以上2倍未満のHis^+復帰変異コロニーを誘発し、かつ濃度依存性を示したものを疑陽性、上記以外を陰性とした。

3．結果および考察

10品目の天然添加物についての試験結果を表2に示した。

セイヨウワサビ抽出物はプレート当たり0.005～0.5μlの濃度範囲で試験した結果、TA100株に対してS9mix非存在下（以下-S9mix）およびS9mix存在下（以下+S9mix）で、それぞれ対照の1.7倍および1.5倍のHis^+復帰変異コロニーを誘発し、かつ濃度依存性を示したため（表3－5、図1）、セイヨウワサビ抽出物のAmes試験による変異原性は疑陽性とした。

納豆菌ガムは2mg/プレートの濃度でTA1537株（+S9mix）に、シソ抽出物は100μlの濃度でTA100株（+S9mix）に対して、1.5倍以上2倍未満のHis^+復帰変異コロニーを誘発した（表4－1、表5－1）。また、ダイズサポニン、オレンジ色素、ニガキ抽出物およびモウソウチク抽出物にも対照の1.5倍以上のHis^+復帰変異コロニーを誘発したプレートがあったため、再度試験を行った。その結果、いずれの試料においてもHis^+復帰変異コロニー数の平均値は対照の1.5倍未満であった（表4－4、5－4、6、7、10、11）。また、上記以外のキチン、フクロノリ抽出物およびレバンはいずれも、代謝活性化の有無に関わらず5種類の試験菌株に対し、対照の1.5倍以上のHis^+復帰変異コロニー数を誘発しなかった（表8、9、12）。したがって、セイヨウワサビ抽出物以外の9品目の天然添加物（納豆菌ガム、ダイズサポニン、オレンジ色素、キチン、シソ抽出物、フクロノリ抽出物、ニガキ抽出物、モウソウチク抽出物およびレバン）

のAmes試験による変異原性は陰性とした。

なお、セイヨウワサビ抽出物以外の9品目の天然添加物は代謝活性化の有無にかかわらず5種類の菌株すべてに対し、試験した濃度範囲では顕著な細胞毒性を示さなかった。

表1　試料

品　目	製品名	ロット	製造者または販売者名
納豆菌ガム	γ-ポリグルタミン酸	Lot No　TT-3	味の素（株）
ダイズサポニン	大豆サポニン	Lot No　DZX-703	（株）トキワ漢方製薬
オレンジ色素	色素サンプル CT-2490	Lot.970214	三栄源エフ・エフ・アイ（株）
キチン	精製キチン粉末	Lot.951212	（株）共和テクノス
シソ抽出物		1996.7.18 製造	（株）梅屋
フクロノリ抽出物	フノリ抽出物	Lot.605101	MRCポリサッカライド
ニガキ抽出物	ニガキ抽出物製剤		クエストジャパン（株）
セイヨウワサビ抽出物	西洋わさび抽出辛味油		山忠わさび（株）
モウソウチク抽出物			日本油脂（株）
レバン	LEV	LEV 96001-3	（株）アドバンス

表2　Ames試験による変異原性試験結果

試料名	濃度範囲（プレート当り）	溶媒	突然変異誘発性[1]									
			TA98		TA100		TA1535		TA1537		TA1538	
			-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
納豆菌ガム	0.1～ 10 mg	蒸留水	−	−	−	−	−	−	−	−	−	−
ダイズサポニン	0.2～100 mg	DMSO[2]	−	−	−	−	−	−	−	−	−	−
オレンジ色素	1 ～100 μl	Ac[3]→DMSO	−	−	−	−	−	−	−	−	−	−
キチン	0.2～ 20 mg	蒸留水	−	−	−	−	−	−	−	−	−	−
シソ抽出物	1 ～200 μl	DMSO	−	−	−	−	−	−	−	−	−	−
フクロノリ抽出物	0.2～ 20 mg	DMSO	−	−	−	−	−	−	−	−	−	−
ニガキ抽出物	1 ～200 μl	DMSO	−	−	−	−	−	−	−	−	−	−
セイヨウワサビ抽出物	0.005～0.5μl	DMSO	−	−	±	±	−	−	−	−	−	−
モウソウチク抽出物	0.2～100 mg	DMSO	−	−	−	−	−	−	−	−	−	−
レバン	0.2～ 20 mg	蒸留水	−	−	−	−	−	−	−	−	−	−

[1] ＋：陽性（溶媒対照の2倍以上のHis⁺復帰変異コロニー数および濃度依存性を示したもの）
　±：疑陽性（溶媒対照の1.5倍以上2倍未満のHis⁺復帰変異コロニー数および濃度依存性を示したもの）
　－：陰性（上記以外のもの）
[2] ジメチルスルホキシド
[3] アセトン

表３－１　セイヨウワサビ抽出物
《試験１》

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	35	38	160	135	15	14	11	14	24	31
0.005	33	38	182	128	12	11	10	16	24	27
0.01	28	39	185	130	13	14	12	12	26	33
0.02	32	48	226	148	9	10	12	15	23	36
0.05	51	43	235	178	9	13	8	15	25	29
0.1	46	44	232	220	6	13	6	15	21	28
0.2	*6*	37	*52*	201	*4*	19	*4*	13	*10*	15
0.5	*0*	*17*	*0*	*44*	*0*	*5*	*0*	*3*	*0*	*12*

値は２プレートの平均値、ただし対照は６プレートの平均値
＊：溶媒対照として DMSO 100μlを使用
▒：対照の1.5倍以上２倍未満
斜体文字：細胞毒性あり

表３－２　セイヨウワサビ抽出物
《試験２》

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数/プレート			
	TA98		TA100	
	-S9	+S9	-S9	+S9
0*	30	40	138	139
0.01	32	38	127	146
0.02	34	38	155	145
0.05	40	42	225	185
0.1	38	45	232	205
0.2	*15*	38	*103*	195

値は２プレートの平均値、ただし対照は６プレートの平均値
＊：溶媒対照としてDMSO 100μlを使用
▒：対照の1.5倍以上２倍未満

表３－３　セイヨウワサビ抽出物
《試験３》

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数/プレート	
	TA100	
	-S9	+S9
0*	143	145
0.01	152	130
0.02	159	142
0.05	238	165
0.1	204	209
0.2	*28*	141

値は３プレートの平均値、ただし対照は６プレートの平均値
＊：溶媒対照としてDMSO 100μlを使用
▒：対照の1.5倍以上２倍未満

表３－４　セイヨウワサビ抽出物
《試験４》

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数/プレート	
	TA100	
	-S9	+S9
0*	115	132
0.02	144	120
0.05	204	203
0.1	178	203
0.2	*97*	186

値は２プレートの平均値、ただし対照は６プレートの平均値
＊：溶媒対照としてDMSO 100μlを使用
▒：対照の1.5倍以上２倍未満

表３－５　セイヨウワサビ抽出物
《試験１、２、３、４の平均値》

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濃　度 (μg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	32(12)	39(12)	139(24)	138(24)	15	14	11	14	24	31
0.005	33(2)	38(2)	182(2)	128(2)	12	11	10	16	24	27
0.01	30(4)	38(4)	154(7)	134(7)	13	14	12	12	26	33
0.02	33(4)	43(4)	170(9)	139(9)	9	10	12	15	23	36
0.05	45(4)	42(4)	233(9)	181(9)	9	13	8	15	25	29
0.1	42(4)	44(4)	232(9)	200(9)	6	13	6	15	21	28
0.2	11(4)	37(4)	65(9)	176(9)	*4*	19	*4*	13	10	15
0.5	*0*(4)	*17*(4)	*0*(9)	*44*(9)	*0*	*5*	*0*	*3*	*0*	*12*

値は２プレートの平均値、ただし対照は６プレート、TA98、TA100は（ ）内のプレート数の平均値
＊：溶媒対照として DMSO 100μlを使用
▒：対照の1.5倍以上２倍未満
斜体文字：細胞毒性あり

表４－１　納豆菌ガム
《試験１》

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	30	39	140	146	14	16	11	16	21	39
0.1	41	42	137	133	15	14	10	19	22	40
0.2	35	37	142	141	9	13	12	13	22	43
0.5	28	39	133	137	14	12	15	22	20	36
1	28	37	142	129	12	18	9	17	21	42
2	30	37	148	138	9	19	10	25	23	48
5	32	40	134	133	12	14	10	15	19	40
10	29	38	138	124	9	15	13	19	17	35

値は２プレートの平均値，ただし対照は６プレートの平均値
＊：溶媒対照として滅菌蒸留水100μlを使用
▒：対照の1.5倍以上２倍未満

表４－２　納豆菌ガム
《試験２》

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数/プレート	
	TA1537	
	-S9	+S9
0*		14
0.5		13
1		16
2		15
5		16

値は３プレートの平均値、ただし対照は６プレートの平均値
＊：溶媒対照として滅菌蒸留水100μlを使用
▒：対照の1.5倍以上２倍未満

表４－３　納豆菌ガム
《試験３》

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数/プレート	
	TA1537	
	-S9	+S9
0*		17
0.5		19
1		17
2		18
5		15

値は２プレートの平均値、ただし対照は６プレートの平均値
＊：溶媒対照として滅菌蒸留水100μlを使用
▒：対照の1.5倍以上２倍未満

000173

表４－４　納豆菌ガム
《試験１、２、３の平均値》

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	30	39	140	146	14	16	11	16(18)	21	39
0.1	41	42	137	133	15	14	10	19(2)	22	40
0.2	35	37	142	141	9	13	12	13(2)	22	43
0.5	28	39	133	137	14	12	15	17(8)	20	36
1	28	37	142	129	12	18	9	17(8)	21	42
2	30	37	148	138	9	19	10	19(8)	23	48
5	32	40	134	133	12	14	10	15(8)	19	40
10	29	38	138	124	9	15	13	19(2)	17	35

値は２プレートの平均値、ただし対照は６プレート、TA1537(+S9)は（ ）内のプレート数の平均値
＊：溶媒対照として滅菌蒸留水100μlを使用

表５－１　シソ抽出物
《試験１》

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	26	38	122	135	13	17	11	14	24	31
1	26	40	113	139	8	18	11	12	27	32
2	30	35	119	136	12	14	9	17	21	31
5	25	41	127	130	12	17	12	14	29	34
10	23	42	131	148	10	16	9	15	24	25
20	24	47	134	136	11	17	13	19	22	31
50	26	39	117	157	16	17	11	17	23	31
100	32	61	124	※238	15	20	10	14	27	39

値は２プレートの平均値、ただし対照は６プレートの平均値
*：溶媒対照として DMSO 100μlを使用
※：対照の1.5倍以上２倍未満

表５－２　シソ抽出物
《試験２》

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数／プレート			
	TA98		TA100	
	-S9	+S9	-S9	+S9
0*		39		141
20		39		142
50		42		150
100		55		196
200		44		171

値は２プレートの平均値、ただし
対照は６プレートの平均値
*：溶媒対照として DMSO 100μlを使用

表５－３　シソ抽出物
《試験３》

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数／プレート			
	TA98		TA100	
	-S9	+S9	-S9	+S9
0*		34		138
20		45		130
50		43		153
100		50		164
200		43		165

値は３プレートの平均値、ただし
対照は６プレートの平均値
*：溶媒対照として DMSO 100μlを使用

表５－４　シソ抽出物
《試験１～３の平均値》

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	26	38(18)	122	138(18)	13	17	11	14	24	31
1	26	40(2)	113	139(2)	8	18	11	12	27	32
2	30	35(2)	119	136(2)	12	14	9	17	21	31
5	25	41(2)	127	130(2)	12	17	12	14	29	34
10	23	42(2)	131	148(2)	10	16	9	15	24	25
20	24	44(7)	134	135(7)	11	17	13	19	22	31
50	26	42(7)	117	153(7)	16	17	11	17	23	31
100	32	54(7)	124	189(7)	15	20	10	14	27	39
200		43(5)		168(5)						

値は２プレートの平均値、ただし対照は６プレート、
TA98(+S9)およびTA100(+S9)は（ ）内のプレート数の平均値
*：溶媒対照として DMSO 100μlを使用

表6　ダイズサポニン

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	31	41(12)	135	140	15	14(12)	11	17	21	38
0.2	30	43(2)	146	139	12	17(2)	13	17	20	38
0.5	30	41(2)	149	148	14	16(2)	13	15	23	31
1	31	49(4)	155	171	11	19(4)	12	16	20	37
2	23	47(4)	135	160	9	15(4)	9	16	16	33
5	25	39(4)	113	151	14	19(4)	7	15	21	29
10	21	36(4)	87	128	14	19(4)	13	16	19	26
20	28	37(4)	61	105	17	16(4)	9	12	16	18
50		38(2)								
100		33(2)								

値は２プレートの平均値、ただし対照は６プレート、
TA98(+S9)およびTA1535(+S9)は（ ）内のプレート数の平均値
*：溶媒対照として DMSO 100μlを使用

表7　オレンジ色素

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	26	43	157	155	15	14(12)	12	14	27	36
1	31	41	162	155	14	15(5)	12	12	27	41
2	36	43	167	159	15	18(5)	13	18	24	34
5	32	49	163	172	13	13(5)	11	16	26	30
10	37	47	145	190	14	16(5)	12	14	26	30
20	30	48	115	141	13	20(5)	10	14	29	28
50	32	34	91	117	14	16(5)	9	16	18	30
100	24	31	89	104	15	15(5)	9	10	16	21

値は２プレートの平均値、ただし対照は６プレート、TA1535(+S9)は（ ）内のプレート数の平均値
*：溶媒対照として DMAO 100μlを使用

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表8　キチン

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	31	42	164	167	14	16	13	15	30	39
0.2	36	47	142	161	14	12	13	14	34	40
0.5	33	47	153	170	11	12	14	18	32	40
1	35	41	156	177	9	12	12	13	32	35
2	35	43	165	154	16	15	14	15	34	39
5	28	49	177	143	12	14	13	16	31	34
10	28	40	165	159	11	12	14	15	36	34
20	26	38	145	148	10	15	11	15	28	27

値は２プレートの平均値、ただし対照は６プレートの平均値
*：溶媒対照として滅菌蒸留水 100μlを使用

表９　フクロノリ抽出物

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	35	38	160	135	15	14	11	14	24	31
0.2	27	35	167	133	13	13	13	19	27	32
0.5	27	40	164	136	15	16	11	12	24	31
1	27	38	172	147	18	15	10	13	25	32
2	25	42	159	156	13	12	13	13	19	28
5	23	41	168	150	12	14	11	15	19	34
10	31	41	172	160	15	14	11	14	21	28
20	30	34	160	152	10	14	9	16	18	28

値は２プレートの平均値、ただし対照は６プレートの平均値
＊：溶媒対照として DMSO 100μlを使用

表１０　ニガキ抽出物

濃　度 (μl/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	26	38(12)	122	138(12)	13	17	11	14	24	31
1	35	39(2)	114	136(2)	13	13	12	16	24	32
2	28	41(2)	114	120(2)	11	13	11	15	27	38
5	29	36(2)	112	133(2)	13	14	9	15	27	31
10	23	41(5)	106	136(5)	11	16	12	15	24	28
20	30	43(5)	114	134(5)	14	14	11	12	24	27
50	24	41(5)	117	158(5)	11	21	9	13	26	37
100	30	51(5)	118	177(5)	12	16	9	13	27	34
200		52(3)		190(3)						

値は２プレートの平均値、ただし対照は６プレート、
TA98(+S9)およびTA100(+S9)は( )内のプレート数の平均値
＊：溶媒対照として DMSO 100μlを使用

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表１１　モウソウチク抽出物

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	35	38	160	138(12)	15	14(12)	10	13	25	34
0.2	29	43	164	142(2)	9	15(2)	5	12	23	29
0.5	26	37	155	140(2)	15	18(2)	8	8	30	26
1	35	40	147	147(2)	16	17(2)	6	11	23	24
2	34	43	158	139(2)	13	16(5)	7	9	17	24
5	36	45	161	150(5)	13	14(5)	6	10	21	12
10	32	33	174	153(5)	12	18(5)	5	9	20	17
20	31	44	172	159(5)	13	16(5)	6	11	17	30
50				143(3)		15(3)				
100				149(3)						

値は２プレートの平均値、ただし対照は６プレートの平均値
TA100(+S9)およびTA1535(+S9)は( )内のプレート数の平均値
＊：溶媒対照として DMSO 100μlを使用

表１２　レバン

濃　度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0*	31	42	164	167	14	16	13	15	30	39
0.2	31	47	157	153	15	16	13	15	30	42
0.5	29	44	155	154	10	17	12	17	34	46
1	31	42	159	147	13	14	10	14	40	43
2	35	43	159	147	12	14	6	15	25	32
5	32	46	150	156	15	18	6	13	26	34
10	35	51	153	157	11	15	7	15	26	28
20	33	40	163	160	11	15	7	19	36	38

値は２プレートの平均値、ただし対照は６プレートの平均値
*：溶媒対照として滅菌蒸留水 100μlを使用

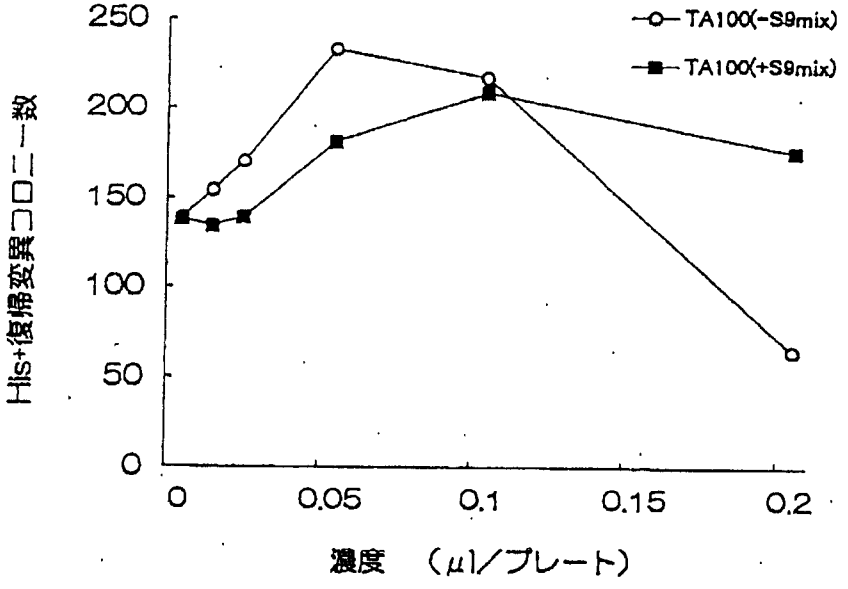

図1　セイヨウワサビ抽出物の変異原性

試料名：セイヨウワサビ抽出物
《試験 1》

濃度 (μl/プレート)	TA98 -S9 (平均)	TA98 +S9 (平均)	TA100 -S9 (平均)	TA100 +S9 (平均)	TA1535 -S9 (平均)	TA1535 +S9 (平均)	TA1537 -S9 (平均)	TA1537 +S9 (平均)	TA1538 -S9 (平均)	TA1538 +S9 (平均)
	His⁺ 復帰変異コロニー数 ／ プレート									
0*	34, 33, 35, 39, 33, 33 (35)	35, 43, 35, 37, 35, 40 (38)	161, 162, 170, 147, 163, 154 (160)	137, 135, 126, 147, 138, 129 (135)	19, 13, 10, 15, 16, 14 (15)	13, 18, 16, 11, 12, 16 (14)	13, 9, 7, 9, 11, 14 (11)	15, 20, 14, 11, 13, 11 (14)	32, 20, 23, 24, 22, 25 (24)	35, 30, 35, 26, 30, 29 (31)
0.005	37, 28 (33)	35, 40 (38)	177, 187 (182)	121, 134 (128)	10, 13 (12)	10, 12 (11)	11, 9 (10)	11, 21 (16)	24, 23 (24)	25, 28 (27)
0.01	22, 33 (28)	41, 37 (39)	203, 166 (185)	125, 134 (130)	14, 11 (13)	15, 13 (14)	10, 14 (12)	13, 11 (12)	26, 25 (26)	35, 31 (33)
0.02	31, 33 (32)	46, 50 (48)	227, 224 (226)	148, 147 (148)	9, 8 (9)	10, 10 (10)	9, 15 (12)	18, 11 (15)	20, 26 (23)	40, 31 (36)
0.05	48, 53 (51)	31, 54 (43)	332, 259 (295)	192, 163 (178)	8, 10 (9)	13, 12 (13)	9, 7 (8)	17, 12 (15)	29, 20 (25)	32, 26 (29)
0.1	49, 43 (46)	37, 50 (44)	303, 260 (282)	207, 232 (220)	4, 7 (6)	11, 15 (13)	5, 6 (6)	17, 13 (15)	22, 19 (21)	25, 30 (28)
0.2	5, 7 (6)	36, 38 (37)	57, 47 (52)	201, 201 (201)	3, 4 (4)	18, 19 (19)	5, 2 (4)	16, 10 (13)	4, 16 (10)	14, 15 (15)
0.5	0, 0 (0)	17, 16 (17)	0, 0 (0)	31, 56 (44)	0, 0 (0)	6, 3 (5)	0, 0 (0)	0, 6 (3)	0, 0 (0)	10, 13 (12)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	523	466	690	1090	239	186	207	100	397	151
試験年月日	'97. 5.13	'97. 5.16	'97. 5.13	'97. 5.16	'97. 8.19	'97. 8.19	'97. 6. 3	'97. 6. 3	'97. 6. 3	'97. 6. 3

*：対照として DMSO 100μl を使用
※：対照の 1.5倍以上 2倍未満
■：対照の 2倍以上
斜体文字：細胞毒性有り

0001179

試料名： セイヨウワサビ抽出物

《試験 2》

濃度 (μl/プレート)	His⁺ 復帰変異コロニー数 / プレート TA98 -S9 (平均)	TA98 +S9 (平均)	TA100 -S9 (平均)	TA100 +S9 (平均)
0*	32, 29, 35, 27, 26, 31 (30)	43, 39, 42, 37, 40, 37 (40)	137, 129, 149, 137, 132, 144 (138)	143, 140, 151, 127, 136, 138 (139)
0.01	33, 31 (32)	38, 37 (38)	126, 128 (127)	153, 138 (146)
0.02	36, 32 (34)	40, 36 (38)	161, 149 (155)	146, 144 (145)
0.05	38, 42 (40)	43, 41 (42)	216, 223 (225)	196, 173 (185)
0.1	37, 39 (38)	43, 47 (45)	197, 226 (212)	223, 186 (205)
0.2	*14, 16 (15)*	36, 39 (38)	*92, 113 (103)*	190, 200 (195)
陽性対照 (μg/プレート)	AF2 (0.1) 421	B(a)P (5) 390	AF2 (0.01) 511	B(a)P (5) 1036
試験年月日	'97. 6. 3	'97. 6. 3	'97. 6. 3	'97. 6. 3

