GR

ORIGINAL SUBMISSION

000001

Japan Cellfoods Co.,Ltd.
SORIO 3-5F,2-2-1 Sakaemachi,Takarazuka,
Hyogo 665-0845,Japan
Phone:+81-797-84-0014 Fax:+81-797-84-9446

GRAS Notification for Pectate Lyase Enzyme Preparation from *Bacillus Subtilis*

Volume 1 of 1

RECEIVED
AUG 29 2002
FOOD ADDITIVES SAFETY

August 22, 2002

000001.001

Cover Letter

Japan Cellfoods Co.,Ltd.
SORIO 3-5F,2-2-1 Sakaemachi,Takarazuka,
Hyogo 665-0845,Japan
Phone:+81-797-84-0014 Fax:+81-797-84-9446

August 16, 2002

Alan Rulis, Ph.D., Director
Office of Food Additive Safety, (HFS 200)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

VIA: Courier

Re: GRAS Notification for Pectate Lyase Enzyme Preparation from *Bacillus subtilis*

Dear Dr. Rulis:

Pursuant to the proposed 21 CFR § 170.36 (c) Japan Cellfoods Co., Ltd claims that the use of pectate lyase enzyme preparations derived from *Bacillus subtilis* is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act because we have determined by scientific procedures that such use is Generally Recognized as Safe (GRAS) as a processing aid.

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

Proposed 21 CFR § 170.36 (c)(1)(i) The name and address of the notifier:

Japan Cellfoods Co., Ltd.
SORIO 3-5F, 2-2-1 Sakaemachi, Takarazuka,
Hyogo 665-0845, Japan

Proposed 21 CFR § 170.36 (c)(1)(ii) The common or usual name of the notified substance:

Pectate lyase enzyme preparation from *Bacillus subtilis*

Proposed 21 CFR § 170.36 (c)(iii) The applicable conditions of use of the notified substance:

The pectate lyase enzyme preparation is intended to be used as a processing aid at minimum levels necessary to achieve the desired effect in accordance with current Good Manufacturing Practices for the preparation of fruit and vegetable purees and concentrates, including the preparation of soybean puree that consist of individual cells of soybean. The purpose of the pectate lyase enzyme preparation is to degrade protopectin in the cell wall.

Proposed 21 CFR § 170.36 (c)(1)(iv) The basis for the GRAS determination:

This GRAS determination is based on scientific procedures.

Proposed 21 CFR § 170.36 (c)(1)(v) Availability of information:

A summary of the data and information supporting this GRAS notification is attached. References and other information that are the basis for this determination are available for review at our facilities in Japan. Alternatively, the documents will be made available at the office of Dr. Clyde A. Takeguchi, Ph.D. at Phoenix Regulatory Associates, Ltd., 21525 Ridgetop Circle, Suite 240, Sterling, VA 20166. If you have any questions or require additional information, please contact Dr. Takeguchi at (703)-406-0906 or by email at phoenix@phoenixrising.com.

Sincerely,

Toru Akazawa, President
Japan Cellfoods Co., Ltd.

Table of Contents

TABLE OF CONTENTS

		Page
Cover Letter		000003
Table of Contents		000006
1.	INTRODUCTION	000009
2.	IDENTITY	000011
2.1	Source of the Enzyme	000011
2.2	EC Number of the Enzyme	000011
2.3	Common Name and Trade Name	000011
2.4	CAS Registry Number	000011
2.5	Properties	000012
2.6	Manufacturing Process	000012
3.	PURITY AND FOOD-GRADE SPECIFICATIONS	000016
3.1	Formulation	000016
3.2	Specifications	000016
3.3	Characteristics	000016
3.4	Composition	000016
4.	APPLICATIONS FOR ENZYME PREPARATION	000019
4.1	Definitions	000019
4.2	Technical Effect	000020
4.3	Application	000020
4.4	Use Level	000020
5.	SAFETY	000022
5.1	Source	000022
5.2	Manufacturing Process	000023
5.3	Enzyme	000023
5.4	Literature Search	000025
6.	EXPOSURE	000027
Conclusion		000027
REFERENCES		000029
APPENDICES		000032

Table of Contents (continued)