*: 対照として DMSO 100μlを使用
▒: 対照の 1.5倍以上 2倍未満
斜体文字：細胞毒性有り

《試験 3》

濃度 (μl/プレート)	His⁺ 復帰変異コロニー数/プレート TA100 -S9 (平均)	TA100 +S9 (平均)
0*	138, 151, 142, 148, 144, 134 (143)	168, 125, 160, 136, 130, 149 (145)
0.01	147, 168, 142 (152)	142, 115, 133 (130)
0.02	166, 148, 164 (159)	145, 131, 151 (142)
0.05	234, 207, 214 (218)	158, 155, 183 (165)
0.1	209, 206, 196 (204)	229, 195, 202 (209)
0.2	*22 44 17 (28)*	138, 140, 145 (141)
陽性対照 (μg/プレート)	AF2 (0.01) 583	B(a)P (5) 926
試験年月日	'97. 6.17	'97. 6.17

*: 対照として DMSO 100μlを使用
▒: 対照の 1.5倍以上 2倍未満
斜体文字：細胞毒性有り

《試験 4》

濃度 (μl/プレート)	His⁺ 復帰変異コロニー数 / プレート TA100 -S9 (平均)	TA100 +S9 (平均)
0*	117, 113, 116, 119, 110, 115 (115)	128, 126, 143, 138, 124, 133 (132)
0.01		
0.02	142, 146 (144)	102, 137 (120)
0.05	210, 193 (202)	208, 198 (203)
0.1	131, 124 (128)	213, 193 (203)
0.2	*111, 83 (97)*	182, 189 (186)
陽性対照 (μg/プレート)	AF2 (0.01) 506	B(a)P (5) 1245
試験年月日	'97. 8.19	'97. 8.19

*: 対照として DMSO 100μlを使用
▒: 対照の 1.5倍以上 2倍未満
斜体文字：細胞毒性有り

試料名 ： 納豆菌ガム
《試験 1》

濃度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	30, 33, 27, 29, 26, 34 (30)	41, 37, 40, 43, 34, 40 (39)	142, 137, 133, 136, 144, 147 (140)	146, 150, 143, 152, 142, 144 (146)	11, 13, 11, 17, 15, 14 (14)	14, 17, 16, 15, 17, 17 (16)	11, 13, 13, 9, 8, 13 (11)	15, 21, 13, 17, 12, 17 (16)	19, 22, 20, 24, 23, 20 (21)	34, 41, 37, 39, 44, 37 (39)
0.1	40, 41 (41)	44, 40 (42)	120, 153 (137)	134, 131 (133)	15, 14 (15)	16, 11 (14)	10, 10 (10)	22, 16 (19)	25, 18 (22)	35, 45 (40)
0.2	32, 38 (35)	36, 37 (37)	140, 143 (142)	152, 130 (141)	8, 9 (9)	10, 16 (13)	12, 12 (12)	14, 11 (13)	21, 23 (22)	34, 52 (43)
0.5	27, 29 (28)	38, 39 (39)	136, 130 (133)	137, 137 (137)	16, 11 (14)	14, 9 (12)	13, 16 (15)	22, 21 (22)	19, 21 (20)	41, 31 (36)
1	29, 27 (28)	35, 39 (37)	147, 36 (142)	144, 113 (129)	11, 13 (12)	16, 19 (18)	11, 6 (9)	18, 15 (17)	22, 20 (21)	45, 38 (42)
2	30, 30 (30)	34, 40 (37)	143, 152 (148)	142, 134 (138)	8, 10 (9)	19, 19 (19)	10, 10 (10)	30, 19 (25)	24, 22 (23)	44, 52 (48)
5	31, 33 (32)	43, 37 (40)	136, 132 (134)	139, 127 (133)	8, 15 (12)	11, 17 (14)	9, 10 (10)	16, 14 (15)	20, 18 (19)	46, 33 (40)
10	33, 25 (29)	40, 36 (38)	143, 133 (138)	130, 118 (124)	7, 11 (9)	17, 13 (15)	12, 14 (13)	22, 15 (19)	13, 21 (17)	36, 33 (35)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	418	370	480	1074	287	216	124	104	323	148
試験年月日	'97. 7.25	'97. 7.25	'97. 7.25	'97. 7.25	'97. 8. 5	'97. 8. 5	'97. 8. 1	'97. 8. 1	'97. 8. 1	'97. 8. 1

*: 対照として蒸留水100μlを使用
▒: 対照の 1.5倍以上 2倍未満

試料名：納豆菌ガム

《試験 2》

濃度 (mg/プレート)	His⁺復帰変異コロニー数/プレート TA1537 -S9 (平均)	+S9 (平均)
0*		10, 13, 17, 15, 11, 20 (14)
0.5		11, 14, 13 (13)
1		18, 13, 17 (16)
2		11, 20, 15 (15)
5		13, 17, 17 (16)
陽性対照 (μg/プレート)	9AA (80)	B(a)P (5) 113
試験年月日		'97. 8. 5

*：対照として蒸留水100μlを使用

《試験 3》

濃度 (mg/プレート)	His⁺復帰変異コロニー数/プレート TA1537 -S9 (平均)	+S9 (平均)
0*		12, 19, 17, 16, 21, 14 (17)
0.5		17, 21, 20 (19)
1		17, 14, 20 (17)
2		23, 13, 19 (18)
5		14, 15, 15 (15)
陽性対照 (μg/プレート)	9AA (80)	B(a)P (5) 166
試験年月日		'97. 9.17

*：対照として蒸留水100μlを使用

試料名 ： シソ抽出物
《試験 1》

濃度 (μl/プレート)	HIs⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	26, 15, 31, 23, 26, 32 (26)	35, 43, 35, 37, 35, 40 (38)	116, 135, 111, 124, 128, 116 (122)	137, 135, 126, 147, 138, 129 (135)	13, 11, 13, 12, 14, 15 (13)	18, 17, 18, 20, 14, 16 (17)	13, 9, 7, 9, 11, 14 (11)	16, 20, 14, 11, 13, 11 (14)	32, 20, 23, 24, 22, 25 (24)	36, 30, 35, 26, 30, 29 (31)
1	23, 28 (26)	43, 37 (40)	104, 122 (113)	135, 143 (139)	8, 8 (8)	17, 18 (18)	11, 10 (11)	9, 14 (12)	24, 30 (27)	31, 33 (32)
2	33, 27 (30)	35, 35 (35)	114, 123 (119)	134, 138 (136)	10, 13 (12)	12, 15 (14)	8, 10 (9)	17, 17 (17)	20, 22 (21)	28, 33 (31)
5	20, 29 (25)	38, 44 (41)	124, 130 (127)	131, 129 (130)	14, 10 (12)	13, 21 (17)	10, 14 (12)	16, 11 (14)	32, 26 (29)	32, 36 (34)
10	29, 16 (23)	41, 43 (42)	130, 132 (131)	146, 150 (148)	10, 10 (10)	19, 13 (16)	11, 7 (9)	18, 12 (15)	24, 23 (24)	24, 26 (25)
20	27, 20 (24)	49, 44 (47)	142, 126 (134)	132, 140 (136)	11, 11 (11)	16, 18 (17)	14, 12 (13)	21, 16 (19)	18, 26 (22)	31, 30 (31)
50	26, 25 (26)	35, 43 (39)	112, 122 (117)	153, 161 (157)	15, 17 (16)	16, 17 (17)	11, 10 (11)	20, 13 (17)	21, 24 (23)	33, 28 (31)
100	33, 30 (32)	80, 62 (61)	117, 131 (124)	209, 227 (218)	12, 17 (15)	19, 20 (20)	8, 11 (10)	14, 13 (14)	27, 27 (27)	42, 36 (39)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	440	466	747	1090	287	216	207	100	397	151
試験年月日	'97. 3.18	'97. 5.16	'97. 3.18	'97. 5.16	'97. 8. 5	'97. 8. 5	'97. 6. 3	'97. 6. 3	'97. 6. 3	'97. 6. 3

*: 対照として DMSO 100μlを使用
※: 対照の 1.5倍以上 2倍未満

試料名 ： シソ抽出物

《試験 2》

濃度 (μl/プレート)	His⁺ 復帰変異コロニー数 / プレート			
	TA98		TA100	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*		37, 40, 42, 41, 35, 40 (39)		127, 146, 133, 142, 138, 158 (141)
20		40, 38 (39)		139, 144 (142)
50		44, 39 (42)		142, 157 (150)
100		53, 56 (55)		189, 203 (196)
200		47, 40 (44)		176, 166 (171)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)
		493		926
試験年月日		'97. 6.17		'97. 6.17

*: 対照として DMSO 100μlを使用

《試験 3》

濃度 (μl/プレート)	His⁺ 復帰変異コロニー数 / プレート			
	TA98		TA100	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*		36, 36, 30, 44, 41, 42 (34)		140, 146, 140, 138, 114, 150 (138)
20		47, 49, 38 (45)		124, 126, 139 (130)
50		42, 43, 45 (43)		143, 160, 157 (153)
100		59, 48, 42 (50)		168, 158, 167 (164)
200		48, 43, 39 (43)		141, 169, 186 (165)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)
		349		1487
試験年月日		'97. 9.17		'97. 9.17

*: 対照として DMSO 100μlを使用

試料名 ： ダイズサポニン
《試験 1》

濃度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	28, 27, 29, 34, 31, 35 (31)	37, 34, 39, 42, 44, 40 (39)	132, 136, 137, 155, 131, 120 (135)	140, 153, 123, 142, 142, 141 (140)	19, 13, 10, 15, 16, 14 (16)	13, 18, 16, 11, 12, 16 (14)	16, 11, 10, 8, 6, 13 (11)	18, 18, 14, 16, 15, 18 (17)	23, 20, 21, 17, 22, 23 (21)	27, 48, 45, 42, 35, 33 (38)
0.2	26, 34 (30)	38, 47 (43)	133, 159 (146)	145, 133 (139)	16, 8 (12)	17, 16 (17)	13, 12 (13)	21, 11 (17)	19, 21 (20)	38, 38 (38)
0.5	28, 32 (30)	44, 38 (41)	166, 131 (149)	143, 152 (148)	17, 10 (14)	13, 18 (16)	12, 13 (13)	17, 12 (15)	24, 22 (23)	33, 29 (31)
1	35, 26 (31)	58, 53 (56)	154, 156 (155)	162, 180 (171)	9, 13 (11)	21, 16 (19)	11, 12 (12)	18, 13 (16)	17, 22 (20)	40, 33 (37)
2	25, 21 (23)	55, 47 (51)	144, 125 (135)	160, 159 (160)	11, 6 (9)	13, 15 (14)	6, 11 (9)	12, 19 (16)	19, 13 (16)	32, 33 (33)
5	24, 26 (25)	43, 34 (39)	117, 109 (113)	157, 145 (151)	11, 17 (14)	21, 20 (21)	7, 6 (7)	15, 14 (15)	15, 27 (21)	29, 29 (29)
10	18, 24 (21)	40, 33 (37)	87, 87 (87)	133, 123 (128)	18, 9 (14)	23, 15 (19)	15, 10 (13)	12, 19 (16)	20, 18 (19)	20, 32 (26)
20	28, 28 (28)	31, 36 (34)	49, 72 (61)	101, 108 (105)	20, 13 (17)	14, 16 (15)	5, 12 (9)	10, 14 (12)	18, 14 (16)	22, 14 (18)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	418	370	480	1074	239	186	124	104	323	148
試験年月日	'97. 7.25	'97. 7.25	'97. 7.25	'97. 7.25	'97. 8.19	'97. 8.19	'97. 8. 1	'97. 8. 1	'97. 8. 1	'97. 8. 1

*: 対照として DMSO 100μlを使用
▒: 対照の 1.5倍以上 2倍未満

試料名 ： ダイズサポニン
《試験 2》

濃度 (mg/プレート)	His⁺ 復帰変異コロニー数 / プレート			
	TA98		TA1535	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*		45, 43, 44, 41, 40, 45 (43)		14, 17, 12, 10, 16, 18 (15)
1		41, 44 (43)		
2		43, 42 (43)		13, 15, 17 (15)
5		36, 44 (40)		18, 20, 15 (18)
10		36, 35 (36)		21, 16, 19 (19)
20		44, 38 (41)		17, 14, 18 (16)
50		34, 42 (38)		
100		26, 39 (33)		
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	NaN_3 (0.5)	2AA (2)
		390		173
試験年月日		'97. 8.19		'97. 9.17

*: 対照として DMSO 100μlを使用

試料名 ： オレンジ色素
《試験 1》

濃度 (μl/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	31, 27, 27, 29, 33, 30 (26)	43, 47, 41, 51, 40, 38 (43)	152, 163, 156, 170, 151, 148 (157)	153, 169, 147, 157, 153, 153 (155)	19, 13, 10, 15, 16, 14 (15)	13, 18, 16, 11, 12, 16 (14)	12, 10, 10, 14, 13, 10 (12)	14, 17, 13, 11, 14, 15 (14)	28, 27, 23, 33, 25, 28 (27)	36, 30, 41, 38, 34, 34 (36)
1	33, 29 (31)	44, 37 (41)	166, 157 (162)	151, 159 (155)	14, 13 (14)	15, 16 (16)	12, 11 (12)	14, 9 (12)	21, 33 (27)	35, 46 (41)
2	35, 36 (36)	40, 45 (43)	162, 172 (167)	157, 161 (159)	16, 13 (15)	24, 18 (21)	13, 13 (13)	17, 19 (18)	27, 20 (24)	34, 34 (34)
5	26, 38 (32)	39, 59 (49)	155, 170 (163)	168, 176 (172)	17, 8 (13)	10, 15 (13)	11, 10 (11)	20, 12 (16)	29, 23 (26)	30, 30 (30)
10	39, 35 (37)	39, 54 (47)	143, 47 (145)	186, 194 (190)	15, 13 (14)	20, 16 (18)	13, 10 (12)	14, 13 (14)	21, 31 (26)	34, 25 (30)
20	30, 29 (30)	40, 55 (48)	122, 108 (115)	141, 140 (141)	12, 14 (13)	20, 20 (20)	11, 8 (10)	11, 16 (14)	28, 29 (29)	25, 30 (28)
50	30, 34 (32)	27, 40 (34)	94, 88 (91)	112, 121 (117)	16, 12 (14)	15, 10 (13)	10, 7 (9)	13, 19 (16)	20, 16 (18)	27, 32 (30)
100	27, 21 (24)	29, 33 (31)	87, 91 (89)	92, 116 (104)	13, 16 (15)	16, 14 (15)	10, 7 (9)	8, 11 (10)	17, 14 (16)	22, 19 (21)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	626	451	857	1606	239	186	241	151	460	172
試験年月日	'97. 6.13	'97. 6.13	'97. 6.13	'97. 6.13	'97. 8.19	'97. 8.19	'97. 6.17	'97. 6.17	'97. 6.17	'97. 6.17

*: 対照として DMSO 100μlを使用
※: 対照の 1.5倍以上 2倍未満

試料名 ： オレンジ色素
《試験 2》

濃 度 (μl/プレート)	His⁺復帰変異コロニー数/プレート TA1535 −S9 (平均)	+S9 (平均)
0*		14, 17, 12, 10, 16, 18 (15)
1		15, 17, 12 (15)
2		16, 17, 14 (16)
5		15, 12, 11 (13)
10		13, 15, 15 (14)
20		20, 19, 19 (19)
50		21, 15, 17 (18)
100		14, 16, 13 (14)
陽性対照 (μg/プレート)	NaN_3 (0.5)	2AA (2) 173
試験年月日	'97. 9.17	'97. 9.17

*: 対照として DMSO 100μlを使用

試料名：キチン

濃度 (mg/プレート)	His⁺ 復帰変異コロニー数 / プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	28, 36, 27, 28, 31, 33 (31)	42, 53, 42, 43, 33, 40 (42)	159, 175, 168, 157, 165, 158 (164)	169, 156, 178, 181, 167, 153 (167)	11, 13, 11, 17, 15, 14 (14)	14, 17, 16, 15, 17, 17 (16)	13, 10, 13, 9, 12, 18 (13)	9, 16, 15, 17, 18, 13 (15)	38, 31, 30, 30, 30, 21 (30)	41, 36, 32, 40, 40, 43 (39)
0.2	36, 36 (36)	44, 50 (47)	143, 141 (142)	159, 163 (161)	11, 16 (14)	11, 13 (12)	11, 15 (13)	12, 16 (14)	31, 36 (34)	37, 42 (40)
0.5	31, 34 (33)	47, 46 (47)	156, 149 (153)	163, 177 (170)	11, 10 (11)	10, 14 (12)	17, 10 (14)	15, 20 (18)	33, 30 (32)	40, 39 (40)
1	30, 39 (35)	49, 33 (41)	150, 162 (156)	181, 172 (177)	12, 5 (9)	11, 12 (12)	12, 11 (12)	12, 13 (13)	38, 25 (32)	31, 39 (35)
2	33, 37 (35)	46, 40 (43)	170, 159 (166)	152, 155 (154)	15, 16 (16)	14, 16 (15)	16, 12 (14)	17, 13 (15)	38, 29 (34)	31, 46 (39)
5	28, 27 (28)	56, 42 (49)	172, 181 (177)	156, 130 (143)	11, 13 (12)	11, 17 (14)	13, 12 (13)	17, 15 (16)	33, 28 (31)	25, 43 (34)
10	24, 32 (28)	38, 42 (40)	166, 164 (165)	158, 160 (159)	14, 7 (11)	10, 14 (12)	14, 13 (14)	14, 15 (15)	36, 36 (36)	35, 33 (34)
20	26, 25 (26)	38, 37 (38)	153, 137 (145)	134, 162 (148)	9, 11 (10)	15, 15 (15)	12, 9 (11)	14, 15 (15)	19, 36 (28)	27, 26 (27)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	626	451	857	1606	287	216	241	151	460	172
試験年月日	'97. 6.13	'97. 6.13	'97. 6.13	'97. 6.13	'97. 8. 5	'97. 8. 6	'97. 6.17	'97. 6.17	'97. 6.17	'97. 6.17

*：対照として蒸留水100μlを使用

試料名：フクロノリ抽出物

濃度 (mg/プレート)	His⁺ 復帰変異コロニー数 / プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	34, 33, 35, 39, 33, 33 (35)	35, 43, 35, 37, 35, 40 (38)	161, 162, 170, 147, 163, 154 (160)	137, 135, 126, 147, 138, 129 (135)	19, 13, 10, 15, 16, 14 (15)	13, 18, 16, 11, 12, 16 (14)	13, 9, 7, 9, 11, 14 (11)	16, 20, 14, 11, 13, 11 (14)	32, 20, 23, 24, 22, 25 (24)	36, 30, 35, 26, 30, 29 (31)
0.2	28, 25 (27)	31, 38 (35)	182, 151 (167)	130, 135 (133)	13, 13 (13)	11, 14 (13)	14, 12 (13)	20, 18 (19)	26, 28 (27)	34, 30 (32)
0.5	22, 31 (27)	42, 38 (40)	186, 141 (164)	133, 139 (136)	15, 15 (15)	16, 16 (16)	10, 11 (11)	10, 13 (12)	27, 20 (24)	30, 32 (31)
1	24, 30 (27)	36, 39 (38)	176, 167 (172)	142, 151 (147)	17, 18 (18)	14, 16 (15)	13, 7 (10)	16, 9 (13)	23, 26 (25)	29, 34 (32)
2	29, 21 (25)	37, 46 (42)	169, 148 (159)	157, 154 (156)	13, 12 (13)	14, 9 (12)	12, 13 (13)	12, 13 (13)	17, 21 (19)	26, 29 (28)
5	18, 28 (23)	44, 38 (41)	165, 171 (168)	174, 126 (150)	15, 8 (12)	13, 15 (14)	12, 9 (11)	18, 11 (15)	19, 18 (19)	31, 36 (34)
10	27, 34 (31)	37, 45 (41)	174, 169 (172)	147, 172 (160)	16, 14 (15)	14, 14 (14)	8, 13 (11)	11, 16 (14)	20, 22 (21)	22, 34 (28)
20	30, 29 (30)	30, 37 (34)	173, 146 (160)	166, 138 (152)	9, 11 (10)	18, 9 (14)	8, 9 (9)	19, 13 (16)	19, 17 (18)	27, 29 (28)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	523	466	690	1090	239	186	207	100	397	151
試験年月日	'97. 5.13	'97. 5.16	'97. 5.13	'97. 5.16	'97. 8.19	'97. 8.19	'97. 6. 3	'97. 6. 3	'97. 6. 3	'97. 6. 3

*: 対照として DMSO 100μl を使用

試料名 ： 二方手抽出物
《試験 1》

濃度 (μl/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	26, 15, 31, 23, 26, 32 (26)	35, 43, 35, 37, 35, 40 (38)	116, 135, 111, 124, 128, 116 (122)	137, 135, 126, 147, 138, 129 (135)	13, 11, 13, 12, 14, 15 (13)	18, 17, 18, 20, 14, 16 (17)	13, 9, 7, 9, 11, 14 (11)	16, 20, 14, 11, 13, 11 (14)	32, 20, 23, 24, 22, 25 (24)	36, 30, 35, 26, 30, 29 (31)
1	33, 37 (35)	37, 41 (39)	110, 117 (114)	146, 125 (136)	12, 13 (13)	16, 9 (13)	10, 14 (12)	17, 15 (16)	23, 24 (24)	34, 30 (32)
2	26, 29 (28)	41, 40 (41)	115, 113 (114)	120, 120 (120)	12, 9 (11)	13, 12 (13)	9, 13 (11)	13, 17 (15)	29, 24 (27)	39, 36 (38)
5	28, 29 (29)	37, 35 (36)	108, 116 (112)	143, 122 (133)	15, 11 (13)	11, 17 (14)	7, 11 (9)	18, 11 (15)	26, 28 (27)	32, 30 (31)
10	24, 22 (23)	41, 47 (44)	113, 99 (106)	121, 126 (124)	7, 15 (11)	16, 16 (16)	9, 14 (12)	19, 11 (15)	23, 24 (24)	30, 25 (28)
20	32, 27 (30)	41, 56 (49)	116, 111 (114)	126, 126 (126)	14, 13 (14)	15, 12 (14)	13, 9 (11)	7, 16 (12)	25, 22 (24)	23, 31 (27)
50	28, 20 (24)	31, 44 (38)	112, 121 (117)	144, 155 (150)	10, 11 (11)	23, 18 (21)	6, 11 (9)	13, 12 (13)	25, 27 (26)	33, 41 (37)
100	34, 25 (30)	52, 54 (53)	110, 126 (118)	157, 210 (184)	13, 11 (12)	19, 12 (16)	10, 9 (9)	11, 15 (13)	31, 23 (27)	29, 38 (34)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	440	466	747	1090	287	216	207	100	397	151
試験年月日	'97. 3.18	'97. 5.16	'97. 3.18	'97. 5.16	'97. 8. 5	'97. 8. 5	'97. 6. 3	'97. 6. 3	'97. 6. 3	'97. 6. 3

*: 対照として DMSO 100μlを使用
※: 対照の 1.5倍以上 2倍未満

試料名 ： ニガキ抽出物
《試験 2》

濃 度 (μl/プレート)	His⁺ 復帰変異コロニー数 ／ プレート			
	TA98		TA100	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*		37, 40, 42, 41, 35, 40 (39)		127, 146, 133, 142, 138, 158 (141)
10		36, 42, 40 (39)		145, 139, 147 (144)
20		40, 37, 39 (39)		138, 138, 143 (140)
50		41, 43, 47 (44)		162, 160, 169 (164)
100		50, 53, 47 (50)		157, 181, 179 (172)
200		54, 53, 49 (52)		177, 191, 203 (190)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5) 493	AF2 (0.01)	B(a)P (5) 926
試験年月日		'97. 6.17		'97. 6.17

*: 対照として DMSO 100μlを使用

試料名：モウソウチク抽出物
《試験 1》

濃度 (mg/プレート)	His⁺ 復帰変異コロニー数 / プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	34, 33, 35, 39, 33, 33 (35)	35, 43, 35, 37, 35, 40 (38)	161, 162, 170, 147, 163, 154 (160)	137, 135, 126, 147, 138, 129 (135)	19, 13, 10, 15, 16, 14 (15)	13, 18, 16, 11, 12, 16 (14)	13, 9, 7, 9, 11, 14 (11)	16, 20, 14, 11, 13, 11 (14)	32, 20, 23, 24, 22, 26 (24)	36, 30, 35, 26, 30, 29 (31)
0.2	25, 32 (29)	43, 43 (43)	179, 148 (164)	133, 150 (142)	11, 7 (9)	16, 13 (15)	13, 10 (12)	11, 18 (15)	29, 23 (26)	36, 33 (35)
0.5	25, 30 (26)	38, 35 (37)	153, 157 (155)	141, 139 (140)	12, 18 (15)	16, 20 (18)	9, 11 (10)	9, 17 (13)	24, 23 (24)	31, 29 (30)
1	32, 38 (35)	46, 33 (40)	160, 133 (147)	133, 160 (147)	16, 15 (16)	19, 15 (17)	13, 7 (10)	12, 13 (13)	27, 21 (24)	37, 36 (37)
2	37, 30 (34)	44, 41 (43)	153, 163 (158)	139, 139 (139)	10, 16 (13)	15, 16 (16)	7, 12 (10)	16, 13 (15)	21, 24 (23)	43, 37 (40)
5	43, 28 (36)	49, 40 (45)	177, 145 (161)	127, 160 (144)	15, 11 (13)	15, 10 (13)	11, 9 (10)	20, 16 (18)	23, 24 (24)	43, 41 (42)
10	38, 25 (32)	28, 38 (33)	187, 160 (174)	154, 163 (159)	10, 14 (12)	15, 26 (21)	13, 8 (11)	20, 15 (18)	24, 26 (25)	30, 26 (28)
20	31, 31 (31)	40, 47 (44)	179, 164 (172)	206, 189 (198)	14, 11 (13)	13, 15 (14)	8, 11 (10)	15, 20 (18)	17, 20 (19)	32, 29 (31)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	523	466	690	1090	239	186	207	100	397	151
試験年月日	'97. 5.13	'97. 5.16	'97. 5.13	'97. 5.16	'97. 8.19	'97. 8.19	'97. 6. 3	'97. 6. 3	'97. 6. 3	'97. 6. 3

*: 対照として DMSO 100μlを使用
※: 対照の 1.5倍以上 2倍未満

試料名：モウソウチク抽出物
《試験 2》

濃度 (mg/プレート)	His⁺ 復帰変異コロニー数 / プレート			
	TA100		TA1535	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*		127, 146, 133, 142, 138, 158 (141)		14, 17, 12 10, 16, 18 (15)
2				15, 13, 20 (16)
5		150, 162, 152 (155)		17, 17, 13 (16)
10		167, 142, 138 (149)		15, 18, 16 (16)
20		122, 144, 136 (134)		20, 17, 13 (17)
50		142, 157, 130 (143)		15, 14, 17 (15)
100		163, 142, 143 (149)		
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	2AA (2)
		926		173
試験年月日		'97. 6.17		'97. 9.17

*: 対照として DMSO 100μlを使用

試料名 ： レバン

濃度 (mg/プレート)	His⁺ 復帰変異コロニー数 ／ プレート									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)	-S9 (平均)	+S9 (平均)
0*	28, 36, 27, 28, 31, 33 (31)	42, 53, 42, 43, 33, 40 (42)	159, 175, 168, 157, 165, 158 (164)	169, 156, 178, 181, 167, 153 (167)	11, 13, 11, 17, 15, 14 (14)	14, 17, 16, 15, 17, 17 (16)	13, 10, 13, 9, 12, 18 (13)	9, 16, 15, 17, 18, 13 (15)	38, 31, 30, 30, 30, 21 (30)	41, 36, 32, 40, 40, 43 (39)
0.2	29, 33 (31)	46, 47 (47)	152, 162 (157)	159, 146 (153)	13, 17 (15)	16, 15 (16)	10, 16 (13)	16, 14 (15)	30, 30 (30)	39, 45 (42)
0.5	30, 27 (29)	40, 48 (44)	155, 155 (155)	156, 152 (154)	9, 11 (10)	13, 21 (17)	11, 12 (12)	21, 13 (17)	33, 35 (34)	43, 48 (46)
1	31, 31 (31)	33, 50 (42)	152, 166 (159)	141, 153 (147)	13, 13 (13)	17, 10 (14)	9, 11 (10)	13, 15 (14)	38, 42 (40)	43, 43 (43)
2	35, 35 (35)	39, 46 (43)	147, 170 (159)	150, 143 (147)	8, 15 (12)	11, 17 (14)	5, 6 (6)	12, 18 (15)	26, 23 (25)	32, 31 (32)
5	34, 30 (32)	46, 46 (46)	159, 141 (150)	169, 142 (156)	10, 19 (15)	21, 15 (18)	6, 6 (6)	16, 11 (13)	25, 26 (26)	31, 37 (34)
10	36, 34 (35)	47, 55 (51)	151, 154 (153)	150, 163 (157)	12, 9 (11)	14, 16 (15)	8, 6 (7)	13, 17 (15)	35, 17 (26)	33, 23 (28)
20	36, 29 (33)	38, 42 (40)	158, 167 (163)	166, 153 (160)	10, 11 (11)	15, 14 (15)	7, 6 (7)	20, 17 (19)	36, 36 (36)	35, 40 (38)
陽性対照 (μg/プレート)	AF2 (0.1)	B(a)P (5)	AF2 (0.01)	B(a)P (5)	NaN_3 (0.5)	2AA (2)	9AA (80)	B(a)P (5)	2NF (2)	B(a)P (5)
	626	451	857	1606	287	216	241	151	450	172
試験年月日	'97. 6.13	'97. 6.13	'97. 6.13	'97. 6.13	'97. 8. 5	'97. 8. 5	'97. 6.17	'97. 6.17	'97. 6.17	'97. 6.17

*: 対照として蒸留水100μlを使用

Appendix IX-5

English Translation of Chromosome Aberration Test of *Bacillus Subtilis* Gum- (PGA)

(see Appendix IX-6)

November 13, 1997

Fiscal 1996 Report

Tests for reevaluating the safety of food additives

Chromosome aberration test of natural additives in cultured mammalian cells

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Purpose

As part of the tests for reevaluating the safety of food additives, at the request of the Food Chemistry Division, Environmental Health Bureau, Ministry of Health and Welfare, the chromosome aberration and *in vitro* micronucleus tests using cultured mammalian cells were conducted for four natural additives distributed through Japan Food Additives Association, to evaluate the clastogenicity of the additives.

Test substances

1. Chitin (thickening agent and stabilizer)
2. Orange color (artificial color)
3. Soybean saponin (emulsifier)
4. "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム") (thickening agent and stabilizer)

All the substances listed above were provided through Japan Food Additives Association.

Test method

Using the Chinese hamster lung fibroblast cell line (CHL/IU), we conducted the *in vitro* chromosome aberration test by 24- or 48-hour continuous treatment without the metabolic activation system and 6-hour treatment with or without the metabolic activation system[1),2)]. In the 24- or 48-hour continuous treatment, 2×10^4 cells were spread out on 6-cm plastic petri dishes and the test substances were added to them on the third day, and after 24 and 48 hours, the chromosomal specimens of the cells were prepared. Five specimen concentrations were set, in principle, (serial dilution with a common ratio of 2), with the highest concentration being that at which cellular proliferation was obviously suppressed in preliminary tests. When no toxicity was observed in preliminary tests, the highest concentration was determined to be 5mg/ml.

In the 6-hour treatment process, the substances were added to the cells three days after cells were spread, which were then treated with the S9 mix or culture medium for six hours. After washing and incubating the cells for another 18 hours, their chromosomal specimens were prepared. We purchased the S9 mix that was prepared from the SD rat liver treated with phenobarbital and 5, 6-benzoflavone from Kikkoman Corporation for use in the tests.

The soluble substances were dissolved in normal saline and added to culture media at a concentration of 10%. The insoluble substances were dissolved in dimethyl sulfoxide (DMSO)

and added to culture media at a concentration of 0.5%. In the case of chitin and "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), because they were used at high concentrations, they were directly suspended in culture media to treat cells.

All chromosomal specimens were coded and 100 metaphase cells per concentration were observed by researchers who were blind to the treatment conditions; the frequency of cells with chromatid-type or chromosome-type structural aberrations (e.g., gaps, breakages, and exchanges.) was determined. In the case of the specimens with an unclear result of either positive or negative, a confirmation test was carried out after optimizing the concentration range to make the final determination. Furthermore, the frequency of polyploids (cells with more than two sets of chromosomes) was also recorded to evaluate the inducibility of numerical aberration. Generally, because 4% or more abnormality was not found in the untreated or solvent-treated control group, the results were categorized in principle as follows: negative (−) for less than 5% frequency, suspected positive (±) for 5% or more but less than 10%, and positive (+) for 10% or more. The results were comprehensively determined by also considering concentration dependence and other factors. Additional tests were conducted to confirm the results when needed.

In the *in vitro* micronucleus test[4)], the same CHL/IU cells as those used in the chromosome aberration test were used. In the 72-hour treatment, 5×10^4 cells were spread out on of 6-cm-diameter petri dishes and the test substances were added to them on the second day. Seventy-two hours after the beginning of the treatment, cell specimens were prepared. In the 6-hour treatment, as in the 72-hour treatment, 5×10^4 cells were spread out on 6-cm-diameter petri dishes and treated with the test substances for six hours with or without the S9 mix on the second day. After the treatment, the culture media were replaced with fresh ones. After 42 hours, cell specimens were prepared.

For the micronucleus test, cells were trypsinized, collected and then centrifuged. After centrifugations the cells were treated hypotonically with 0.075 M KCl for 10 minutes at room temperature. After the hypotonic treatment, the cells were half-fixed by adding 0.5~1ml of a fixative solution (1:3 acetic alcohol). After another centrifugation, the resuspension of cells in the same fixative solution was repeated 3 times or more. After the cells were sufficiently fixed, the fixative solution was changed to methanol containing 1% acetic acid and slide specimens of cells were prepared by the air-dry method, similarly to the chromosome specimens.

The specimens for the micronucleus test were stained by the simple acridine orange staining method. A drop of 40μg/ml acridine orange solution (in PBS) was placed onto a slide on which

a cover glass was immediately placed and extra dye solution was removed with tissue paper. The specimens were examined under a fluorescence microscope equipped with the B excitation light source system and 20x objective lens.

One thousand cells that were well stained and whose cytoplasm was completely intact were used for observation to determine the induction frequency of micronucleate cells. Cells with micronuclei were categorized as follows:

1) mononuclear cells with a very small micronucleus (MN-1)
2) mononuclear cells with a typical micronucleus (MN-2)
3) mononuclear cells with a large micronucleus (MN-3)
4) mononuclear cells with multiple micronuclei (Mu-MN)
5) mononuclear cells with micronuclei other than those listed above (MN-T)
6) multinuclear cells (including severely damaged cells) (Mu-N)
7) multinuclear cells with a micronucleus (Mu+MN)
8) metaphase cell (MP)

Results

The results of the *in vitro* chromosome aberration test by 24- or 48-hour continuous treatment using CHL/IU cells without the metabolic activation system and the confirmation tests are shown in Table 1. In addition, the results of the *in vitro* chromosome aberration test by 6-hour treatment with or without the metabolic activation system and the confirmation tests are shown in Table 2.

Chitin did not induce chromosomal aberrations both in the 24- and 48-hour continuous treatment and short-term treatment, regardless of the presence of the metabolic activation system. In terms of numerical aberrations, the frequency of polyploids did not increase at all.

Orange color showed cytotoxicity at the highest concentration in the continuous treatment; thus, it was impossible to examine 100 metaphase cells. However, the induction of numerical and structural aberrations of chromosomes was not observed. In the case of short-term treatment, because cytotoxicity was not found in the highest-concentration group in the first test, the confirmation test was conducted by increasing the concentration to the maximum. As a result, at a moderate concentration of 2.5mg/ml without metabolic activation system, 14% of the cells showed structural aberrations such as chromatid-type exchanges. However, it was impossible to examine 100 cells at the highest concentration of 5mg/ml owing to cytotoxicity, and no aberrations were induced at a low concentration of 1.25mg/ml. Meanwhile, in the presence of

the metabolic activation system, numerical and structural aberrations of chromosomes were not induced at any concentration up to 5mg/ml.

In the case of soybean saponin, although it was impossible to examine the cells under all treatment conditions due to its cytotoxicity at the highest concentration, the induction of both numerical and structural aberrations of chromosomes was not found. The clastogenicity observed in the presence of the metabolic activation system is considered to be a nonspecific response associated with cytotoxicity, as suggested by the fact that the number of cells observed was 33.

In the case of "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), because it was contaminated with bacteria, the test was conducted in the presence of penicillin and streptomycin. In the first continuous treatment, because cytotoxicity was not observed, two additional tests at high concentrations were conducted. As a result of the tests at all concentrations up to the maximum of 5mg/ml, cytotoxicity was observed at the highest concentration but both numerical and structural aberrations of chromosomes were not observed at lower concentrations. On the other hand, although 6% chromosomal aberrations were identified at the highest concentration of 5mg/ml in the short-term treatment with the metabolic activation system, the aberration types were limited to gaps and chromatid-type exchanges. In addition, chromosomal aberrations were induced in the absence of the metabolic activation system.

The results of the micronucleus test are shown in Tables 3 and 4. The results showed almost the same tendency as those of the chromosome aberration test.

Chitin, orange color, and "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム") were applied at all concentrations up to the maximum of 5mg/ml in the continuous and short-term treatments regardless of the presence of the metabolic activation system and the results were negative. Although soybean saponin was applied at all concentrations up to 5mg/ml in the continuous treatment, micronuclei were not observed at the concentration of 2.5mg/ml or higher owing to cytotoxicity, and micronuclei were not induced at the concentrations of less than 2.5mg/ml. However, only when metabolic activation system was present in the short-term treatment were micronuclei induced at one-half of the concentration that made the observation impossible owing to cytotoxicity (2.5mg/ml). This positive result is more likely the same nonspecific response associated with cytotoxicity as that observed as suspected positive in the chromosome aberration test.

Discussion

In the case of chitin, the investigation was conducted at any of the concentrations used up to the maximum or at the concentration that made it impossible to observe chromosomes owing to cytotoxicity, under all treatment conditions. However, numerical and structural aberrations of chromosomes were not induced.

Orange color in the presence of the metabolic activation system in the continuous treatment and short-term treatments did not induce chromosomal aberrations at any of the concentrations used up to the concentration that showed cytotoxicity or the highest concentration. However, in the absence of the metabolic activation system in short-term treatment, 14% of cells in the group treated with 2.5mg/ml orange color showed structural aberrations. However, apparent cytotoxicity was observed in the group treated at 5mg/ml, whereas no aberrations were induced in the group treated at 1.25mg/ml. Therefore, considering that there was no concentration dependence of response and that the introduction of the metabolic activation system did not induce aberrations, this induction of the aberrations is more likely associated with cytotoxicity and its *in vivo* adverse effects is considered to be small.

In the case of soybean saponin, mild chromosome structural aberrations and the induction of micronuclei that were considered to be associated with cytotoxicity were observed in the presence of the metabolic activation system. However, their *in vivo* adverse effects is considered to be small, given that they were induced at a high concentration and that a concentration-response correlation was not found.

In the case of "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), 6% of the cells treated at the highest concentration of 5mg/ml showed aberrations only in the short-term treatment with the metabolic activation system. In accordance with the determination criteria, the result was suspected positive. However, given that no chromosomal aberrations were induced under any other treatment conditions, that the concentration was very high, that the frequency of aberrations was not high, and other factors, it is considered that "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム") does not have *in vivo* adverse effects.

Conclusion

For the four test substances we examined this year, numerical and structural aberrations of chromosomes were not induced under almost all treatment conditions. For orange color, soybean saponin and "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), there were weak positive or suspected positive results in some tests. However, considering that the concentrations used were high, that no concentration-response relationship was identified, and other factors, the biological significance of these responses is small and that these substances have no *in vivo* significant adverse effects.

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平成８年度報告書

食品添加物安全性再評価等の試験

天然添加物の哺乳類培養細胞を用いる染色体異常試験

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目的

食品添加物安全性再評価等の試験検査の一環として，厚生省生活衛生局食品化学課の依頼により，日本食品添加物協会を通じて配布された天然添加物4種について，哺乳類培養細胞を用いる染色体異常試験および in vitro小核試験を実施し，染色体異常誘発性を評価する．

被験物質

1．　キチン（増粘安定剤）
2．　オレンジ色素（着色料）
3．　大豆サポニン（乳化剤）
4．　納豆菌ガム（増粘安定剤）

上記検体は，全て日本食品添加物協会を通じて供与された．

試験方法

チャイニーズ・ハムスター肺由来の線維芽細胞株（CHL/IU）を用い，代謝活性化系非存在下での24，48時間連続処理法および代謝活性化系の存在／非存在下での6時間処理法による染色体異常試験を行った[1,2]．24, 48 時間連続処理法においては，細胞2 x 10^4個を6 cmのプラスチックシャーレに播き，3日目に検体を加え，24時間および48時間後に染色体標本を作製した．検体濃度は予備試験によって，細

胞増殖が明らかに抑制される濃度を最高に，原則として５濃度（公比2で段階希釈）を設定した．予備試験で毒性が観察されない場合には，5 mg/mlを最高濃度とした．

6時間処理法においては，細胞播種後3日目に被験物質をS9 mixまたは培養液と共に6時間処理し，洗浄後さらに18時間培養した後，染色体標本を作製した．S9はフェノバルビタールおよび 5, 6-ベンゾフラボンで処理したSDラットの肝から調製したものを，キッコーマン(株)より購入して用いた．

検体は水溶性の場合は生理食塩水に溶解し，培養液に10%の割合で添加した．非水溶性の場合はDMSO(dimethyl sulfoxide)に溶解し，培養液に0.5%の割合で添加した．また，キチンと納豆菌ガムに関しては高用量で処理するため，培養液に直接懸濁して細胞を処理した．

染色体標本は全てコード化し，処理の条件が分からない状態で，濃度当り100個の分裂中期像について観察し，染色分体型あるいは染色体型の構造異常（ギャップ，切断，交換など）をもつ細胞の出現頻度を記録した．結果が明白な陽性または陰性を示さなかった検体について，用量範囲を最適化して確認試験を行い，最終判定を下した．さらに，数的異常の誘発性を評価するために倍数体（染色体数が倍化した細胞）についても併せて記録した．結果の判定は，未処理及び溶媒処理の対照群では通常4%以上の異常はみられないため，原則として，5%未満を陰性(－)，5%以上10%未満を疑陽性(±)，10%以上を陽性(＋)とし，用量依存性等を考慮して総合的に行った．なお，必要に応じて追加再実験を行い，結果の確認を行った．

In vitro 小核試験[1]は染色体異常試験に用いたのと同じCHL/IU細胞を用いた．72時間処理法の場合には 5×10^4 個の細胞を直径6 cmのシャーレに播種し，2日目に被験物質を加え，処理開始72時間後に

標本を作製した．6時間処理法は，72時間処理法と同様5×10^4個の細胞を直径 6 cm のシャーレに播種し，2日目に被験物質で6時間S9 mixの存在，非存在下で処理した．処理後新鮮な培地と置き換え，42時間後に標本を作製した．

小核試験の標本は，細胞をトリプシン処理して回収し，遠沈後0.075 M KCl，室温10分間の低張処理を施した．低張処理終了後0.5～1 mlの固定液(1:3 acetic alcohol)を加えて半固定を行った．遠沈後同上の固定液に再懸濁する操作を３回以上繰り返した．充分固定を行った後，固定液を1%の酢酸を含むメタノールに置き換え，空気乾燥法によって染色体標本と同様にスライド標本を作製した．

小核試験用標本は，アクリジンオレンジ簡易染色法で染色した．すなわち，40μg/mlのアクリジンオレンジ溶液(PBS)を1滴滴下し，直ちにカバーグラスをのせ，余分な色素溶液をティシュペーパを用いて取り除いた．観察はＢ励起照明系を備えた蛍光顕微鏡を用い，20倍の対物レンズを用いて行った．

標本の観察は，染色状態が良好で，かつ，細胞質が完全に保存されている細胞のみを対象とし，1000個の細胞を観察して小核細胞の誘発頻度を求めた．なお，小核を有する細胞の判定は次の分類にしたがって行った．

1）非常に小さい小核を持った単核細胞(MN-1)

2）典型的な小核を持った単核細胞(MN-2)

3）大きな小核を持った単核細胞(MN-3)

4）複数個の小核を持った単核細胞(Mu-MN)

5）形状にかかわらず小核を持った単核細胞(MN-T)

6）多核細胞（傷害を強く受けている細胞を含む）(Mu-N)

7）小核を持った多核細胞(Mu+MN)

8）分裂中期細胞(MP)

結果

チャイニーズ・ハムスター培養細胞(CHL/IU)を用いる代謝活性化系非存在下での24，48時間連続処理法の結果を確認試験結果と共に表1に示す．また，代謝活性化系の存在／非存在下での6時間処理法による結果を確認試験結果と共に表2に示す．

キチンは24時間および48時間の連続処理，また短時間処理においても代謝活性化系の存在に関わりなく，染色体異常を誘発しなかった．また，数的異常に関しても倍数体の出現頻度の上昇は全く観察されなかった．

オレンジ色素は，連続処理法においては最高用量で細胞毒性のため100分裂中期像の観察が不可能であった．しかし，染色体の数的および構造異常の誘発は観察されなかった．短時間処理は最初の試験で最高用量群において細胞毒性が観察されなかったので，限界用量まで用量を高めて再試験を行った．その結果，代謝活性化系の非存在下の中用量（2.5 mg/ml）において染色分体型交換をはじめとする構造異常が14%の細胞に観察された．しかし，高用量（5 mg/ml）では細胞毒性のため100細胞の観察は不可能であり，低用量（1.25 mg/ml）においては異常の誘発は認められなかった．ただし，代謝活性化系の存在下においては，5 mg/mlの限界用量まで染色体の数的ならびに構造異常の誘発は観察されなかった．