APPENDICES

Title	Tab
Improvement of the strain for the production of PECTINASE XP-534	1
Sakamoto, Tatsuji, Hours, Roque Alberto, and Sakai, Takuo, Purification, Characterization, and Production of Two Pectic Transeliminases with Protopectinase Activity from *Bacillus subtilis*, *Biosci. Biotech. Biochem.*, 58(2), 353-358, 1994.	2
Technical Information: UniCell Beans®, CEL 0032, Japan Cellfoods Co., Ltd. Takarazuka, Japan, October 2000	3
Test of Pectinase XP-534 Concentration for Acute Oral Toxicity in Mice, June 12, 2000. Japan Food Research Laboratories	4

1. Introduction

1. INTRODUCTION

The pectate lyase enzyme preparation (commercial name Monalisa Enzyme in the U.S., Pectinase XP-534 in Japan) is derived from the submerged fermentation of a nontoxigenic, nonpathogenic strain of *Bacillus subtilis*.

The pectate lyase enzyme preparation is for use as a processing aid in the production of fruit and vegetable products. For example, the enzyme preparation degrades the protopectin in the cell walls of soybeans, resulting in intact single soybean cells that can be dried and used in various food applications. The enzyme preparation can also be used in combination with the traditional multi-component pectinase products, which consist of pectin depolymerase, pectate lyase, pectin lyase, and pectinesterase.

The information provided in this notification includes the identity of the source, the manufacturing process (Section 2), and food-grade specifications (Section 3) of the enzyme. Section 5 includes a discussion of the safety of the production strain, the enzyme, and the manufacturing process, including an evaluation of dietary exposure to the enzyme preparation. This information is the basis of our general recognition of safety (GRAS) determination of this pectate lyase enzyme preparation by scientific procedures.

2. Identity

2. IDENTITY

2.1. Source of the Enzyme

Bacillus subtilis IFO 3134 was purchased from the Institute of Fermentation (Osaka, Japan) and maintained at 4°C on agar slants. This strain is a non-toxic, non-pathogenic strain of *B. subtilis*. The strain used to produce the enzyme was isolated after exposure to nitrosoguanidine (see flow diagram, Appendix 1).

2.2. EC Number of the Enzyme: EC 4.2.2.2

2.3. Common Name and Trade Name

2.3.1. Common Name

Pectate lyase

2.3.2. Synonyms

Endo pectate lyase (or transeliminase)
Pectic acid lyase
Polygalacturonate lyase
Endogalacturonate transeliminase
Endo-α-1,4-polygalacturonic acid lyase
Polygalacturonic acid lyase
α-1,4-D-endopolygalacturonic acid lyase
PPase-N (research)

2.3.3. Trade Name

Pectinase XP-534 (in Japan)
Monalisa Enzyme (in USA)

2.4. CAS Registry Number: CAS 9015-75-2

Pectate Lyase Enzyme from *Bacillus Subtilis* (continued)
2. Identity (continued)

2.5. Properties

PPase-N (Appendix 2)

MW (gel filtration)	32,000
MW (SDS-PAGE)	43,000
Isoelectric point	9.4
Optimum pH (at 37°C)	8.0
Optimum temp. (at pH 6.0)	60°C
pH stability (37°C, 16 hr)	3-10, ≥ 70% activity
Thermo stability (pH 6, 0.5 hr)	40-50°C, ≥ 70% activity

2.6. Manufacturing Process

The pectate lyase enzyme is manufactured at the Nagase ChemteX Corporation (Nagase) facility in Fukuchiyama, Kyoto, Japan, by submerged culture fermentation of *B. subtilis* 3134, which is a standard enzyme purification method for enzymes excreted into the fermentation medium.[1, 2, 3, Appendix 2] Raw materials used for fermentation (see 2.6.1) and for purification of the enzyme (see 2.6.2) are food grade or are of a purity commonly used for fermentation. The conformance of raw materials to specifications are checked by the Nagase Quality Control Department. All equipment is cleaned and maintained according to good manufacturing practices.

Pectate Lyase Enzyme from *Bacillus Subtilis* (continued)
2. Identity (continued)

2.6.1. Raw Materials for Fermentation

The raw materials used in the fermentation process are food grade or commercial grade commonly used for fermentation of food and food ingredients.

2.6.2 Raw Materials for the Purification

The raw materials used for the purification of the enzyme are food grade ingredients.

Pectate Lyase Enzyme from *Bacillus Subtilis* (continued)
2. Identity (continued)

2.6.3. Manufacturing Flow Diagram

3. Purity and Food-Grade Specifications

Pectate Lyase Enzyme from *Bacillus Subtilis* (continued)

3. PURITY AND FOOD-GRADE SPECIFICATIONS

3.1 Formulation

The enzyme preparation is standardized with dextrin.