大豆サポニンは，全ての処理条件下において最高用量で細胞毒性により観察が不能となったが，染色体の数的，構造異常共に誘発は観察されなかった．代謝活性化系存在下にみられた染色体異常誘発性は，観察細胞が33であることからもわかるように，細胞毒性に関連した非特異的反応であったと考えられる．

納豆菌ガムに関しては，検体にバクテリアのコンタミネーションが認められたためペニシリンおよびストレプトマイシンを添加した条件下において試験を行った．最初の連続処理では細胞毒性が観察されなかったため，さらに高用量の試験を２回追加した．その結果，限界用量である5 mg/mlまで試験したが，最高用量で細胞毒性が観察され，それ以下の用量では染色体の数的，構造異常共に観察されなかった．一方，短時間処理法の代謝活性化系の存在下において，最高用量の5 mg/mlで6%の異常が認められたが，異常の種類としてはギャップと染色分体型交換のみであった．また，代謝活性化系の非存在下においては異常の誘発は観察されなかった．

小核試験の結果を表3および4にまとめる．結果は染色体異常試験とほぼ同じ傾向を示した．

キチンおよびオレンジ色素および納豆菌ガムに関しては連続処理，短時間処理では代謝活性化の有無に関わらず限界用量である5 mg/mlまで処理したが，結果は陰性であった．大豆サポニンは連続処理法では５mg/mlまで処理したが，2.5 mg/ml以上では細胞毒性のため小核の観察はできなかった．また，それ以下の用量では小核の誘発は観察されなかった．しかし，短時間処理で，代謝活性化系を組み込んだ場合にのみ，細胞毒性により観察が不能となる用量（2.5 mg/ml）の1/2用量で小核の誘発が観察された．この陽性結果は染色体異常試験における疑陽性の結果が細胞毒性に関係した非特異的な反応である可能性が強い．

考察

キチンに関しては全ての処理条件下において限界用量または細胞毒性により染色体の観察ができなくなる用量まで検討したが，染色体の構造および数的異常の誘発は観察されなかった．

オレンジ色素は連続処理法および短時間処理の代謝活性化系存在下においては，細胞毒性が認められる用量または限界用量まで異常の誘発は認められなかった．しかし，短時間処理の代謝活性化系非存在下において，2.5 mg/ml処理群において構造異常が14%の細胞に観察された．しかし，倍量の5 mg/mlでは明らかな細胞毒性が観察され，また，1/2量の1.25 mg/ml処理群においては異常の誘発は全く観察されなかった．従って，用量依存性の無いこと，また，代謝活性化系の導入により異常の誘発が観察されなかった点を考慮すると，この異常の誘発は細胞毒性と関連したものである可能性が強く，生体に対する影響は少ないものと考えることができる．

大豆サポニンは代謝活性化系の存在下において細胞毒性に関係すると考えられる弱い染色体の構造異常および小核の誘発が観察されたが，高用量である点，用量相関性が認められない点を考えあわせると，生体に対する影響は少ないものと考えることができる．

納豆菌ガムは短時間処理，代謝活性化系の存在下においてのみ，限界用量の5 mg/ml処理により6%の細胞に異常が観察された．判定基準からすれば疑陽性であるが，他の処理条件では全く染色体異常を誘発しなかったこと，非常に高用量であること，異常の出現頻度が高くないこと，等を考えあわせると生体に対する影響を心配する必要はないものと考えられる．

まとめ

本年度試験した4種の検体は，ほとんどの処理条件下で染色体の数的および構造異常の誘発は認められなかった．オレンジ色素，大豆サポニンおよび納豆菌ガムでは，一部の試験系において弱い陽性または疑陽性の結果を示したが，処理用量の高い点，用量反応関係が認められない点，等を考慮するとこれらの反応は生物学的な意義は低く，いずれの検体も生体において影響が現れるものとは考えがたい．

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表1　チャイニーズ・ハムスター培養細胞を用いる染色体異常試験（連続処理法）

被験物質名	S9 mix	時間* (h)	溶媒**	濃度 (mg/ml)	観察細胞数	倍数体 (%)	判定	染色体構造異常を持つ細胞の出現頻度*** ctg	ctb	cte	frg	csb	cse	total	判定
キチン	—	24-0	MEM	0(None)	100	4.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0(Solvent)	100	1.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				1.25	100	1.0	—	0.0	0.0	1.0	0.0	0.0	2.0	3.0	—
				2.5	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				5.0	100	0.0	—	0.0	1.0	0.0	0.0	0.0	0.0	1.0	—
	—	48-0	MEM	0(None)	100	0.0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				0(Solvent)	100	1.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				1.25	100	1.0	—	0.0	0.0	0.0	0.0	0.0	1.0	1.0	—
				2.5	100	2.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				5.0	100	1.0	—	1.0	0.0	1.0	0.0	1.0	0.0	3.0	—
オレンジ色素	—	24-0	DMSO	0(None)	100	4.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0(Solvent)	100	2.0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				0.0625	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				0.125	100	1.0	—	1.0	0.0	0.0	0.0	2.0	0.0	3.0	—
				0.25	100	1.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				0.5	—	—	#TOX	—	—	—	—	—	—	—	TOX
	—	48-0	DMSO	0(None)	100	0.0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				0(Solvent)	100	0.0		0.0	0.0	2.0	0.0	0.0	0.0	2.0	
				0.0625	100	1.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				0.125	100	0.0	—	0.0	0.0	0.0	0.0	0.0	1.0	1.0	—
				0.25	100	3.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
				0.5	63	0.0	—	0.0	1.0	0.0	0.0	0.0	0.0	1.0	—
					(100			0.0	0.6	0.0	0.0	0.0	0.0	0.6	—)

表1の続き

被験物質名	S 9 mix	時間* (h)	溶媒**	濃度 (mg/ml)	観察 細胞数	倍数体 (%)	判定	染色体構造異常を持つ細胞の出現頻度*** ctg	ctb	cte	frg	csb	cse	total	判定
大豆サポニン	—	24-0	Saline	0(None)	100	1.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0(Solvent)	100	0.0		2.0	0.0	0.0	0.0	0.0	0.0	2.0	
				0.25	100	0.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
				0.5	100	2.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
				1.0	100	1.0	—	0.0	0.0	1.0	0.0	0.0	0.0	1.0	—
				2.0	—	—	TOX	—	—	—	—	—	—	—	TOX
	—	48-0	Saline	0(None)	100	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0(Solvent)	100	1.0		0.0	0.0	0.0	0.0	0.0	1.0	1.0	
				0.25	100	0.0	—	0.0	0.0	0.0	0.0	2.0	1.0	3.0	—
				0.5	100	3.0	—	0.0	1.0	2.0	0.0	0.0	0.0	3.0	—
				1.0	100	0.0	—	1.0	0.0	0.0	0.0	1.0	1.0	3.0	—
				2.0	42	0.0	—	0.0	1.0	3.0	0.0	0.0	0.0	4.0	—
					(100			0.0	2.4	7.1	0.0	0.0	0.0	9.5	±)
納豆菌ガム	—	24-0	MEM+P	0(None)	100	0.0		2.0	1.0	0.0	0.0	0.0	0.0	3.0	
				0(Solvent)	100	0.0		0.0	1.0	0.0	0.0	0.0	0.0	1.0	
				0.0725	100	0.0	—	1.0	1.0	0.0	0.0	0.0	0.0	2.0	—
				0.1	100	0.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
				0.125	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	—	48-0	MEM+P	0(None)	100	0.0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				0(Solvent)	100	1.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0.0725	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				0.1	100	1.0	—	0.0	1.0	0.0	0.0	0.0	0.0	1.0	—
				0.125	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—

表1の続き

被験物質名	S 9 mix	時間* (h)	溶媒**	濃度 (mg/ml)	観察細胞数	倍数体 (%)	判定	染色体構造異常を持つ細胞の出現頻度*** ctg	ctb	cte	frg	csb	cse	total	判定
納豆菌ガム	—	24-0	MEM+P	0(None)	100	0.0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				0(Solvent)	100	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0.125	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				0.25	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				0.5	100	1.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
	—	48-0	MEM+P	0(None)	100	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0(Solvent)	100	1.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0.125	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				0.25	100	1.0	—	1.0	1.0	0.0	0.0	0.0	0.0	2.0	—
				0.5	100	0.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
	—	24-0	MEM+P	0(None)	100	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0(Solvent)	100	0.0		1.0	1.0	0.0	0.0	0.0	0.0	2.0	
				0.625	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				1.25	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				2.5	100	0.0	—	4.0	3.0	0.0	0.0	0.0	0.0	6.0	±
				5.0	—	—	TOX	—	—	—	—	—	—	—	TOX
	—	48-0	MEM+P	0(None)	100	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0(Solvent)	100	1.0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				0.625	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				1.25	100	0.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
				2.5	100	0.0	—	0.0	1.0	0.0	0.0	0.0	0.0	1.0	—
				5.0	—	—	TOX	—	—	—	—	—	—	—	TOX

*処理時間-回復時間，**MEM：MEM+10%CSで直接懸濁後培養Mediumと交換処理，
MEM+P：MEM+10%CS+1%Penicillin Streptomycinで直接懸濁後培養Mediumと交換処理，DMSO：ジメチルスルフォキサイド，Saline：生理食塩水，
***ctg：染色分体ギャップ（染色体ギャップを含める），ctb：染色分体切断，cte：染色分体交換，frg：断片化，csb：染色体切断，cse：染色体交換，
#TOX：生存細胞殆どない

表2　チャイニーズ・ハムスター培養細胞を用いる染色体異常試験（短時間処理法）

被験物質名	S 9 mix	時間* (h)	溶媒**	濃度 (mg/ml)	観察細胞数	倍数体 (%)	判定	染色体構造異常を持つ細胞の出現頻度*** ctg	ctb	cte	frg	csb	cse	total	判定
キチン	−	6-18	MEM	0(Solvent)	100	0.0		0.0	0.0	0.0	0.0	0.0	1.0	1.0	
				1.25	100	0.0	−	1.0	0.0	0.0	0.0	0.0	0.0	1.0	−
				2.5	100	1.0	−	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
				5.0	100	0.0	−	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
	+	6-18	MEM	0(Solvent)	100	1.0		1.0	1.0	0.0	0.0	0.0	0.0	2.0	
				1.25	100	0.0	−	0.0	0.0	0.0	0.0	1.0	0.0	1.0	−
				2.5	100	1.0	−	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
				5.0	100	2.0	−	2.0	0.0	0.0	0.0	0.0	0.0	2.0	−
オレンジ色素	−	6-18	DMSO	0(Solvent)	100	1.0		1.0	0.0	1.0	0.0	0.0	0.0	2.0	
				0.125	100	0.0	−	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
				0.25	100	0.0	−	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
				0.5	100	2.0	−	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
	+	6-18	DMSO	0(Solvent)	100	0.0		2.0	0.0	0.0	0.0	0.0	0.0	2.0	
				0.125	100	1.0	−	1.0	0.0	0.0	0.0	0.0	0.0	1.0	−
				0.25	100	1.0	−	1.0	0.0	0.0	0.0	1.0	0.0	3.0	−
				0.5	100	1.0	−	2.0	0.0	0.0	0.0	0.0	0.0	2.0	−
	−	6-18	MEM	0(Solvent)	100	0.0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				1.25	100	0.0	−	1.0	0.0	0.0	0.0	0.0	0.0	1.0	−
				2.5	100	4.0	−	1.0	5.0	9.0	0.0	0.0	0.0	14.0	+
				5.0	51	1.0	−	1.0	3.0	2.0	0.0	0.0	0.0	5.0	±
					(100			2.0	5.9	3.9	0.0	0.0	0.0	9.8	±)
	+	6-18	MEM	0(None)	100	0.0		2.0	0.0	0.0	0.0	0.0	0.0	2.0	
				1.25	100	0.0	−	1.0	1.0	0.0	0.0	0.0	0.0	2.0	−
				2.5	100	2.0	−	1.0	1.0	2.0	0.0	0.0	0.0	4.0	−
				5.0	100	4.0	−	0.0	0.0	1.0	0.0	0.0	0.0	1.0	−

表2の続き

被験物質名	S 9 mix	時間* (h)	溶媒**	濃度 (mg/ml)	観察細胞数	倍数体 (%)	倍数体 判定	染色体構造異常を持つ細胞の出現頻度*** ctg	ctb	cte	frg	csb	cse	total	判定
大豆サポニン	—	6-18	Saline	0(Solvent)	100	0.0		0.0	0.0	0.0	0.0	0.0	1.0	1.0	
				0.3125	100	2.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				0.625	100	2.0	—	0.0	1.0	0.0	0.0	0.0	0.0	1.0	—
				1.25	100	1.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
				2.5	100	2.0	—	2.0	0.0	0.0	0.0	0.0	0.0	2.0	—
				5.0	—	—	#TOX	—	—	—	—	—	—	—	TOX
	+	6-18	Saline	0(Solvent)	100	0.0		0.0	1.0	0.0	0.0	0.0	0.0	1.0	
				0.3125	100	0.0	—	1.0	0.0	0.0	0.0	0.0	0.0	1.0	—
				0.625	100	1.0	—	0.0	1.0	1.0	0.0	0.0	0.0	2.0	—
				1.25	33	0.0	—	0.0	3.0	5.0	0.0	0.0	0.0	7.0	±
					(100			0.0	9.1	15.2	0.0	0.0	0.0	21.0	+)
				2.5	—	—	TOX	—	—	—	—	—	—	—	TOX
				5.0	—	—	TOX	—	—	—	—	—	—	—	TOX
納豆菌ガム	—	24-0	MEM+P	0(None)	100	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				1.25	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				2.5	100	1.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
				5.0	100	0.0	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	+	48-0	MEM+P	0(None)	100	0.0		4.0	0.0	0.0	0.0	0.0	0.0	4.0	
				1.25	100	0.0		2.0	1.0	1.0	0.0	0.0	0.0	4.0	—
				2.5	100	0.0	—	2.0	0.0	0.0	0.0	0.0	0.0	2.0	—
				5.0	100	0.0	—	2.0	4.0	0.0	0.0	0.0	0.0	6.0	±

*処理時間-回復時間，**MEM：MEM+10%CSで直接懸濁後培養Mediumと交換処理，
MEM+P：MEM+10%CS+1%Penicillin Streptomycinで直接懸濁後培養Mediumと交換処理，DMSO：ジメチルスルフォキサイド，Saline：生理食塩水，
***ctg：染色分体ギャップ（染色体ギャップを含める），ctb：染色分体切断，cte：染色分体交換，frg：断片化，csb：染色体切断，cse：染色体交換，
#TOX：生存細胞殆どない

表3　チャイニーズ・ハムスター培養細胞を用いる in vitro 小核試験　（連続処理法）

被験物質名	S9	処理時間	濃度(mg/ml)	細胞数	MN-1	MN-2	MN-3	Mu-MN	MN-T	Mu-N	Mu+MN	MP
キチン	−	72-0	0(MEM)	1000	0	4	1	0	5	2	2	17
			0.3125	1000	0	4	0	0	4	2	0	16
			0.625	1000	1	1	0	0	1	5	1	11
			1.25	1000	1	2	0	0	2	2	0	8
			2.5	1000	0	2	0	1	3	6	1	10
			5.0	1000	2	2	0	2	4	5	0	11
オレンジ色素	−	72-0	0(DMSO)	1000	1	7	0	0	7	11	1	14
			0.03125	1000	1	5	0	0	5	3	1	11
			0.0625	1000	0	2	1	0	3	6	1	5
			0.125	1000	0	5	0	0	5	1	0	14
			0.25	1000	0	5	0	0	5	5	1	12
			0.5	1000	0	18	1	0	19	6	3	11
	−	72-0	0(MEM)	1000	0	3	0	0	3	5	0	15
			0.3125	1000	2	5	3	1	9	10	0	21
			0.625	1000	1	10	4	2	16	9	0	5
			1.25	#TOX	−	−	−	−	−	−	−	−
			2.5	TOX	−	−	−	−	−	−	−	−
			5.0	TOX	−	−	−	−	−	−	−	−
大豆サポニン	−	72-0	0(Saline)	1000	0	1	1	0	2	5	0	10
			0.3125	1000	2	3	0	0	3	6	2	8
			0.625	1000	1	6	2	0	8	37	3	18
			1.25	1000	0	9	0	2	11	67	6	4
			2.5	TOX	−	−	−	−	−	−	−	−
			5.0	TOX	−	−	−	−	−	−	−	−

表3の続き

被験物質名	S9	処理時間	濃度(mg/ml)	細胞数	MN-1	MN-2	MN-3	Mu-MN	MN-T	Mu-N	Mu+MN	MP
納豆菌ガム	−	72-0	0(MEM+P)	1000	3	1	0	0	1	1	0	2
			0.0625	1000	1	3	1	1	5	2	0	8
			0.125	1000	0	5	0	0	5	10	0	10
			0.25	1000	0	2	1	0	3	6	1	8
			0.5	1000	1	3	0	0	3	2	1	11
			1.0	1000	3	3	0	0	3	4	1	7
	−	72-0	0(MEM+P)	1000	2	1	0	0	1	3	0	3
			0.625	1000	2	4	0	0	4	12	0	2
			1.25	1000	1	8	0	0	8	8	0	1
			2.5	1000	1	10	3	1	14	6	1	1
			5.0	TOX	−	−	−	−	−	−	−	−

MN-1: 非常に小さい小核を持った単核細胞
MN-2: 典型的な小核を持った単核細胞
MN-3: 大きな小核を持った単核細胞
Mu-MN: 複数個の小核を持った単核細胞
MN-T: 形状にかかわらず小核を持った単核細胞
Mu-N: 多核細胞（損傷を多く受けている細胞も含む）
Mu+MN: 小核を持った多核細胞
MP: 分裂中期細胞
#TOX: 生存細胞殆ど無い

表4　チャイニーズ・ハムスター培養細胞を用いる in vitro 小核試験　（短時間処理法）

被験物質名	S9	処理時間	濃度(mg/ml)	細胞数	MN-1	MN-2	MN-3	Mu-MN	MN-T	Mu-N	Mu+MN	MP
キチン	－	6-42	0(MEM)	1000	0	2	0	0	2	2	2	10
			0.3125	1000	1	5	0	0	5	4	0	11
			0.625	1000	0	5	2	1	8	8	0	11
			1.25	1000	1	8	0	0	8	6	3	11
			2.5	1000	2	9	0	1	10	2	0	24
	＋	6-42	0(MEM)	1000	0	5	0	0	5	2	0	19
			0.3125	1000	0	3	0	0	3	7	2	10
			0.625	1000	0	2	0	0	2	2	1	14
			1.25	1000	0	4	0	0	4	9	1	21
			2.5	1000	1	6	0	1	7	5	0	17
	－	6-42	0(MEM)	1000	1	2	1	0	3	2	0	12
			2.5	1000	3	16	1	0	17	6	1	14
			5.0	1000	0	6	0	0	6	3	0	12
	＋	6-42	0(MEM)	1000	0	0	1	2	3	1	2	22
			2.5	1000	1	4	1	1	6	2	0	18
			5.0	1000	4	8	0	0	8	2	1	29
オレンジ色素	－	6-42	0(DMSO)	1000	3	4	1	0	5	4	0	11
			0.0625	1000	0	3	0	1	4	3	1	12
			0.125	1000	0	4	0	0	4	5	3	11
			0.25	1000	0	4	1	2	7	3	3	14
			0.5	1000	2	6	0	3	9	5	0	21
	＋	6-42	0(DMSO)	1000	1	5	1	3	9	5	0	5
			0.0625	1000	1	12	0	0	12	12	2	2
			0.125	1000	0	6	2	0	8	9	0	3
			0.25	1000	1	4	0	0	4	8	0	8
			0.5	1000	0	4	0	0	4	0	1	8
	－	6-42	0(MEM)	1000	1	2	1	0	3	2	6	12
			0.625	1000	5	6	0	0	6	5	2	9
			1.25	1000	0	5	1	2	8	10	0	14
			2.5	1000	1	12	2	1	15	10	4	18
			5.0	1000	4	2	0	0	2	8	2	4
	＋	6-42	0(MEM)	1000	0	0	1	2	3	1	2	22
			0.625	1000	1	3	0	0	3	0	1	22
			1.25	1000	1	10	0	2	12	7	0	14
			2.5	1000	0	8	1	1	10	3	2	8
			5.0	1000	1	12	2	0	14	16	1	18

表4の続き

被験物質名	S9	処理時間	濃度(mg/ml)	細胞数	MN-1	MN-2	MN-3	Mu-MN	MN-T	Mu-N	Mu+MN	MP
大豆サポニン	−	6-42	0(Saline)	1000	2	5	0	0	5	1	0	6
			0.625	1000	2	8	0	0	8	2	0	9
			1.25	1000	0	6	0	0	6	7	1	8
			2.5	1000	2	14	0	0	14	12	4	14
			5.0	#TOX	−	−	−	−	−	−	−	−
	+	6-42	0(Saline)	1000	1	1	0	0	1	3	0	7
			0.625	1000	1	6	1	0	7	7	2	14
			1.25	1000	12	70	6	27	103	40	29	18
			2.5	TOX	−	−	−	−	−	−	−	−
			5.0	TOX	−	−	−	−	−	−	−	−
納豆菌ガム	−	6-42	0(MEM+P)	1000	0	2	1	0	3	5	0	11
			0.3125	1000	0	4	0	0	4	6	1	8
			0.625	1000	0	3	2	0	5	6	0	5
			1.25	1000	1	10	1	0	11	5	0	6
			2.5	1000	1	6	0	0	6	5	2	12
			5.0	1000	0	4	0	0	4	6	0	6
	+	6-42	0(MEM+P)	1000	0	2	0	0	2	5	0	4
			0.3125	1000	0	3	0	0	3	2	0	6
			0.625	1000	0	5	0	0	5	5	1	4
			1.25	1000	1	6	0	0	6	13	1	2
			2.5	1000	1	12	1	0	13	7	2	2
			5.0	1000	0	11	0	0	11	22	0	4

MN-1: 非常に小さい小核を持った単核細胞
MN-2: 典型的な小核を持った単核細胞
MN-3: 大きな小核を持った単核細胞
Mu-MN: 複数個の小核を持った単核細胞
MN-T: 形状にかかわらず小核を持った単核細胞
Mu-N: 多核細胞（損傷を多く受けている細胞も含む）
Mu+MN: 小核を持った多核細胞
MP: 分裂中期細胞
#TOX: 生存細胞殆ど無い

Appendix IX-7

English Translation of Mutagenicity Test (Micronucleus Test) of *Bacillus subtilis* Gum – (PGA)

(See Appendix IX-8)

Fiscal 1996

Tests for developing standards of food additives

Study of mutagenicity test (micronucleus test)

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Abstract

Four natural food additives utilized in Japan, namely, "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), soybean saponin, bitterwood (*Picrasma quassioides*) extract and horseradish (*Armoracia rusticana*) extract, were orally administered to mice twice using a gastric sonde. After that, micronuclei inducibility in the bone marrow of the mice was investigated. The results showed that micronuclei were not induced by these natural additives.

I. Introduction

As part of the reevaluation test for the safety of natural food additives used in Japan, following the study by Hachiya et al.[1)] in the previous fiscal year, we investigated micronuclei inducibility in the mouse bone marrow, a study funded by the National Institute of Health Sciences. In this paper, we report the test results for the four food additives that were assigned to Akita University in fiscal 1996.

II. Samples

The four food additives subjected to the micronucleus tests are shown in Table 1. These samples were obtained through Japan Food Additives Association from February to late July 1997. Table 1 shows information obtained from materials attached to the samples, such as trade names, the manufacturers (the providers), lot numbers, and compositions of the samples.

"Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム") and bitterwood extract were administered after they were dissolved in distilled water at the maximum concentration of 0.3g/ml. Soybean saponin is insoluble, but it was possible to prepare a suspension in distilled water at a concentration of up to 0.12g/ml. Horseradish extract was dissolved in olive oil at a concentration of up to at least 0.3g/ml. Prepared solutions of bitterwood extract and horseradish extract at 10ml/kg and "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム") and soybean saponin at 20ml/kg were orally administered to mice using a gastric sonde.

III. Method

Animals

ddY male mice (9 weeks old) from Japan SLC Inc. were used. They were reared in polycarbonate cages in a conventional environment in which temperature was maintained at 22±3℃ and humidity at 60~70% in a 12-hour light/dark cycle.

Acute toxicity test

In the preliminary toxicity test to determine the doses, the samples were administered to mice twice at the following doses: "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), 3g/kg; soybean saponin, 2.4g/kg; bitterwood extract, 0.1, 1.0, 1.6 and 3.0g/kg; and horseradish extract, 0.1, 0.14, 0.225, 0.37, 0.6 and 1.0g/kg. We observed the survival of the mice for seven days. According to the LD_{50} test by Lorke[2)], we calculated 50% lethal dose (LD^2_{50}) for two administrations of each sample.

Micronucleus test

In the micronucleus test, three doses were set using the common ratio of 2, with either 3g/kg, or the technically feasible highest dose (2.4g/kg) or 0.6 × LD^2_{50}, whichever is lower, as the highest dose. The micronucleus test was implemented in accordance with the standard protocol[3] except that the samples were administered to the experimental group twice in 24 hours. Six mice were used for each group. Twenty-four hours following the second administration of the samples to the experimental group and following the single administration of Mitomycin C at 2mg/kg to the positive control group, bone marrow smears from the femurs were prepared. The Giemsa-stained smears were coded and 1000 polychromatic erythrocytes (PCE) per animal were observed by the blind method to determine the frequency of micronuclei and polychromatic erythrocytes in all erythrocytes. The frequency of micronuclei in polychromatic erythrocytes was determined using the Kastenbaum-Bowman test[4].

IV. Results and Discussion

The results of the preliminary toxicity test are shown in Table 2. For "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), since no animal died following the two administrations at 3g/kg and the LD^2_{50} was 3g/kg or more, it was determined that the doses of 0.75, 1.5 and 3g/kg were appropriate for administration twice in the micronucleus test. For soybean saponin, no animal died following the two administrations at 2.4g/kg, which was the technically feasible highest dose; therefore, it was determined that the doses of 0.6, 1.2 and 2.4g/kg were appropriate for administration twice in the micronucleus test. For bitterwood extract, since no animal died following the two administrations at 0.1~3g/kg, it was determined that the doses of 0.75, 1.5 and 3g/kg were appropriate for administration twice in the micronucleus test. For horseradish extract, since the animals survived following the administration at 0.1g/kg but they died when administered at 0.14g/kg, LD^2_{50} was estimated to be 0.12g/kg. Therefore, the doses of 0.018, 0.035 and 0.07g/kg were determined to be appropriate for administration twice in the micronucleus test.

Table 3 shows the results of the micronucleus test of the four food additives. The frequency of micronuclei in polychromatic erythrocytes in the negative control group ranged from 0.12 to 0.15%, whereas those for the groups administered "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), soybean saponin, bitterwood extract and horseradish extract ranged from 0.12 to 0.20%, 0.12% to 0.23%, 0.13% to 0.18%, and 0.12% to 0.23%, respectively. These frequencies in the administered groups did significantly differ from that in the negative control group.

On the other hand, the group administered with Mitomycin C, which was the positive control

group, showed a significant micronuclei inducibility of 4.78% ($p<0.01$).

V. Conclusion

After oral administration of the four food additives (i.e., "Bacillus natto (*Bacillus subtilis*) Gum"("納豆菌ガム"), soybean saponin, bitterwood extract and horseradish extract) to mice, micronuclei inducibility was not observed in the bone marrow of the mice.

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平成 8 年度
食品添加物規格基準作成等の試験検査
変異原性試験（小核試験）に関する研究

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摘　　要

食品添加物などとしてわが国で用いられている天然添加物4品目：納豆菌ガム，ダイズサポニン，ニガキ抽出物，セイヨウワサビ抽出物について，経口ゾンデによる胃内2回投与の後，マウス骨髄における小核誘発性を検索した。その結果，これらの小核誘発性は認められなかった。

Ⅰ．緒　言

わが国で食品添加物などとして用いられている天然添加物の安全性を再評価する試験の一環として，前年度の蜂谷ら[1]に引き続き国立衛生試験所受託研究費により，マウス骨髄における小核誘発性の有無を検索した。本稿では平成8年度の秋田大学担当分4品目についてその結果を報告する。

Ⅱ．被験品目

小核試験の対象品目は表1に示す4品目である。これらの検体はすべて日本食品添加物協会を通じ，1997年2月～7月下旬に入手した。表1のうち，検体の商品名，製造者（提供者），ロット番号，組成などは検体に添付された資料などから転記した。

納豆菌ガムおよびニガキ抽出物は 0.3 g/ml

を最高に，いずれも蒸留水に溶解したものを投与に用いた。ダイズサポニンは不溶性で，蒸留水を用いたとき 0.12 g/ml までの濃度において懸濁液の調整が可能であった。セイヨウワサビ抽出物は，オリーブ油を用いて少なくとも 0.3 g/ml の濃度まで溶解した。これらの調整液のうち，ニガキ抽出物とセイヨウワサビ抽出物は 10 ml/kg を，納豆菌ガムとダイズサポニンは 20 ml/kg を，経口ゾンデでマウス胃内へ投与した。

Ⅲ．試験方法

動物

動物は日本エスエルシー株式会社ddY雄マウス（9週齢）を用い，温度22±3℃，湿度60～70%，明暗12時間のconventional環境下，ポリカーボネート製ケージで飼育した。

急性毒性試験

投与量を決定する予備毒性試験では，納豆菌ガムは，3 g/kgの用量で，ダイズサポニンは 2.4 g/kgの用量で，ニガキ抽出物は 0.1 g/kg，1.0 g/kg，1.6 g/kg，3.0 g/kg の用量で，セイヨウワサビ抽出物は0.1g/kg，0.14 g/kg，0.225 g/kg，0.37 g/kg，0.6 g/kg，1.0 g/kg の用量でそれぞれ2回ずつ投与した。7日間にわたってこれらの生存状況を観察し，Lorke[2] の LD_{50} 概算法に準じて2回投与の50% 致死量(LD^2_{50})を求めた。

小核試験

小核試験における1回あたりの投与量は 3 g/kg あるいは操作上可能な上限の投与量(2.4 g/kg)あるいは 0.6 × LD^2_{50} のうちのいずれか低いほうを最高に，公比2で3段階を設定した。小核試験実施方法は，実験群において検体を24時間間隔で2回投与したほかは標準プロトコール[3]に準じた。動物は各群

6匹ずつ使用し，実験群については2回目の投与の後，陽性対照群では Mitomycin C：2 mg/kg を1回投与したのち，それぞれ24時間後に大腿骨の骨髄塗抹標本を作成した。Giemsa 染色した標本はコード化し，盲検法で1匹あたり多染性赤血球（PCE）1000細胞を観察して小核含有頻度および全赤血球中の多染性赤血球頻度を求めた。小核含有多染性赤血球頻度は Kastenbaum-Bowman[4] によって検定した。

Ⅳ．結果および考察

予備毒性試験の結果は表2に示すように，納豆菌ガムは 3 g/kg の2回投与で死亡例がみられず，この LD^{2}_{50} は 3 g/kg以上であった。よって，小核試験での投与量を0.75，1.5，3 g/kgの各2回投与と決定した。ダイズサポニンは，操作上可能な最高投与量の2.4 g/kg の2回投与で死亡例がみられず，小

核試験での投与量を 0.6 g/kg, 1.2 g/kg, 2.4 g/kg の各2回投与と決定した。ニガキ抽出物は 0.1～3 g/kgの2回投与で死亡例がみられず，小核試験での投与量を 0.75, 1.5, 3 g/kg の各2回投与とした。セイヨウワサビ抽出物は，0.1 g/kg の投与では生存したが，0.14 g/kg 以上で死亡したため，LD^2_{50}は 0.12 g/kg と推定した。これにもとづき，小核試験の投与量は，0.018, 0.035, 0.07 g/kg 各2回投与とした。

4品目の小核試験の結果を表3に示す。陰性対照群の小核含有多染性赤血球頻度は，0.12～0.15％の範囲にあるのに対し，納豆菌ガム投与群では0.12～0.20％，ダイズサポニン投与群では0.12～0.23，ニガキ抽出物投与群では，0.13～0.18％，セイヨウワサビ抽出物投与群では0.12～0.23％となった。これら各投与群の頻度は陰性対照群と比較して有意差を認めなかった。

一方，陽性対照群であるマイトマイシンC

投与群では4.78%と有意の小核誘発性を示した（$p < 0.01$）。

V．結　　論

納豆菌ガム，ダイズサポニン，ニガキ抽出物，セイヨウワサビ抽出物の4品目については経口投与によるマウス骨髄の小核誘発性は認められなかった。

文　　献

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表1. 平成８年度　食品添加物安全性再評価の試験品目

試験	品名	形状(密度)	担体(溶解性)	商品名	製造者 (lot.No.あるいはdate)
小核試験	納豆菌ガム	フレーク状[1]	蒸留水（溶解）	γ-ポリグルタミン酸	味の素（株）(TT-3)
	ダイズサポニン	粉末	蒸留水（懸濁）	大豆サポニン	（株）トキワ漢方製薬 (DZX-703)
	ニガキ抽出物	液体[2](0.91)	蒸留水（溶解）	ニガキ抽出物製剤	クエストジャパン（株）(不明)
	セイヨウワサビ抽出物	液体(1.00)	オリーブ油（溶解）	西洋わさび抽出辛味油	山忠わざび（株）(不明)

[1]含量85.5%

[2]ニガキ抽出物（粉末，原体）0.3%，ブドウ糖 0.7%，95%エタノール 50.0%，精製水 49.0%，

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表2. 小核被験品目についての２回投与による50%致死量（LD^2_{50}）

品目	一回あたり投与量(mg/kg)	投与回数	生存数/投与数（一週間）	推定LD^2_{50}
納豆菌ガム	3000	×2	3/3	＞3000 mg/kg
ダイズサポニン	2400	×2	3/3	＞2400 mg/kg
ニガキ抽出物	100	×2	3/3	
	160	×2	3/3	
	1000	×2	3/3	
	3000	×2	3/3	＞3000 mg/kg
セイヨウワサビ抽出物	100	×2	3/3	120 mg/kg
	140	×2	0/1	
	225	×2	0/1	
	370	×1	0/1	
	600	×1	0/1	
	1000	×1	0/3	

表3. 天然添加物についてのマウス小核試験結果

被験物質	投与量 mg/kg/回	投与スケジュール[1]	動物数	多染性赤血球頻度 %±SD (min/max)	多染性赤血球観察数	小核含有多染性赤血球 数	小核含有多染性赤血球 %±SD (min/max)
納豆菌ガム	0	×2(24hr), 24hr	6	41.9±9.2 (27.8/50.7)	6000	9	0.15±0.12 (0.00/0.30)
	750	×2(24hr), 24hr	6	36.8±7.1 (25.1/44.0)	6000	12	0.20±0.14 (0.00/0.40)
	1500	×2(24hr), 24hr	6	34.8±5.8 (28.6/45.2)	6000	7	0.12±0.10 (0.00/0.30)
	3000	×2(24hr), 24hr	6	35.9±8.9 (22.0/45.9)	6000	9	0.15±0.14 (0.00/0.40)
ダイズサポニン	0	×2(24hr), 24hr	6	46.3±10.1 (35.2/60.3)	6000	9	0.15±0.16 (0.00/0.40)
	600	×2(24hr), 24hr	6	42.9±10. 3(33.3/61.6)	6000	12	0.20±0.13 (0.00/0.30)
	1200	×2(24hr), 24hr	6	40.4±4.5 (34.3/44.8)	6000	7	0.12±0.12 (0.00/0.30)
	2400	×2(24hr), 24hr	6	43.3±10.3 (32.4/60.9)	6000	14	0.23±0.21 (0.00/0.50)
ニガキ抽出物	0	×2(24hr), 24hr	6	47.7±9.3 (36.5/60.1)	6000	7	0.12±0.13 (0.00/0.30)
	750	×2(24hr), 24hr	6	44.1±10.5 (29.4/55.1)	6000	8	0.13±0.10 (0.00/0.30)
	1500	×2(24hr), 24hr	6	45.5±7.9 (36.2/56.5)	6000	11	0.18±0.31 (0.00/0.80)
	3000	×2(24hr), 24hr	6	45.2±8.5 (38.3/61.4)	6000	9	0.15±0.14 (0.00/0.40)
セイヨウワサビ抽出物	0	×2(24hr), 24hr	6	42.2±12.1 (26.6/62.5)	6000	8	0.13±0.14 (0.00/0.30)
	18	×2(24hr), 24hr	6	42.7±8.0 (28.5/52.7)	6000	14	0.23±0.08 (0.10/0.30)
	35	×2(24hr), 24hr	6	40.6±11.5 (24.7/56.0)	6000	8	0.13±0.10 (0.00/0.30)
	70	×2(24hr), 24hr	6	42.2±10.9 (27.0/54.3)	6000	7	0.12±0.12 (0.00/0.30)
マイトマイシンC	2	×1,24hr	6	35.9±6.9 (26.0/46.9)	6000	287*	4.78±1.83 (2.50/7.10)

[1] ×投与回数（投与間隔），最終投与後のサンプリング時間
* $P<0.01$

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付 表

小 核 試 験 検 鏡 結 果

No 97-1
検体名 : 納豆菌ガム
機関名 : 秋田大学
動 物 : マウス／ddY(SLC)／雄／9wo／ip
実験番号 : 9706B

D;mg/kg	SCHEDULE	MOUSE#	MNPCE;%	MNNCE;%	MNE;%	PCE;%	BW:g	CODE
0	x2, 24hr	Na-0 -1	0.00	0.00	0.00	50.0	37.6	5778
		Na-0 -2	0.00	0.08	0.04	43.8	35.4	9035
		Na-0 -3	0.20	0.10	0.15	50.7	37.1	9552
		Na-0 -4	0.30	0.25	0.27	45.3	39.5	5415
		Na-0 -5	0.20	0.12	0.14	27.8	37.3	6336
		Na-0 -6	0.20	0.20	0.20	33.9	39.5	3916
		MEAN	0.15	0.12	0.13	41.9	37.7	
		STD	0.12	0.09	0.10	9.2	1.6	
		MIN	0.00	0.00	0.00	27.8	35.4	
		MAX	0.30	0.25	0.27	50.7	39.5	
		TOTAL	9			6000		
750	x2, 24hr	Na-1 -1	0.00	0.10	0.08	25.1	38.4	6322
		Na-1 -2	0.10	0.06	0.08	37.7	37.9	9575
		Na-1 -3	0.40	0.07	0.21	42.6	37.4	7246
		Na-1 -4	0.30	0.07	0.16	39.5	36.2	3798
		Na-1 -5	0.20	0.24	0.22	44.0	36.4	7250
		Na-1 -6	0.20	0.23	0.22	31.8	36.9	7618
		MEAN	0.20	0.13	0.16	36.8	37.2	
		STD	0.14	0.08	0.07	7.1	0.9	
		MIN	0.00	0.06	0.08	25.1	36.2	
		MAX	0.40	0.24	0.22	44.0	38.4	判定
		TOTAL	12			6000		—
1500	x2, 24hr	Na-2 -1	0.30	0.08	0.18	45.2	38.5	83
		Na-2 -2	0.10	0.14	0.13	32.4	38.5	2801
		Na-2 -3	0.10	0.14	0.13	31.5	37.2	2338
		Na-2 -4	0.10	0.24	0.20	28.6	38.0	7972
		Na-2 -5	0.10	0.12	0.11	37.4	37.0	6038
		Na-2 -6	0.00	0.10	0.07	34.0	38.2	9379
		MEAN	0.12	0.14	0.14	34.8	37.9	
		STD	0.10	0.05	0.05	5.8	0.7	
		MIN	0.00	0.08	0.07	28.6	37.0	
		MAX	0.30	0.24	0.20	45.2	38.5	判定
		TOTAL	7			6000		—
3000	x2, 24hr	Na-3 -1	0.10	0.08	0.09	44.7	35.8	3089
		Na-3 -2	0.10	0.08	0.09	22.0	38.9	8180
		Na-3 -3	0.40	0.09	0.19	31.2	41.3	7340
		Na-3 -4	0.20	0.06	0.11	36.6	37.6	3465
		Na-3 -5	0.10	0.08	0.09	45.9	35.0	5280
		Na-3 -6	0.00	0.11	0.07	34.9	34.0	4945
		MEAN	0.15	0.08	0.11	35.9	37.1	
		STD	0.14	0.02	0.04	8.9	2.7	
		MIN	0.00	0.06	0.07	22.0	34.0	
		MAX	0.40	0.11	0.19	45.9	41.3	判定
		TOTAL	9			6000		—

No 97-2
検体名 : ダイズサポニン
機関名 : 秋田大学
動 物 : マウス／ddY(SLC)／雄／9wo／ip
実験番号 : 9706B

D;mg/kg	SCHEDULE	MOUSE#	MNPCE;%	MNNCE;%	MNE;%	PCE;%	BW:g	CODE
0	x2, 24hr	D -0 -1	0.40	0.00	0.19	47.7	39.7	4121
		D -0 -2	0.00	0.34	0.20	40.5	37.6	1731
		D -0 -3	0.10	0.46	0.24	60.3	38.2	6347
		D -0 -4	0.30	0.00	0.17	55.9	35.4	1273
		D -0 -5	0.00	0.11	0.07	35.2	39.1	1053
		D -0 -6	0.10	0.06	0.08	38.1	39.8	6167
		MEAN	0.15	0.16	0.16	46.3	38.3	
		STD	0.16	0.19	0.07	10.1	1.7	
		MIN	0.00	0.00	0.07	35.2	35.4	
		MAX	0.40	0.46	0.24	60.3	39.8	
		TOTAL	9			6000		
600	x2, 24hr	D -1 -1	0.30	0.28	0.28	35.6	40.7	8790
		D -1 -2	0.00	0.00	0.00	33.3	38.8	5375
		D -1 -3	0.30	0.06	0.16	39.0	41.3	9121
		D -1 -4	0.10	0.00	0.04	40.7	34.7	7793
		D -1 -5	0.30	0.00	0.14	47.2	38.6	1904
		D -1 -6	0.20	0.16	0.18	61.6	40.0	6105
		MEAN	0.20	0.08	0.13	42.9	39.0	
		STD	0.13	0.11	0.10	10.3	2.4	
		MIN	0.00	0.00	0.00	33.3	34.7	
		MAX	0.30	0.28	0.28	61.6	41.3	判定
		TOTAL	12			6000		—
1200	x2, 24hr	D -2 -1	0.00	0.06	0.04	38.7	37.5	1136
		D -2 -2	0.10	0.05	0.07	34.3	40.5	2663
		D -2 -3	0.20	0.00	0.09	44.5	40.6	1925
		D -2 -4	0.00	0.08	0.04	43.6	40.2	9247
		D -2 -5	0.30	0.08	0.18	44.8	35.8	8642
		D -2- 6	0.10	0.06	0.07	36.5	41.9	9315
		MEAN	0.12	0.06	0.08	40.4	39.4	
		STD	0.12	0.03	0.05	4.5	2.3	
		MIN	0.00	0.00	0.04	34.3	35.8	
		MAX	0.30	0.08	0.18	44.8	41.9	判定
		TOTAL	7			6000		—
2400	x2, 24hr	D -3 -1	0.50	0.16	0.37	60.9	37.5	4250
		D -3 -2	0.40	0.34	0.36	36.3	42.6	7145
		D -3 -3	0.20	0.06	0.11	37.9	43.0	9654
		D -3 -4	0.00	0.09	0.05	47.4	40.5	9463
		D -3 -5	0.00	0.00	0.00	32.4	39.4	9710
		D -3- 6	0.30	0.25	0.27	45.2	40.4	9723
		MEAN	0.23	0.15	0.19	43.3	40.6	
		STD	0.21	0.13	0.16	10.3	2.0	
		MIN	0.00	0.00	0.00	32.4	37.5	
		MAX	0.50	0.34	0.37	60.9	43.0	判定
		TOTAL	14			6000		—