3.2. Specifications

Enzyme activity*	650,000U/g±10%
Heavy metal	Not more than 40 ppm
Arsenic	Not more than 4 ppm
Total viable count	Not more than 10,000/g
Coliforms	Negative

*One unit of enzyme activity was defined as the activity that liberates 1 μmole of unsaturated galacturonate per ml of the reaction mixture under the conditions of the reaction (see Appendix 2). The activity was assayed by measurement of the increase in A_{235} of the reaction mixture.

3.3. Characteristics

Pale brown powder

3.4 Composition

3.4.1. Chemical Composition of Pectinase XP-534

Pectinase XP-534 Lot number	Kjeldahl protein (%)	Carbohydrates (%)	Water (%)	Fat (%)	Ash (%)
2184777	1.2	93.7	4.8	0.0	0.3
2163425	1.6	92.4	5.4	0.0	0.6
2149270	2.0	90.7	6.3	0.0	1.0
2156352	1.7	91.0	6.5	0.0	0.8
2028022*	1.6	87.6	10.1	0.0	0.7
Average	1.6	91.1	6.6	0.0	0.7

*The unformulated enzyme (without dextrin) from lot 2028022 was used for the toxicological study.

Pectate Lyase Enzyme from *Bacillus Subtilis* (continued)
3. Purity and Food-Grade Specifications (continued)

3.4.2. Total Organic Solids (TOS)

$$TOS(\%) = 100 - A - W - D = 100 - 0.7 - 6.6 - 90 = 2.7\ \%$$

A = ash
W = water
D = diluent (Dextrin)

4. Applications for Enzyme Preparation

4. APPLICATIONS FOR ENZYME PREPARATION

4.1. Definitions[4, 5]

Pectic substances.—complex plant carbohydrates characterized by the presence of galacturonic acid units.

Protopectin.—water insoluble, unhydrolyzed pectic substances fixed in the plant tissue.

Pectin.—water soluble, methylated pectic substances, usually from protopectin treated with protopectinase, acids or other reagents.

Pectic acid.—pectic substances obtained by hydrolysis of methyl ester groups from pectin; polygalacturonic acid essentially free of methyl ester groups.

Protopectinase.—hydrolyzes or dissolves protopectin and results in the separation of plant cells from each other (maceration).

Pectase.—converts pectin into pectic acid.

Pectinase.—degrades pectin and pectic acid.

Pectin esterase.—removes methoxyl groups from pectin (pectin methylesterase).

Pectin depolymerase.—degrades the α-1,4-bond by hydrolysis or transelimination.

Polymethylgalacturonase.—hydrolyzes the α-1,4-bond next to a free caroboxyl group in pectin by hydrolysis.

Polygalacturonase.—hydrolyzes the α-1,4-bond in pectic acid.

Pectin lyase.—degrades the α-1,4-bond in pectin by transelimination.

Pectate lyase.—degrades the α-1,4-bond in pectic acid by transelimination.

Endo-type enzyme causes random cleavage and the exo-type enzyme causes sequential cleavage of pectic substances.

4.2. Technical Effect

The function of the pectate lyase enzyme preparation is the maceration of the plant tissue by degrading the protopectin in cell walls and separation of the individual plant cells from each other. Pectate lyases degrade glycosidic linkages of pectate next to free carboxyl groups by β elimination.[5, 6, 7] The endo-type enzymes split the pectate chain at random, resulting in a reduction in viscosity of the solution with a small increase in reducing end groups. The endo-type enzyme degrades pectate (polygalacturonate) to Δ4-5 oligogalacturonates with the unsaturation at the non-reducing end.

4.3. Application

The pectate lyase enzyme preparation will be used as a processing aid in the food industry primarily in the maceration of fruit and vegetable pulp applications. Japan Cellfoods Co., Ltd. currently uses the enzyme preparation to macerate soybean resulting in a pulp consisting mainly of intact single-cells (UniCell Beans®) that are dried and can be used in various applications (Appendix 3). In Japan, the UniCell Beans® flour are used in bread at a level of 3-10%, and tofu (3-10%). The enzyme preparation can also be used in combination with the traditional multi-enzyme pectinase products.

4.4. Use Level

The enzyme preparation is used at minimum levels necessary to achieve the desired effect in accordance with current Good Manufacturing Practice procedures. The recommended use level is 0.1% of the weight of the soybean.