No 97-3
検体名 : ニガキ抽出物
機関名 : 秋田大学
動 物 : マウス／ddY(SLC)／雄／9wo／ip
実験番号 : 9706B

D;mg/kg	SCHEDULE	MOUSE#	MNPCE;%	MNNCE;%	MNE;%	PCE;%	BW:g	CODE
0	x2, 24hr	Ni-0 -1	0.00	0.17	0.09	46.1	39.5	2167
		Ni-0 -2	0.20	0.00	0.07	36.5	39.8	1663
		Ni-0 -3	0.30	0.16	0.22	44.5	36.1	2675
		Ni-0 -4	0.00	0.41	0.17	57.8	36.4	3831
		Ni-0 -5	0.00	0.00	0.00	60.1	33.7	3990
		Ni-0 -6	0.20	0.28	0.25	41.5	36.4	3646
		MEAN	0.12	0.17	0.14	47.7	37.0	
		STD	0.13	0.16	0.10	9.3	2.3	
		MIN	0.00	0.00	0.00	36.5	33.7	
		MAX	0.30	0.41	0.25	60.1	39.8	
		TOTAL	7			6000		
750	x2, 24hr	Ni-1 -1	0.10	0.33	0.21	52.2	35.5	81
		Ni-1 -2	0.00	0.00	0.00	46.7	35.7	6633
		Ni-1 -3	0.30	0.25	0.28	55.1	37.1	973
		Ni-1 -4	0.10	0.00	0.03	33.1	34.0	8424
		Ni-1 -5	0.20	0.17	0.18	29.4	34.4	1145
		Ni-1 -6	0.10	0.09	0.10	47.9	37.5	8212
		MEAN	0.13	0.14	0.13	44.1	35.7	
		STD	0.10	0.13	0.11	10.5	1.4	
		MIN	0.00	0.00	0.00	29.4	34.0	
		MAX	0.30	0.33	0.28	55.1	37.5	判定
		TOTAL	8			6000		—
1500	x2, 24hr	Ni-2 -1	0.20	0.00	0.11	56.5	34.5	726
		Ni-2 -2	0.00	0.00	0.00	50.6	38.4	5250
		Ni-2 -3	0.00	0.06	0.04	36.2	36.5	8360
		Ni-2 -4	0.00	0.06	0.04	37.2	36.2	9469
		Ni-2 -5	0.10	0.09	0.10	48.2	34.2	6837
		Ni-2 -6	0.80	0.16	0.44	44.2	31.1	774
		MEAN	0.18	0.06	0.12	45.5	35.2	
		STD	0.31	0.06	0.16	7.9	2.5	
		MIN	0.00	0.00	0.00	36.2	31.1	
		MAX	0.80	0.16	0.44	56.5	38.4	判定
		TOTAL	11			6000		—
3000	x2, 24hr	Ni-3 -1	0.00	0.07	0.04	39.7	33.2	3056
		Ni-3 -2	0.10	0.00	0.06	61.4	35.0	2701
		Ni-3 -3	0.10	0.15	0.13	42.5	38.2	8405
		Ni-3 -4	0.20	0.12	0.15	38.3	37.3	8853
		Ni-3 -5	0.40	0.14	0.25	41.8	36.0	3950
		Ni-3 -6	0.10	0.27	0.19	47.4	37.5	4432
		MEAN	0.15	0.13	0.14	45.2	36.2	
		STD	0.14	0.09	0.08	8.5	1.9	
		MIN	0.00	0.00	0.04	38.3	33.2	
		MAX	0.40	0.27	0.25	61.4	38.2	判定
		TOTAL	9			6000		—

No 97-4
検体名 : セイヨウワサビ抽出物
機関名 : 秋田大学
動 物 : マウス／ddY(SLC)／雄／9wo／ip
実験番号 : 9706B

D;mg/kg	SCHEDULE	MOUSE#	MNPCE;%	MNNCE;%	MNE;%	PCE;%	BW:g	CODE
0	x2, 24hr	S -0 -1	0.30	0.30	0.30	37.4	39.2	9574
		S -0 -2	0.30	0.09	0.19	47.7	38.6	5691
		S -0 -3	0.10	0.14	0.12	41.5	38.7	93
		S -0 -4	0.00	0.00	0.00	62.5	41.4	3996
		S -0 -5	0.10	0.18	0.16	26.6	36.6	1976
		S -0 -6	0.00	0.12	0.08	37.6	39.8	7206
		MEAN	0.13	0.14	0.14	42.2	39.1	
		STD	0.14	0.10	0.10	12.1	1.6	
		MIN	0.00	0.00	0.00	26.6	36.6	
		MAX	0.30	0.30	0.30	62.5	41.4	
		TOTAL	8			6000		
18	x2, 24hr	S -1 -1	0.10	0.00	0.04	43.2	34.8	9269
		S -1 -2	0.20	0.00	0.06	28.5	35.8	5196
		S -1 -3	0.30	0.00	0.12	41.6	35.5	5316
		S -1 -4	0.30	0.09	0.19	46.9	37.7	6031
		S -1 -5	0.20	0.08	0.13	43.4	39.8	9440
		S -1 -6	0.30	0.33	0.32	52.7	42.1	6786
		MEAN	0.23	0.08	0.14	42.7	37.6	
		STD	0.08	0.13	0.10	8.0	2.8	
		MIN	0.10	0.00	0.04	28.5	34.8	
		MAX	0.30	0.33	0.32	52.7	42.1	判定
		TOTAL	14			6000		—
35	x2, 24hr	S -2 -1	0.10	0.08	0.09	43.4	39.8	6654
		S -2 -2	0.10	0.40	0.25	50.3	36.3	1793
		S -2 -3	0.30	0.51	0.39	56.0	40.3	855
		S -2 -4	0.20	0.13	0.15	24.7	33.8	3987
		S -2 -5	0.10	0.11	0.11	36.4	39.4	282
		S -2 -6	0.00	0.10	0.07	33.2	40.5	2604
		MEAN	0.13	0.22	0.18	40.6	38.4	
		STD	0.10	0.18	0.12	11.5	2.7	
		MIN	0.00	0.08	0.07	24.7	33.8	
		MAX	0.30	0.51	0.39	56.0	40.5	判定
		TOTAL	8			6000		—
70	x2, 24hr	S -3 -1	0.10	0.00	0.05	51.0	39.5	6892
		S -3 -2	0.20	0.00	0.08	42.5	40.1	1947
		S -3 -3	0.00	0.00	0.00	47.1	37.9	975
		S -3 -4	0.30	0.00	0.16	54.3	37.0	550
		S -3 -5	0.10	0.04	0.05	27.0	36.8	9518
		S -3 -6	0.00	0.05	0.03	31.4	37.0	5355
		MEAN	0.12	0.01	0.06	42.2	38.1	
		STD	0.12	0.02	0.06	10.9	1.4	
		MIN	0.00	0.00	0.00	27.0	36.8	
		MAX	0.30	0.05	0.16	54.3	40.1	判定
		TOTAL	7			6000		—

No 97-5
検体名 : mitomycin C
機関名 : 秋田大学
動 物 : マウス／ddY(SLC)／雄／9wo／ip
実験番号 : 9706B

D;mg/kg	SCHEDULE	MOUSE#	MNPCE;%	MNNCE;%	MNE;%	PCE;%	BW:g	CODE
2	x1, 24hr	MMC -1	2.50	0.20	0.97	33.5	39.7	4775
		MMC -2	4.40	0.21	1.65	34.3	38.5	2429
		MMC -3	6.40	0.09	3.05	46.9	35.8	4195
		MMC -4	7.10	0.22	2.67	35.5	40.4	6934
		MMC -5	5.30	0.18	1.51	26.0	37.9	4829
		MMC -6	3.00	0.26	1.33	39.0	39.9	3737
		MEAN	4.78	0.19	1.86	35.9	38.7	
		STD	1.83	0.06	0.81	6.9	1.7	
		MIN	2.50	0.09	0.97	26.0	35.8	
		MAX	7.10	0.26	3.05	46.9	40.4	判定
		TOTAL	287			6000		p<0.01

Appendix IX-9

English Translation of Japanese Publication, "A 90 Day Subchronic Oral Toxicity Study of *Bacillus subtilis* Gum in F344 Rats"

(see Appendix IX-11)

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Biological test report

Virulence of *Bacillus AJ 12757*

October 25, 2001

Reserch Center for Clinical Pharmacology
The Kitasato Institute

1. Title of the study:

Virulence of *Bacillus AJ 12757*

2. Experimental number: NO568

3. Client for the experiment:

Hiroyuki Tanimoto
Food Research & Development Laboratories
Ajinomoto Co., Inc.
1-1, Suzuki-ku, Kawasaki-shi, Kawasaki-cho, 210-8681

4. Testing facility:

Preclinical Division
Research Center for Clinical Pharmacology
The Kitasato Institute,
5-9-1, Sirogane, Minato-ku, Tokyo, 108-8642

5. Responsible person for the experiment:

Kanki Komiyama

Summary

Pathogenicity and toxin productivity of *Bacillus AJ 12757* were compared to that of control strain *Bacillus cereus* on a request of Ajinomoto Co., Inc.

Each culture broth was separated to the cells and the supernatant by centrifugation. Each harvested cells were prepared with 10-fold diluted series and were administrated intraperitoneally in mice. Then survival and death of mice was investigated. The body weight of each survived mouse was measured chronologically, and 14 days after administration they were sacrificed and examined macroscopically. On the other hand, each supernatant was sterilized by filtration and 2-fold diluted series were prepared The enterotoxic activity of the culture filtrate was assayed by measuring the CRET-RPLA test.

As the results, LD_{50} values of *Bacillus AJ 12757*and *Bacillus cereus* were estimated to be above 10^8 and $10^{6.9}$CFU/mL, respectively. Although in some groups, body weight of survived mice were decreased by two days after administration and were increased in all groups thereafter. No abnormality of each organs was detected in all groups by the macroscopically examination of sacrificed mice.

Toxin titer was detected in a 2-fold of *Bacillus AJ 12757*supernatant diluters. On the other hand, toxin titer was detected in a 3200-fold of *Bacillus cereus* supernatant diluters. This 2-fold titer were non-specific agglutinin, therefore *AJ 12757* strain was differentiated with *cereus* on the determinative microbiology.

From these results, it can be concluded that *Bacillus AJ 12757* strains are very weak pathogenic and non-produce of toxin.

I . Objective

In order to conform safety of *Bacillus AJ 12757*, LD_{50} values and toxin productivity were investigated.

II . Strains tested

1-1. Strains:

1) *Bacillus AJ 12757*

1-2. Supplier of strains tested:
Healthy & Nutritional Food Development Section
Food Research & Development Laboratories Ajinomoto Co., Inc.

1-3. Storage conditions:
Culture broth and supernatant of the culture broth was stored in a refrigerator (4 C)

III . Control strain
1. *Bacillus cereus JCM 2152*

IV. Preparation of cells of *Bacillus AJ 12757* and *Bacillus cereus*.

1) *Bacillus AJ 12757* was cultured by Amino Acid Technology Group Amino Acid Department Amino Science Division, Ajinomoto Co., Inc..
2) *Bacillus cereus* was shake cultured in 1% glucose contained Brain Heart Infusion (BHI) medium at 35℃ for 18h. Thereafter culture broth 1.0mL was added in new 1% glucose contained BHI medium 100mL and was shake cultured at 35℃ for 6h.
3) Each culture broth was separated to the cells and the supernatant by centrifugation (3,000 rpm, 30 min). Serial 10-fold dilutions of each harvested cells were performed with sterile saline, and $10^8 \sim 10^1$CFU/ml of *Bacillus AJ 12757* and $10^{10} \sim 10^4$ CFU/ml of *Bacillus cereus* were prepared with 10% mucin solution. Serial 2-fold dilutions of each supernatant were prepared with sterile saline.

V . Methods

1) LD_{50} value measurement

①Animal used

Animal, Strain : ddY mice(SPF), 4-weeks old, male
Date of supply : August 30, September 27, 2001
Supplier : SLC Japan Inc. (3371-8, Koto, Hamamatsu-shi, Sizuoka)

②Breeding conditions

Temperature: 23±2℃
Humidity: 55±15 %
Housing : Five mice were placed in each polycarbonate cage (24 × 17 × 12 cm), in which commercial diet (CL-2, CILEA Japan Inc.) and water were available *ad libitum*.

③ Experimental Procedure

After mice were acclimatized for 7 days prior to testing, test strains and control strain (prepared as described above) were administrated intraperitoneally in mice at a volume of 0.5ml.

④ Observation

After administrated , viability of mice was observed daily for two weeks.

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The body weight of each mouse was recorded before administration, and that of survived mice twice a week after the administration. .

After 2 weeks observation, the mice were sacrificed and each organ was examined macroscopically.

④CRET-RPLA method

A kit for the detection of *Bacillus cereus* enterotoxin (diagnosis by reversed passive latex agglutination, Denka Seikenn Co., Ltd.) was used. Diluted solution of the culture supernatant of each strain was prepared on the multiple well plates. Sensitize latex were added to the serial diluents, and then latex agglutination of each well was observed after 18 h incubation at room temperature.

VI. Results

1) LD_{50} values (Table 1, 2 and 3)

In the *Bacillus AJ 12757* cells treated groups, all mice were survived for 14 days in the $10^8 \sim 10^1$ CFU/ml of cells treated groups. The mortality rate were 0% in the all groups. LD_{50} value of *Bacillus AJ 12757* was estimated to be above 10^8 CFU/ml.

In the *Bacillus cereus* cells treated groups, 10 of 10 in the group of 10^{10} and 10^9 CFU/ml of cells treatment, 9 of 10 in the group of 10^8CFU/ml of cells treatment and 7 of 10 in the group of 10^7CFU/ml of cells treatment, mice was died respectively. The mortality rate were 100%, 100%, 90% and 70%, respectively. In the $10^6 \sim 10^4$ CFU/ml cells treated groups, all mice were survived for 14 days. LD_{50} value of *Bacillus cereus* was estimated to be $10^{6.9}$ CFU/ml.

2) Body weight transition of the survived mice

Results are shown in Table 4.

3) Macroscopically examination of the survived mice

Results are shown in Table 5.

4) CRET -RPLA test

Agglutination reaction occured by the addition of until 2-fold diluted supernatant of *Bacillus AJ 12757* culture broth. Agglutination reaction occurred by the addition of until 3200-fold diluted supernatant of *Bacillus cereus* culture broth.

VI. Discussion

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LD_{50} value of *Bacillus AJ 12757* was estimated to be above 10^8 CFU/ml. In a group treated with $10^8 \sim 10^6$CFU/mL cells and group treated with supernatant of culture broth, body weight of the survived mice were decreased in two days after administration. But they were recovered thereafter and gained body weight almost the same rate as the control group till 14 days. Decrease of body weight in there groups, were stress by the administration of the cells. Toxin was detected in until 2-fold diluted supernatant by CRET-RPLA test. This toxin was nonspecific agglutinin titer therefore *Bacillus AJ 12757* was differentiated with *Bacillus cereus* on the determinative microbiology. By the macroscopically examination

of sacrificed mice, concerning all organs, no abnormality was observed from all groups. From these results, LD_{50} value of the strain was low, body weight decrease by the administration of the cells was low, and no *cereus* like toxin was detected in the supernatant by CRET -RPLA test, thus, it can be concluded that *Bacillus AJ 12757* is very weak pathogenic and non-produce of toxin.

LD_{50} value of *Bacillus cereus* was estimated to be $10^{6.9}$ CFU/ml. In the all group body weight of the survived mice were almost same rate as the control group till 14 days. By the macroscopically examination of sacrificed mice, concerning all organs, no abnormality was observed from all groups. Toxin was detected in until 3200-fold diluted supernatant by CRET-RPLA test. From these results, LD_{50} value of the strain was low, body weight decrease by the administration of the cells was low, and toxin was detected in until 3200-fold diluted supernatant by CRET-RPLA test, thus, it can be concluded that *Bacillus cereus* is very weak pathogenic and non-produce of toxin.

In summary, it can be concluded that *Bacillus AJ 12757* are very weak pathogenic and not produced toxin. Compared with *Bacillus cereus*, this strains tested can be concluded to be very weak pathogenic, and non-produce of toxin.

Ⅶ References

1. Gakuyu-kai, Institute for Medical Sciences (ed.): Summary of bacteriology exercise (in Japanese), Maruzen Press, Japan

2. Thompson,N.E., et al, Isolation and some properties of an enterotoxin produced by *Bacillus cereus*. Infect. Immun., 43, 887 (1984)

I admit that this study report is produced on the basis of the experimental data I performed with two other co-workers.

October25, 2001

Responsible person for the experiment (b) (6)

Kanki Komiyama Ph.D.

Table1. NumbNunbeer of dead mice administratted testing and control strain

(Dead/Total)

	Concentration of administrated cells (CFU/ml)									
Strain	10^{10}	10^{9}	10^{8}	10^{7}	10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	10^{1}
AJ 12757	NT	NT	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
B.cereus	10/10	10/10	9/10	7/10	0/10	0/10	0/10	NT	NT	NT

NT : not tested

Table 2. Mortality rate caused by the administretion of testing and control strain

(Death rate (%)=Dead/Total×100)

	Concentration of administrated cells (CFU/ml)									
Strain	10^{10}	10^{9}	10^{8}	10^{7}	10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	10^{1}
AJ 12757	NT	NT	0	0	0	0	0	0	0	0
B.cereus	100	100	90	70	0	0	0	NT	NT	NT

NT : not tested

Table 3. LD_{50} value of testing and control strain

Strain	LD_{50} value
AJ 12757	10^{9}CFU/ml<
B.cereus	$10^{4.9}$CFU/ml

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Table 4-1. Body weight of mice administrated acillus AJ 12757(g)

Dose	Number of mouse	Days after administration 0	2	6	9	14
10^8 CFU/ml	1	27.7	23.4	28.1	30.2	31.9
	2	25.8	22.1	27.1	29.8	33.3
	3	30.3	26.8	31.8	34.5	37.1
	4	30.1	27.4	30.0	32.7	34.8
	5	27.0	22.4	27.0	29.9	34.0
	6	27.4	23.2	27.2	29.8	32.8
	7	27.6	23.6	28.6	31.5	33.6
	8	28.5	25.6	30.4	32.9	35.4
	9	30.5	27.1	32.1	35.0	37.2
	10	28.1	24.2	29.1	31.5	34.9
	average	28.3	24.6	29.1	31.8	34.5
	SD	1.54	1.98	1.89	1.94	1.74
10^7 CFU/ml	1	24.3	23.3	27.5	29.6	31.6
	2	28.4	27.1	31.2	32.9	34.4
	3	29.8	28.3	31.5	33.8	35.9
	4	29.6	27.8	31.7	33.5	35.4
	5	28.8	27.9	30.7	32.3	33.2
	6	26.2	26.0	29.1	30.6	32.2
	7	29.6	29.3	33.1	35.8	37.7
	8	27.3	25.5	29.0	30.7	32.9
	9	28.0	26.4	31.0	33.9	35.6
	10	27.3	26.5	29.7	32.5	34.6
	average	27.9	26.8	30.4	32.6	34.3
	SD	1.71	1.70	1.63	1.86	1.89
10^6 CFU/ml	1	28.4	27.6	30.7	32.3	34.7
	2	26.3	25.6	30.1	32.4	34.4
	3	26.6	26.1	30.0	31.9	32.8
	4	29.3	29.2	32.3	34.9	37.4
	5	27.6	27.3	31.3	34.0	36.2
	6	27.6	28.8	33.0	34.4	37.7
	7	31.0	27.5	31.6	33.1	35.6
	8	29.3	27.9	30.7	32.1	32.6
	9	26.2	25.7	28.9	30.2	31.9
	10	28.2	27.9	31.6	34.2	36.5
	average	28.1	27.3	31.0	33.0	35.0
	SD	1.53	1.23	1.20	1.43	2.04
10^5 CFU/ml	1	26.8	27.1	29.6	31.4	32.6
	2	27.5	27.9	31.1	33.5	35.7
	3	26.1	26.7	29.4	31.7	34.3
	4	25.9	27.0	29.6	31.3	33.3
	5	28.3	28.8	31.5	33.2	35.3
	6	27.0	27.3	30.6	33.3	35.2
	7	25.8	26.2	29.3	30.9	33.2
	8	28.3	28.9	32.4	34.5	36.2
	9	27.2	27.8	30.6	32.3	33.8
	10	26.4	27.0	30.1	32.2	33.3
	average	26.9	27.5	30.4	32.4	34.3
	SD	0.92	0.88	1.02	1.14	1.23

Table 4-2. Body weight of mice administrated *Bacillus AJ 12757* (g)

Dose	Number of mouse	Days after administration 0	2	6	9	14
10^4 CFU/ml	1	26.8	26.9	30.0	31.4	33.1
	2	29.4	29.7	31.7	33.2	35.0
	3	29.6	28.5	31.7	33.3	34.4
	4	25.9	26.5	29.4	30.8	32.7
	5	28.9	28.7	31.4	32.4	34.2
	6	29.4	29.2	31.8	33.1	34.9
	7	30.3	29.3	32.1	34.1	37.2
	8	30.9	31.0	33.6	34.5	36.3
	9	25.8	26.3	28.7	30.0	31.8
	10	31.1	32.5	35.1	36.9	39.5
	average	28.8	28.8	31.5	33.0	34.9
	SD	1.96	1.98	1.91	1.96	2.27
10^3 CFU/ml	1	30.0	29.8	32.6	34.6	36.6
	2	25.7	26.3	29.3	31.6	33.4
	3	28.5	27.9	29.0	31.4	33.0
	4	29.9	30.9	33.3	35.9	38.7
	5	28.0	28.7	31.3	33.5	36.1
	6	26.0	26.3	29.1	31.4	33.2
	7	28.4	28.9	31.6	33.8	35.7
	8	27.3	27.2	29.4	31.2	33.1
	9	32.0	33.1	36.0	38.9	40.7
	10	27.9	28.8	31.3	33.7	36.5
	average	28.4	28.8	31.3	33.6	35.7
	SD	1.90	2.09	2.25	2.45	2.61
10^2 CFU/ml	1	29.2	30.0	33.6	36.2	38.2
	2	28.6	28.9	32.3	34.7	36.8
	3	27.7	29.1	32.2	34.1	35.6
	4	26.8	27.6	30.5	32.3	33.4
	5	31.7	33.1	37.0	39.6	42.3
	6	29.6	30.9	34.7	36.4	37.3
	7	26.4	27.0	30.7	33.5	36.0
	8	30.7	30.9	34.2	36.3	37.6
	9	29.6	29.4	32.6	34.7	36.4
	10	25.4	26.1	30.2	32.0	34.4
	average	28.6	29.3	32.8	35.0	36.8
	SD	1.99	2.06	2.15	2.25	2.42
10^1 CFU/ml	1	28.7	28.7	31.7	33.1	34.4
	2	30.1	30.0	32.2	33.7	35.4
	3	26.5	25.9	28.7	29.9	30.7
	4	28.0	27.6	30.9	32.7	33.8
	5	28.1	28.5	31.2	32.1	33.5
	6	30.7	30.5	33.1	35.7	37.4
	7	28.1	28.7	30.8	32.8	34.4
	8	30.0	30.1	31.8	33.5	35.4
	9	32.9	34.0	38.1	40.3	42.3
	10	29.9	30.1	34.2	36.5	38.0
	average	29.3	29.4	32.2	34.0	35.5
	SD	1.79	2.13	2.51	2.87	3.13

Table 4-3. Body weight of mice administrated *Bacillus AJ 12757* (g)

Dose	Number of mouse	Days after administration 0	2	6	9	14
Mucin	1	27.1	26.6	29.6	31.0	32.9
	2	29.3	28.5	32.3	32.4	34.0
	3	28.4	28.6	31.9	32.9	35.0
	4	28.4	28.6	31.5	32.5	34.3
	5	28.5	28.8	32.0	34.4	36.4
	6	28.6	28.6	31.2	32.8	34.4
	7	29.2	29.3	32.5	33.8	35.3
	8	27.7	27.3	30.3	31.5	33.5
	9	29.0	29.0	32.4	34.3	36.2
	10	31.5	31.9	34.0	35.3	36.8
	average	28.8	28.7	31.8	33.1	34.9
	SD	1.18	1.38	1.22	1.34	1.29

Table 4-4. Body weight of mice administrated *Bacillus AJ 12757* supernatant

Sample Dose	Number of mouse	Days after administration 0	2	6	9	14
Supernatant non-diluted	1	28.1	25.8	29.9	32.4	34.7
	2	28.0	26.0	30.7	32.7	34.6
	3	25.6	25.3	27.9	29.9	31.7
	4	27.3	25.6	29.8	32.6	34.6
	5	28.1	26.4	29.9	32.4	33.6
	6	27.8	26.4	30.6	32.8	35.0
	7	29.6	29.6	33.6	36.7	39.6
	8	27.9	26.1	30.3	33.1	35.2
	9	26.4	25.5	28.8	30.8	33.5
	10	31.2	30.9	35.8	38.6	41.9
	average	28.0	26.7	30.7	33.2	35.4
	SD	1.55	1.90	2.30	2.58	3.01
Supernatant 2-fold diluted	1	27.0	27.1	30.1	32.2	34.2
	2	27.0	26.8	30.0	32.2	34.2
	3	30.1	30.1	33.7	36.8	38.2
	4	26.4	27.0	29.2	31.0	32.3
	5	29.0	30.0	34.9	37.9	41.2
	6	27.2	28.2	30.2	32.3	33.5
	7	27.9	27.6	30.9	32.9	35.3
	8	27.3	28.5	30.6	32.8	33.9
	9	29.4	30.0	33.7	36.6	38.4
	10	26.9	28.1	29.7	31.2	32.9
	average	27.8	28.3	31.3	33.6	35.4
	SD	1.25	1.29	2.01	2.52	2.89
Medium	1	27.3	28.1	29.9	31.7	33.4
	2	30.6	31.0	33.3	35.4	36.8
	3	27.7	28.1	30.5	32.2	32.8
	4	29.5	30.2	32.0	33.6	34.8
	5	27.7	28.6	30.5	32.3	34.0
	6	29.2	30.5	33.6	36.6	38.7
	7	29.9	30.4	33.2	35.4	36.5
	8	29.7	30.1	32.6	33.7	34.0
	9	29.6	29.3	31.2	32.8	34.3
	10	27.4	28.1	31.0	32.9	34.5
	average	28.8	29.4	31.8	33.6	35.0
	SD	1.21	1.13	1.34	1.62	1.80

Table 5-1. Relative body weight of mice administrated *Bacillus AJ 12757*

Dose	Number of mouse	Days after administration				
		0	2	6	9	14
10^8 CFU/ml	1	1.00	0.85	1.01	1.09	1.15
	2	1.00	0.85	1.05	1.15	1.29
	3	1.00	0.88	1.05	1.14	1.22
	4	1.00	0.91	1.00	1.09	1.16
	5	1.00	0.83	1.00	1.11	1.26
	6	1.00	0.85	0.99	1.09	1.20
	7	1.00	0.86	1.03	1.14	1.22
	8	1.00	0.90	1.06	1.15	1.24
	9	1.00	0.89	1.05	1.15	1.22
	10	1.00	0.86	1.04	1.12	1.24
	average	1.00	0.87	1.03	1.12	1.22
	SD	0	0.03	0.03	0.04	0.04
10^7 CFU/ml	1	1.00	0.96	1.13	1.22	1.30
	2	1.00	0.96	1.10	1.16	1.21
	3	1.00	0.95	1.06	1.13	1.20
	4	1.00	0.94	1.07	1.13	1.20
	5	1.00	1.97	1.07	1.12	1.16
	6	1.00	0.99	1.11	1.17	1.23
	7	1.00	0.99	1.12	1.21	1.28
	8	1.00	0.93	1.06	1.13	1.21
	9	1.00	0.94	1.11	1.21	1.27
	10	1.00	0.97	1.09	1.19	1.27
	average	1.00	0.96	1.09	1.17	1.23
	SD	0	0.02	0.03	0.04	0.04
10^6 CFU/ml	1	1.00	0.97	1.08	1.14	1.22
	2	1.00	0.97	1.14	1.23	1.31
	3	1.00	0.98	1.13	1.20	1.23
	4	1.00	0.99	1.10	1.19	1.27
	5	1.00	0.99	1.13	1.23	1.31
	6	1.00	1.04	1.19	1.25	1.36
	7	1.00	0.89	1.02	1.07	1.15
	8	1.00	0.95	1.05	1.09	1.11
	9	1.00	0.98	1.10	1.15	1.22
	10	1.00	0.99	1.12	1.21	1.30
	average	1.00	0.98	1.11	1.18	1.25
	SD	0	0.04	0.05	0.06	0.08
10^5 CFU/ml	1	1.00	1.01	1.11	1.17	1.22
	2	1.00	1.01	1.13	1.22	1.30
	3	1.00	1.02	1.12	1.22	1.31
	4	1.00	1.05	1.15	1.21	1.29
	5	1.00	1.02	1.11	1.17	1.25
	6	1.00	1.01	1.13	1.23	1.30
	7	1.00	1.02	1.14	1.20	1.29
	8	1.00	1.02	1.15	1.22	1.28
	9	1.00	1.02	1.12	1.19	1.24
	10	1.00	1.02	1.14	1.22	1.26
	average	1.00	1.02	1.13	1.20	1.27
	SD	0	0.01	0.01	0.02	0.03

Table 5-2. Relative body weight of mice administrated *Bacillus AJ 12757*

Dose	Number of mouse	Days after administration 0	2	6	9	14
10^4 CFU/ml	1	1.00	1.00	1.12	1.17	1.24
	2	1.00	1.01	1.08	1.13	1.19
	3	1.00	0.96	1.07	1.13	1.16
	4	1.00	1.02	1.13	1.19	1.26
	5	1.00	1.00	1.09	1.12	1.19
	6	1.00	1.00	1.08	1.13	1.19
	7	1.00	0.97	1.06	1.13	1.23
	8	1.00	1.00	1.09	1.12	1.17
	9	1.00	1.02	1.11	1.17	1.23
	10	1.00	1.04	1.13	1.19	1.27
	average	1.00	1.00	1.10	1.15	1.21
	SD	0	0.02	0.03	0.03	0.04
10^3 CFU/ml	1	1.00	0.99	1.09	1.15	1.22
	2	1.00	1.02	1.14	1.23	1.16
	3	1.00	0.98	1.02	1.10	1.13
	4	1.00	1.03	1.12	1.20	1.13
	5	1.00	1.02	1.12	1.20	1.12
	6	1.00	1.01	1.12	1.21	1.17
	7	1.00	1.02	1.11	1.19	1.21
	8	1.00	1.00	1.08	1.14	1.13
	9	1.00	1.03	1.12	1.21	1.21
	10	1.00	1.04	1.12	1.21	1.19
	average	1.00	1.01	1.10	1.18	1.17
	SD	0	0.02	0.04	0.04	0.04
10^2 CFU/ml	1	1.00	1.03	1.15	1.24	1.31
	2	1.00	1.01	1.13	1.21	1.29
	3	1.00	1.05	1.16	1.23	1.28
	4	1.00	1.03	1.14	1.20	1.25
	5	1.00	1.04	1.17	1.25	1.33
	6	1.00	1.04	1.17	1.23	1.26
	7	1.00	1.03	1.17	1.27	1.36
	8	1.00	1.01	1.12	1.18	1.22
	9	1.00	0.99	1.10	1.17	1.23
	10	1.00	1.03	1.19	1.26	1.35
	average	1.00	1.03	1.15	1.23	1.29
	SD	0	0.02	0.03	0.03	0.05
10^1 CFU/ml	1	1.00	1.00	1.10	1.15	1.20
	2	1.00	1.00	1.07	1.12	1.17
	3	1.00	0.98	1.08	1.13	1.16
	4	1.00	0.99	1.10	1.17	1.21
	5	1.00	1.01	1.11	1.14	1.19
	6	1.00	0.99	1.08	1.17	1.22
	7	1.00	1.02	1.10	1.17	1.23
	8	1.00	1.00	1.06	1.12	1.18
	9	1.00	1.03	1.16	1.23	1.28
	10	1.00	1.01	1.14	1.22	1.27
	average	1.00	1.00	1.10	1.16	1.21
	SD	0	0.02	0.03	0.04	0.04

Table 5-3. Relative body weight of mice administrated *Bacillus AJ 12757*

Dose	Number of mouse	Days after administration				
		0	2	6	9	14
Mucine	1	1.00	0.98	1.09	1.15	1.21
	2	1.00	0.97	1.10	1.11	1.16
	3	1.00	1.01	1.12	1.16	1.23
	4	1.00	1.01	1.11	1.14	1.20
	5	1.00	1.01	1.12	1.21	1.28
	6	1.00	1.00	1.09	1.15	1.20
	7	1.00	1.00	1.11	1.16	1.21
	8	1.00	0.98	1.09	1.14	1.21
	9	1.00	1.00	1.11	1.18	1.25
	10	1.00	1.01	1.08	1.12	1.17
	average	1.00	1.00	1.10	1.15	1.21
	SD	0	0.01	0.01	0.03	0.03

Table 5-4. Relative body weight of mice administrated *Bacillus A.* 12757supernatant

Sample Dose	Number of mouse	Days after administration				
		0	2	6	9	14
Supernatant non-diluted	1	1.00	0.92	1.07	1.16	1.23
	2	1.00	0.93	1.09	1.17	1.24
	3	1.00	0.99	1.09	1.17	1.24
	4	1.00	0.94	1.09	1.19	1.27
	5	1.00	0.94	1.06	1.15	1.20
	6	1.00	0.95	1.10	1.18	1.26
	7	1.00	1.00	1.13	1.24	1.34
	8	1.00	0.94	1.09	1.19	1.26
	9	1.00	0.96	1.09	1.17	1.27
	10	1.00	0.99	1.15	1.24	1.34
	average	1.00	0.95	1.10	1.19	1.26
	SD	0	0.03	0.03	0.03	0.05
Supernatant 2-fold diluted	1	1.00	1.00	1.11	1.19	1.26
	2	1.00	0.99	1.11	1.19	1.27
	3	1.00	1.00	1.12	1.22	1.27
	4	1.00	1.02	1.10	1.17	1.22
	5	1.00	1.03	1.20	1.31	1.42
	6	1.00	1.04	1.11	1.19	1.23
	7	1.00	0.99	1.11	1.18	1.27
	8	1.00	1.05	1.12	1.20	1.24
	9	1.00	1.02	1.15	1.25	1.31
	10	1.00	1.04	1.10	1.16	1.22
	average	1.00	1.02	1.13	1.21	1.27
	SD	0	0.02	0.03	0.04	0.06
Medium	1	1.00	1.03	1.10	1.16	1.23
	2	1.00	1.01	1.09	1.16	1.20
	3	1.00	1.01	1.10	1.16	1.18
	4	1.00	1.02	1.09	1.14	1.18
	5	1.00	1.03	1.01	1.17	1.23
	6	1.00	1.05	1.15	1.25	1.32
	7	1.00	1.02	1.11	1.19	1.22
	8	1.00	1.02	1.10	1.13	1.15
	9	1.00	0.99	1.05	1.11	1.16
	10	1.00	1.03	1.13	1.20	1.26
	average	1.00	1.02	1.10	1.17	1.21
	SD	0	0.02	0.03	0.04	0.05

Table 6-1. Body weight of mice administrated *Bacillus cereus* (g)

Dose	Number of mouse	Days after administration				
		0	3	7	10	14
10^{10} CFU/ml	1	26.8	Died			
	2	26.3	〃			
	3	27.4	〃			
	4	25.0	〃			
	5	27.3	〃			
	6	25.3	〃			
	7	26.7	〃			
	8	25.7	〃			
	9	26.9	〃			
	10	22.3	〃			
	average	26.0				
	SD	1.51				
10^{9} CFU/ml	1	25.1	Died			
	2	26.4	〃			
	3	27.7	〃			
	4	25.6	〃			
	5	26.9	〃			
	6	25.6	〃			
	7	26.3	〃			
	8	23.2	〃			
	9	23.7	〃			
	10	27.5	〃			
	average	25.8				
	SD	1.49				
10^{8} CFU/ml	1	27.6	Died			
	2	26.9	〃			
	3	30.4	〃			
	4	25.6	〃			
	5	25.4	27.6	31.0	33.4	35.1
	6	26.1	Died			
	7	25.2	〃			
	8	25.2	〃			
	9	27.4	〃			
	10	29.2	〃			
	average	26.9	27.6	31.0	33.4	35.1
	SD	1.78				
10^{7} CFU/ml	1	26.3	Died			
	2	24.8	〃			
	3	25.2	26.6	30.1	32.1	34.1
	4	26.6	Died			
	5	26.5	25.4	30.6	33.6	36.8
	6	27.4	Died			
	7	23.7	〃			
	8	25.6	25.0	30.3	34.0	37.3
	9	24.7	Died			
	10	25.8	〃			
	average	25.7	25.7	30.3	33.2	36.1
	SD	1.10	0.84	0.24	0.95	1.73

Table 6-2. Body weight of mice administrated *Bacillus cereus* (g)

Dose	Number of mouse	Days after administration				
		0	3	7	10	14
10^6 CFU/ml	1	25.0	26.7	25.9	30.4	33.6
	2	28.1	28.4	28.9	33.7	38.5
	3	26.0	25.7	25.8	30.4	33.6
	4	26.6	27.3	29.1	33.9	35.5
	5	24.4	21.7	23.2	28.5	33.4
	6	25.6	26.9	26.4	31.0	34.1
	7	26.8	27.4	27.2	30.8	33.4
	8	25.6	24.7	25.1	30.0	33.4
	9	27.2	28.1	28.3	33.1	35.9
	10	27.2	25.3	26.9	32.0	34.1
	average	26.3	26.2	26.7	31.4	34.5
	SD	1.14	1.99	1.83	1.76	1.63
10^5 CFU/ml	1	26.7	28.8	29.1	34.2	37.6
	2	27.0	28.7	28.3	33.7	36.3
	3	26.9	28.8	30.1	33.3	34.9
	4	26.2	28.2	28.5	33.5	36.8
	5	25.8	27.8	28.2	33.0	36.3
	6	23.9	25.3	26.6	30.4	33.3
	7	24.4	26.7	27.1	32.2	35.0
	8	24.0	25.4	25.6	30.1	32.4
	9	27.2	30.2	30.3	34.6	37.5
	10	27.1	28.8	29.8	34.3	38.1
	average	25.9	27.9	28.4	32.9	35.8
	SD	1.35	1.58	1.56	1.57	1.90
10^4 CFU/ml	1	25.5	27.0	27.4	31.2	35.6
	2	25.9	27.5	28.8	33.8	36.3
	3	26.8	29.3	29.0	33.1	35.6
	4	26.3	28.3	28.8	34.4	39.6
	5	25.7	27.4	27.0	30.8	33.9
	6	25.5	27.3	26.9	31.4	34.9
	7	25.6	27.4	26.6	31.3	33.9
	8	26.3	28.0	27.6	31.9	34.4
	9	27.3	30.0	31.8	35.6	38.3
	10	26.1	28.4	28.6	33.4	36.2
	average	26.1	28.0	28.2	32.7	35.9
	SD	0.58	0.95	1.52	1.61	1.86
Mucin	1	24.9	26.6	27.0	31.1	33.9
	2	25.9	28.2	27.5	30.9	32.2
	3	25.2	27.2	25.1	30.3	33.7
	4	24.2	26.0	26.3	30.6	34.2
	5	27.9	29.1	29.2	32.4	35.4
	6	27.8	28.5	31.6	34.1	37.0
	7	24.3	25.9	26.9	31.3	33.6
	8	25.4	26.9	27.9	31.3	33.1
	average	25.7	27.3	27.7	31.5	34.1
	SD	1.4	1.2	2.0	1.2	1.5

Table 6-3. Body weight of mice administrated *Bacillus cereus* supernatant (g)

Dose	Number of mouse	Days after administration				
		0	3	7	10	14
Supernatant non-diluted	1	26.8	25.9	27.8	32.1	37.7
	2	26.0	26.1	26.6	31.0	35.3
	3	25.6	26.9	26.4	30.1	32.6
	4	25.7	23.0	24.5	29.8	34.7
	5	25.7	24.0	26.7	29.6	31.4
	6	27.6	26.7	27.0	31.2	34.6
	7	26.5	25.2	26.5	30.2	33.4
	8	27.1	26.2	26.3	31.2	34.7
	9	25.8	25.3	25.9	30.4	32.9
	10	26.1	25.5	26.3	31.5	33.1
	average	26.3	25.5	26.4	30.7	34.0
	SD	0.69	1.19	0.83	0.81	1.74
Supernatant 2-fold diluted	1	24.9	26.4	25.8	30.1	31.6
	2	29.2	31.1	31.4	34.5	36.7
	3	25.0	26.1	26.3	26.5	23.9
	4	23.9	26.0	26.4	31.2	34.2
	5	26.2	26.9	26.2	30.2	31.2
	6	28.4	30.7	31.3	34.1	36.3
	7	27.3	29.6	29.8	34.2	35.8
	8	25.2	26.3	27.9	31.2	31.9
	9	27.0	29.1	28.8	34.3	36.6
	10	25.8	27.3	26.8	30.8	32.2
	average	26.3	28.0	28.1	31.3	33.1
	SD	1.67	1.97	2.14	2.57	3.89
Medium	1	24.5	26.7	26.1	29.8	32.4
	2	25.5	27.9	27.5	31.6	35.3
	3	28.1	31.4	30.7	34.9	37.8
	4	26.3	28.8	27.9	32.1	35.7
	5	27.2	29.9	28.9	34.4	37.2
	average	26.3	28.9	28.2	32.6	35.7
	SD	1.42	1.80	1.69	2.10	2.12

Table 7-1. Relative body weight of mice administrated *Bacillus cereus*

Dose	Number of mouse	Days after administration				
		0	3	7	10	14
10^{10} CFU/ml	1	1.00	Died			
	2	1.00	〃			
	3	1.00	〃			
	4	1.00	〃			
	5	1.00	〃			
	6	1.00	〃			
	7	1.00	〃			
	8	1.00	〃			
	9	1.00	〃			
	10	1.00	〃			
	average	1.00				
	SD	0.00				
10^{9} CFU/ml	1	1.00	Died			
	2	1.00	〃			
	3	1.00	〃			
	4	1.00	〃			
	5	1.00	〃			
	6	1.00	〃			
	7	1.00	〃			
	8	1.00	〃			
	9	1.00	〃			
	10	1.00	〃			
	average	1.00				
	SD	0.00				
10^{8} CFU/ml	1	1.00	Died			
	2	1.00	〃			
	3	1.00	〃			
	4	1.00	〃			
	5	1.00	1.08	1.22	1.32	1.38
	6	1.00	Died			
	7	1.00	〃			
	8	1.00	〃			
	9	1.00	〃			
	10	1.00	〃			
	average	1.00	1.08	1.22	1.32	1.38
	SD	0.00				
10^{7} CFU/ml	1	1.00	Died			
	2	1.00	〃			
	3	1.00	1.06	1.20	1.28	1.36
	4	1.00	Died			
	5	1.00	0.96	1.15	1.27	1.39
	6	1.00	Died			
	7	1.00	〃			
	8	1.00	0.98	1.18	1.33	1.46
	9	1.00	Died			
	10	1.00	〃			
	average	1.00	1.00	1.18	1.29	1.40
	SD	0.00	0.05	0.03	0.03	0.05

Table 7-2. Relative body weight of mice administrated *Bacillus cereus*

Dose	Number of mouse	Days after administration				
		0	3	7	10	14
10^6 CFU/ml	1	1.00	1.07	1.03	1.22	1 .34
	2	1.00	1.01	1.03	1.20	1.37
	3	1.00	0.99	0.99	1.17	1.29
	4	1.00	1.02	1.09	1.27	1.39
	5	1.00	0.89	0.95	1.17	1.37
	6	1.00	1.05	1.03	1.21	1.33
	7	1.00	1.02	1.01	1.15	1.25
	8	1.00	0.96	0.98	1.17	1.30
	9	1.00	1.04	1.04	1.22	1.32
	10	1.00	0.93	0.99	1.17	1.25
	average	1.00	1.00	1.02	1.19	1.32
	SD	0.00	0.06	0.04	0.04	0.04
10^5 CFU/ml	1	1.00	1.08	1.09	1.28	11.41
	2	1.00	1.06	1.05	1.25	1.34
	3	1.00	1.07	1.12	1.24	1.30
	4	1.00	1.07	1.09	1.28	1.41
	5	1.00	1.07	1.09	1.28	1.40
	6	1.00	1.06	1.11	1.28	1.40
	7	1.00	1.10	1.11	1.32	1.44
	8	1.00	1.06	1.07	1.25	1.35
	9	1.00	1.11	1.11	1.27	1.38
	10	1.00	1.06	1.10	1.26	1.41
	average	1.00	1.08	1.09	1.27	1.38
	SD	0.00	0.02	0.02	0.02	0.04
10^4 CFU/ml	1	1.00	1.06	1.07	1.22	1.40
	2	1.00	1.06	1.11	1.30	1.40
	3	1.00	1.09	1.08	1.24	1.33
	4	1.00	1.07	1.09	1.31	1.50
	5	1.00	1.07	1.05	1.20	1.32
	6	1.00	1.07	1.05	1.23	1.37
	7	1.00	1.07	1.04	1.22	1.32
	8	1.00	1.06	1.05	1.21	1.31
	9	1.00	1.10	1.17	1.31	1.41
	10	1.00	1.09	1.10	1.28	1.39
	average	1.00	1.07	1.08	1.25	1.37
	SD	0.00	0.01	0.04	0.04	0.06
Mucin	1	1.00	1.07	1.08	1.25	1.36
	2	1.00	1.09	1.06	1.20	1.25
	3	1.00	1.08	1.00	1.20	1.34
	4	1.00	1.07	1.09	1.26	1.41
	5	1.00	1.04	1.05	1.16	1.27
	6	1.00	1.03	1.14	1.23	1.33
	7	1.00	1.07	1.11	1.28	1.38
	8	1.00	1.06	1.10	1.23	1.30
	average	1.00	1.06	1.08	1.23	1.33
	SD	0.00	0.02	0.04	0.04	0.06

Table 7-3. Relative body weight of mice administrated *Bacillus cereus* super

Dose	Number of mouse	Days after administration				
		0	3	7	10	14
Supernatant non-diluted	1	1.00	0.97	1.04	1.20	1.41
	2	1.00	1.00	1.03	1.19	1.36
	3	1.00	1.05	1.03	1.18	1.27
	4	1.00	0.89	0.95	1.16	1.35
	5	1.00	0.94	1.04	1.15	1.22
	6	1.00	0.97	0.98	1.13	1.25
	7	1.00	0.95	1.00	1.14	1.26
	8	1.00	0.97	0.97	1.15	1.28
	9	1.00	0.98	1.01	1.18	1.28
	10	1.00	0.98	1.01	1.21	1.27
	average	1.00	0.97	1.01	1.17	1.29
	SD	0.00	0.04	0.03	0.03	0.06
Supernatant 2-fold diluted	1	1.00	1.06	1.04	1.21	1.27
	2	1.00	1.06	1.07	1.18	1.26
	3	1.00	1.04	1.05	1.06	0.96
	4	1.00	1.09	1.11	1.31	1.43
	5	1.00	1.03	1.00	1.15	1.19
	6	1.00	1.08	1.10	1.20	1.28
	7	1.00	1.08	1.09	1.26	1.31
	8	1.00	1.05	1.11	1.24	1.27
	9	1.00	1.08	1.07	1.27	1.36
	10	1.00	1.06	1.04	1.19	1.25
	average	1.00	1.06	1.07	1.21	1.26
	SD	0.00	0.02	0.04	0.07	0.12
Medium	1	1.00	1.09	1.06	1.21	1.32
	2	1.00	1.10	1.08	1.24	1.38
	3	1.00	1.11	1.09	1.24	1.34
	4	1.00	1.09	1.06	1.22	1.36
	5	1.00	1.10	1.06	1.36	1.37
	average	1.00	1.10	1.07	1.23	1.35
	SD	0.00	0.01	0.01	0.02	0.02

Table 8-1. Macroscopical examination of survived mice administrated *Bacillus AJ 12757*

Dose	Number of mouse	Findings	Dose	Number of mouse	Findings	Dose	Number of mouse	Findings
	1	Normal		1	Normal		1	Normal
	2	〃		2	〃		2	〃
	3	〃		3	〃		3	〃
10^{6}	4	〃	10^{5}	4	〃	10^{2}	4	〃
CFU/ml	5	〃	CFU/ml	5	〃	CFU/ml	5	〃
	6	〃		6	〃		6	〃
	7	〃		7	〃		7	〃
	8	〃		8	〃		8	〃
	9	〃		9	〃		9	〃
	10	〃		10	〃		10	〃
	1	Normal		1	Normal		1	Normal
	2	〃		2	〃		2	〃
	3	〃		3	〃		3	〃
10^{7}	4	〃	10^{4}	4	〃	10^{1}	4	〃
CFU/ml	5	〃	CFU/ml	5	〃	CFU/ml	5	〃
	6	〃		6	〃		6	〃
	7	〃		7	〃		7	〃
	8	〃		8	〃		8	〃
	9	〃		9	〃		9	〃
	10	〃		10	〃		10	〃
	1	Normal		1	Normal		1	Normal
	2	〃		2	〃		2	〃
	3	〃		3	〃		3	〃
10^{8}	4	〃	10^{3}	4	〃		4	〃
CFU/ml	5	〃	CFU/ml	5		Mucin	5	〃
	6	〃		6	〃		6	〃
	7	〃		7	〃		7	〃
	8	〃		8	〃		8	〃
	9	〃		9	〃		9	〃
	10	〃		10	〃		10	〃
					〃			

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Table 8-2. Macroscopical examination of survived mice administrated *B.cereus*

Dose	Nunber of mouse	Findings	Dose	Nunber of mouse	Findings	Dose	Nunber of mouse	Findings
	1	Died		1	Died		1	Normal
	2	〃		2	〃		2	〃
	3	〃		3	Normal		3	〃
10^{8}	4	〃	10^{7}	4	Died	10^{4}	4	〃
CFU/ml	5	〃	CFU/ml	5	Normal	CFU/ml	5	〃
	6	〃		6	Died		6	〃
	7	〃		7	〃		7	〃
	8	〃		8	Normal		8	〃
	9	〃		9	Died		9	〃
	10	〃		10	〃		10	〃
	1	Died		1	Normal		1	Normal
	2	〃		2	〃		2	〃
	3	〃		3	〃		3	〃
10^{7}	4	〃	10^{6}	4	〃	Mucin	4	〃
CFU/ml	5	〃	CFU/ml	5	〃		5	〃
	6	〃		6	〃		6	〃
	7	〃		7	〃		7	〃
	8	〃		8	〃		8	〃
	9	〃		9	〃			
	10	〃		10	〃			
	1	Died		1	Normal			
	2	〃		2	〃			
	3	〃		3	〃			
10^{6}	4	〃	10^{5}	4	〃			
CFU/ml	5	Normal	CFU/ml	5	〃			
	6	Died		6	〃			
	7	〃		7	〃			
	8	〃		8	〃			
	9	〃		9	〃			
	10	〃		10	〃			

Table 8-3. Macroscopical examination of survived mice administrated supernatant of culture

Sanple	Nunber of mouse	Findings	Sanple	Nunber of mouse	Findings
Supernatant of *AJ 12757*	1	Normal	Supernatant of B.cereus	1	Normal
	2	〃		2	〃
	3	〃		3	〃
	4	〃		4	〃
	5	〃		5	〃
	6	〃		6	〃
	7	〃		7	〃
	8	〃		8	〃
	9	〃		9	〃
	10	〃		10	〃
2-fold of Supernatan of *AJ 12757*	1	Normal	Supernatant of B.cereus	1	Normal
	2	〃		2	〃
	3	〃		3	〃
	4	〃		4	〃
	5	〃		5	〃
	6	〃		6	〃
	7	〃		7	〃
	8	〃		8	〃
	9	〃		9	〃
	10	〃		10	〃
Medium of *AJ 12757*	1	Normal	Medium of B.cereus	1	Normal
	2	〃		2	〃
	3	〃		3	〃
	4	〃		4	〃
	5	〃		5	〃
	6	〃			
	7	〃			
	8	〃			
	9	〃			
	10	〃			

no. 010026

Analytical Report

To: Yasuhiro Mihara
Fermentation & Biotechnology Laboratories,
Ajinomoto Co.,Inc.

1, Test strain

(1) B1 (AJ 12757)

2、Order

(1) DNA-DNA hybridization

(2) G+C content

3, Results

Strain	G+C content (mol%)	% Reassociation with DNA from: B1
B1	41.8	100
Bacillus subtilis HSCC 185^T	42.3	92

Strain history

HSCC 185 from JCM 1465

(b) (6)

Signiture: [redacted]

R & D Department, Higeta Shoyu Co., Ltd.
2-8 Chuo-cho, Choshi, Chiba, 288-8680, Japan
Phone : 0479-22-1180
FAX : 0479-24-3422

000284

Michael W. Pariza Consulting LLC
7102 Valhalla Trail
Madison, WI 53719

Michael W. Pariza, Member

December 5, 2008

Dr. Robert G. Bursey
President
Ajinomoto Corporate Services LLC
1120 Connecticut Ave., N.W.
Suite 1010
Washington, D.C. 20036

Dear Dr. Bursey:

I am writing in regard to your request for an evaluation of the safety of Ajinomoto's *Bacillus AJ 12757*, to be used in the manufacture of poly gamma glutamic acid (PGA). PGA, a peptide polymer of varying length consisting of L and D glutamic acid residues, has a long history of safe consumption by humans as a component of fermented soy beans in a product called 'natto.' In evaluating the safety of *Bacillus AJ 12757* for the manufacture of this ingredient I considered the biology of the organism, information provided by Ajinomoto consisting of a report on the identity of the organism from the Higeta Shoyu Company, Ltd., a Biological Test Report from the Kitasato Institute, and other information on related *Bacillus* strains that is publically available in the peer-reviewed scientific literature.

The report from Ajinomoto Company, Inc., indicates that *Bacillus AJ 12757* is most closely related to *B. subtilis* and *B. amyloliquefaciens*, both of which have long histories of safe use as production organisms for food-grade ingredients. The Biological Test Report from the Kitasato Institute describes an extensive animal study in which mice were injected with 10^8 viable *Bacillus AJ 12757* organisms without suffering any detectable pathological effects. This test is more than adequate to ensure that the organism does not produce toxic substances.

From these considerations, I conclude that Ajinomoto's *Bacillus AJ 12757* strain is safe for use in the manufacture of food-grade poly gamma glutamic acid (PGA).
Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject product by you or others.

Sincerely,

(b) (6)

Michael W. Pariza
Member, Michael W. Pariza Consulting LLC

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Japanese Journal of Allergology Links

[A case of "late-onset" anaphylaxis caused by fermented soybeans; Natto]

[Article in Japanese]

Suzuki S, Nakamura Y, Kawano Y, Nishioka K.

Medical Center for Allergic and Immune Disease, Yokohama City Minato Red Cross Hospital. szshintr@yahoo.co.jp

Natto is a Japanese traditional food made from fermented soybeans. We report a case of anaphylaxis caused by natto and review the literature. The patient was a 22-year-old man who showed systemic eruption with itching and pectoralgia about 10 hours after eating a meal containing natto. Results of skin tests for soybean allergen were negative, and the allergen remained unidentified. We then used a food elimination trial to confirm the allergy. However the patient did not refrain from eating natto, and he had three anaphylactic reactions might have been caused by natto. Each event occurred 10 to 14 hours after he ate a meal containing natto. We performed detailed examinations to determine the allergen. First, the prick-by-prick tests with natto and its characteristic viscous yarn-like surface were done and yielded positive results. Next, a provocation test with commercial natto (50 g) was performed and caused systemic eruption and pectoralgia about 9 hours after ingestion of the natto. The patients'plasma histamine level was elevated during the anaphylactic event. Anaphylaxis caused by natto was diagnosed. Recent studies have shown that the anaphylaxis caused by natto is of late-onset. Late-onset anaphylaxis can be considered one of IgE-mediated allergic reactions. The viscous surface of natto contains poly-gamma-glutamic acid (PGA). The hypothesized mechanism of late-onset anaphylaxis is delayed absorption or release of PGA into the bowel. In our case, the patient ate heated natto, we therefore speculate that suspect allergens were heat resistant. Patients with natto allergy must not eat natto, whether or not it is cooked or heated. Natto may induce allergic reactions up to a half-day after ingestion; thus, the clinical course and patient's diet must be considered during medical examination.

000286

XI-1-1

Natto has recently gained popularity as a health food in foreign countries. The existence or natto allergy should be more widely recognized.

PMID 16883111 [PubMed - indexed for MEDLINE]

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EXPERT OPINION

ALLERGENICITY ASSESSMENT OF POLYGLUTAMIC ACID

For
AJINOMOTO CO.

March 9, 2009

Steve L. Taylor, Ph.D.
Consultant
Professor and Director
Food Allergy Research & Resource Program
University of Nebraska
Lincoln NE 68583-0919 USA

Polyglutamic acid (PGA) is a new food ingredient that has been developed by Ajinomoto Co. PGA is produced by a fermentation process. PGA is composed primarily of the polymer but PGA is not a typical polypeptide. Many of the glutamic acid residues in PGA are in the D-isomer form, whereas glutamic acid mostly exists in nature as the L-isomer. Furthermore, the peptide linkages between the glutamic acid molecules in PGA are gamma linkages instead of the much more typical alpha linkages.

In terms of an allergenicity assessment of PGA, several issues merit exploration.

(1) Since soybean protein is incorporated into the fermentation medium and since PGA is found naturally in a popular Japanese fermented soy product called natto, does PGA pose any risk to soybean-allergic individuals?
(2) PGA is a novel polypeptide as outlined above. Would any likelihood exist that PGA might induce allergic sensitization in susceptible consumers?
(3) Would any carryover of soy protein or soy protein fragments occur as a result of the use of soy protein in the fermentation medium?

Risk to Soy-Allergic Consumers

In my opinion, PGA presents no risk to soybean-allergic consumers. This opinion is based upon several facts. Soybeans are considered as a commonly allergenic food. A diversity of protein allergens have been identified in soybeans including conglycinin, glycinin, an oleosin protein sometimes known as P34, profilin, a Bet v 1 (birch pollen)-cross-reactive protein, and Kunitz trypsin inhibitor. PGA is not closely related to any of these known soy allergens. PGA is a polymer of glutamic acid and the poly-glutamic acid sequence was subjected to a sequence homology search using the University of Nebraska AllergenOnline database. PGA was 38% homologous to beta-conglycinin over an 80-amino acid window. Beta-conglycinin is a known soybean allergen (Ogawa et al., 1995). This type of bioinformatics approach is frequently used in the allergenicity assessment of novel proteins and homology above 35% over an 80-amino acid window is

considered as minimal for potential significance (Goodman et al., 2008). However, the polyglutamic acid sequence of PGA matches with a low-complexity region of beta-conglycinin that is unlikely to be involved in binding to soy-specific IgE antibodies in soy-allergic humans. Furthermore, the gamma linkages involved in PGA are quite distinct for the alpha linkage comparisons to known allergens using the AllergenOnline database. Thus, PGA has a much different structure from the sequence that matches with the segment of beta-conglycinin in AllergenOnline.

However, to be more certain that PGA would not present a risk to soy-allergic consumers, a serum screening assay was conducted. Serum screening is advocated as the next step in the allergenicity assessment when sequence homology above 35% over an 80-amino acid window is identified by bioinformatics (Goodman et al., 2005). A radioallergosorbent (RAST) inhibition test was employed. In the RAST inhibition test, a soy protein extract is bound to solid phase. These bound soy proteins would bind to IgE antibodies in the sera of soy-allergic individuals. PGA was unable to inhibit the binding of the soy-specific IgE antibodies to the soy protein solid phase. Thus, the soy-specific IgE antibodies did not recognize any epitopes on PGA. This result constitutes rather strong evidence that PGA would not be elicit allergic reactions in soy-allergic consumers.

Furthermore, soy ELISA tests have been conducted in Japan (Morishita et al., 2008). These soy ELISAs are specific for the detection of intact soy proteins, although it is not known if these tests detect all of the known soy allergens. No intact soy proteins were identified in PGA by the soy ELISA. While this evidence is not terribly strong, it does serve to corroborate the RAST inhibition test result to some extent.

Risk of Allergic Sensitization to PGA

As previously noted, PGA is a rather unique polypeptide especially with the respect to the gamma linkages for the glutamic acid residues and the presence of a preponderance of D-glutamic acid isomers. The gamma linkages in PGA tend to render this polypeptide resistant to digestion. Digestive stability, especially pepsin resistance, is thought to correlate to some extent with the potential allergenicity of food proteins (Astwood et al., 1996). Because of its digestive stability, PGA might be able to reach the intestinal tract of susceptible consumers in a form that is sufficiently large to be immunoreactive. PGA feeding studies conducted by Ajinomoto confirm that 70-80% of PGA appears in the feces of rats after ingestion. The molecular weight of the PGA fragments found in the rat feces was approximately 5300 daltons corresponding to 35-40 glutamic acid residues. While such short fragments are unlikely to elicit allergic sensitization, the possibility cannot be totally excluded.

PGA occurs naturally in natto, a popular fermented soy product in Japan. Nine cases of natto allergy have been identified in Japan in recent years among subjects who could safely consume other soy-based foods (Suzuki et al., 2006; Inomata et al., 2007). Thus, these natto-allergic individuals do not fit the immunogenic profile of typical soy-allergic consumers. They appear to be sensitized to a protein that is uniquely found in natto. The allergens involved in these cases of natto allergy have not been specifically identified although several IgE-binding proteins with molecular weights of 26, 28, and 38 kD have been observed (Inomata et al., 2007). These IgE-binding proteins could either be PGA or some fermentation product derived from one or more soybean proteins. Thus, existing

natto-allergic consumers might or might not be at risk from consumption of PGA. However, natto is not a very common allergenic food.

The possibility does exist that PGA might elicit allergic sensitization among susceptible consumers who ingest PGA-containing foods. However, that same concern could exist for virtually every novel protein introduced into the human diet. Should allergic sensitization of PGA occur, these affected consumers would be able to avoid PGA because it will be declared on product ingredient statements. With the low level of anticipated use for PGA (0.2 – 0.4% as a salt substitute), the likelihood of sensitization to PGA would seem low and certainly much lower than the likelihood of sensitization to natto among consumers of that product. Oral challenges of the natto-allergic consumers indicated that reactions would occur following ingestion of 50 g of natto but not after consumption of 10 g (Inomata et al., 2007). PGA use will be sufficiently low that such levels of exposure are quite unlikely.

Soy Protein Carryover from the Fermentation Medium

No evidence exists to suggest that soy proteins or fragments of soy proteins could be carried over from the fermentation medium into the PGA product. The soy ELISA test failed to detect any residues of intact soy protein. The PGA product was unable to inhibit the binding of soy-specific IgE antibodies from the sera of soy-allergic individuals to soy protein bound to a solid phase in the RAST inhibition test. This result indicates that no intact soy proteins or fragments of soy proteins of sufficient size to bind to IgE antibodies were found in PGA.

Conclusion

In my expert opinion, PGA will not pose any risk to soy-allergic consumers. It is not homologous to known soy allergens and does not bind to soy-specific IgE antibodies present in the sera of soy-allergic subjects. Furthermore, no evidence exists that any soy protein or fragments of soy protein are carried over from the fermentation medium to the PGA ingredient.

However, some possibility exists that susceptible individuals might become sensitized to PGA through oral exposure. Based on the low level of anticipated exposure, this risk seems small. If some small number of consumers develop PGA allergy, any risk can be easily managed by product avoidance through labeling of the ingredient.

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February 16 2009

Allergen content test report by ELISA method

【Reception date of specimen】 February 9. 2009
【Specimen No.】 FE09-02-030
【Material】 Sample A Lot No.07.4 28
【Test items】 Soybean
【Reagent kit for testing】 FASTKIT ELISA Ver. II Soybean
(Nippon Meat Packers. Inc.)

【Test results】

Item	Dilution rate	Measured values (ng/mL)	Protein content (μg/g = ppm)
Soybean	400	Less than 2.5	Less than 1.0

* The extract obtained from the specimen was measured in triplicate. and the average value is indicated.

【Measuring range】 2 5~50ng/mL (1~20ppm)

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Pages 000298 - 000304 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

UNIVERSITY OF Nebraska Lincoln

INSTITUTE OF AGRICULTURE AND NATURAL RESOURCES
FOOD ALLERGY RESEARCH AND RESOURCE PROGRAM

Analysis of Polyglutamic Acid Product Using Radioallergosorbent Test Inhibition (RAST-inhibition)

March 5, 2009

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TECHNICAL REPORT

Sample: Two (2) samples of polyglutamic acid product were provided to FARRP by Dr. Robert Bursey of Ajinomoto Corporate Services in Washington DC for soy RAST-inhibition analysis to determine potential allergenicity for soy-allergic consumers. The lot numbers provided for the two samples were 07428 and 07510

METHODS
Extraction

A standard extract of raw Vinton soybeans was prepared by mixing pulverized soybeans 1:10 (w/v) with 0.01 M phosphate buffer containing 0.85% sodium chloride and 0 02% sodium azide, pH 7 4 (PBS) overnight at 4°C . Extracts of the polyglutamic acid products were prepared in the same fashion. Extracts were clarified by centrifugation The soluble protein level of each extract was determined by the Lowry method (1)

Soy radioallergosorbent test-inhibition (RAST-inhibition)

The RAST-inhibition method uses serum from soy-allergic individuals and detects IgE (immunoglobulin E)-binding. RAST-inhibition is a competitive radioimmunoassay. The protocol for the assay was performed generally as described in Hefle et al (2). Soy proteins from an extract of raw Vinton soybeans were bound to a solid phase (cyanogen bromide-activated Sepharose®, Amersham Biosciences Corp., Piscataway, NJ). Various dilutions of the standard raw Vinton soybean extract and the polyglutamic acid product extracts were separately mixed with the soy solid phase and pooled sera from soy-allergic individuals After incubation and washing, the amount of IgE bound to the solid phase was determined using radioactive iodine labeled anti-human IgE antibody and then counted in a gamma counter. In this method, the amount of IgE bound to the solid phase is inversely proportional to the amount of soy-reacting IgE-binding material in the polyglutamic acid product extracts.

RESULTS AND CONCLUSION

The resulting RAST-inhibition curve for soy is shown in Figure 1. The standard curve generated from the raw Vinton soybean extract has a slope of 30.51. The extracts of the polyglutamic acid products did not exhibit any inhibition of soy-specific IgE to the soy proteins bound to solid phase. Thus, polyglutamic acid does not bind with soy-specific IgE from the sera of soy-allergic individuals. Based upon this information, polyglutamic acid is unlikely to present any risk to soy-allergic individuals. Double-blind, placebo-controlled oral food challenges of soy-allergic individuals with polyglutamic acid might be considered as further evidence of the lack of allergenicity of polyglutamic acid.

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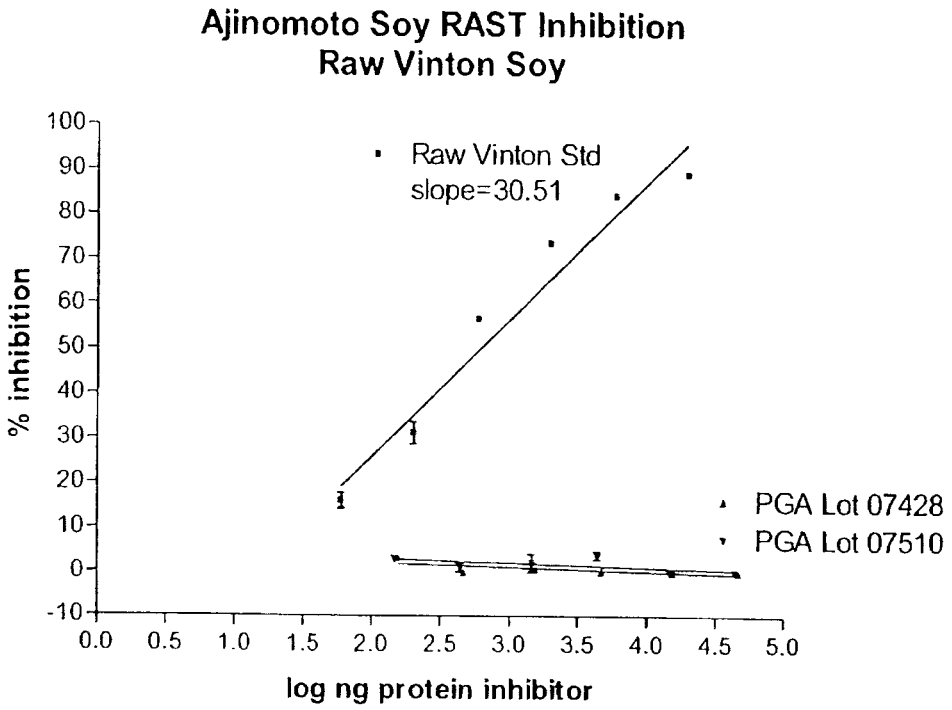
Figure 1


Ajinomoto Soy RAST Inhibition
Raw Vinton Soy
Raw Vinton Std
slope=30.51
PGA Lot 07428
PGA Lot 07510
% inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
log ng protein inhibitor

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000298 - 000304	Morishita, Naoki; Kamiya, Kumiko; Matsumoto, Takashi; Sakai, Shinobu; Teshima, Reiko; Urisu, Atsuo; Moriyama, Tatsuya; Ogawa, Tadashi; Akiyama, Hiroshi; Morimatsu, Fumiki	Reliable Enzyme-Linked Immunosorbent Assay for the Determination of Soybean Proteins in Processed Foods	2008	Journal of Agricultural and Food Chemistry	Volume 56, Number 16, pgs 6818-6824

NA- Not applicable