5. Safety

5. SAFETY

5.1. Source

In 1999, FDA affirmed that carbohydrase and protease enzyme preparations from either *B. subtilis* or *B. Amyloliquefaciens* are considered as generally recognized as safe (GRAS) for use as direct food ingredients.[2] FDA stated that nontoxigenic and nonpathogenic strains of *B. subtilis* are widely available and had have been safely used in a variety of food applications, including the documented consumption of *B. subtilis* in the Japanese food, natto, a fermented soy bean food. FDA concluded that carbohydrase and protease enzyme preparations derived from *B. subtilis* were in common use in food prior to January 1, 1958.

Three articles obtained from the literature review (see 5.4) discussed the use of *B subtilis* in traditionally fermented bean products. Isu and Ofuya[8] used five strains of pure cultures of *B. subtilis* isolated and identified from locally produced 'ugba,' used as a food snack or soup condiment. The pure culture mixture was compared for changes in pH, amino-nitrogen and protease activity as fermentation indicators. Results demonstrated that the use of the pure culture innocula significantly reduced the fermentation time and improved the quality of the product compared to traditional natural fermentation of African oil bean into 'ugba.'

Omafuvbe et al.[9] isolated and identified predominantly *B. subtilis*, *B. licheniformis*, and *B. pumilus* from the traditional production of 'daddawa' from soybean. They reported that the *Bacillus* species produced extracellular enzymes which hydrolyzed the organic components of the fermenting beans resulting in increased soluble products.

Kiers et al.[10] stated that *Bacillis subtilis* is an important starter culture for Asian and African fermented legume products, such as Japanese natto, Thai thua-nao, Indian kinema and West African dawadawa. They reported that the fermentation process increases the nutrient availability of the fermented food products. *In vitro* digestion of *Bacillus*-fermented soy bean increased from 29% up to 33-43% after fermentation for 48 hours. These articles support the history of safe use of food products fermented by *B. subtilis*.

The *B. subtilis* strain used for the production of the pectate lyase enzyme preparation is a strain obtained by exposure of a nontoxigenic and nonpathogenic strain of *B. subtilis* from the Institute of Fermentation, Osaka, Japan, to nitrosoguanidine. Exposure to a traditional mutagenic agent does not result in a toxigenic or pathogenic organism.

Pectate Lyase Enzyme from *Bacillus Subtilis* (continued)
5. Safety (continued)

Japan Cellfoods concludes that the *B. subtilis* production strain is nontoxigenic and nonpathogenic and that the use of this strain in food applications and as a source of enzymes is safe.

5.2. Manufacturing Process

The manufacturing process is based on generally available and accepted methods used for production of microbial enzymes used to exclude contaminating organisms.[1, 2, 3] Further, FDA stated in the 1999 final rule on carbohydrase and protease from *B. subtilis*, that these enzymes are excreted into the fermentation media in the submerged culture method is processed in an identical manner as the manufacturing process for pectate lyase.[2] Thus, Japan Cellfoods concludes that the manufacturing process is substantially equivalent to other processes for manufacturing commercial microbial enzymes for food use.

5.3. Enzyme

Commercially, pectate lyase is one of several enzymes present in commercial pectinase products used in food processing.[11] In general, bacterial lyases are specific for galacturonoate polymers in which a low proportion of C-6 is methylated (pectate). Bacterial pectate lyases have been described in species of *Clostridium*[12], *Xanthomona*[13,14], *Pseudomonas*[15], *Bacillus*[7, 16, 17], *and Erwinia*[18,19].

Commercial pectinase derived from *Aspergillus niger* is listed in the *Food Chemicals Codex*[10] and includes four enzymes, pectate lyase (EC 4.2.2.2), pectin depolymerase , pectin lyase, and pectinesterase. FDA recently responded to a GRAS notification (GRN 000089)[20] from the Enzyme Technical Association (ETA) regarding pectinase from *A. niger* that contain the four enzymes listed above, stating that it had no further questions at this time. ETA provided published information that fungal pectinase was used in processing fruit juices, purees, and coffee beans prior to 1957.

Pectate lyases are well-characterized enzymes.[7, 11-19] The presence of pectate lyase in commercial fungal pectinase preparations supports the use of pectate lyase in food but its use as an enzyme preparation containing primarily pectate lyase has been limited to other industrial uses. These enzymes have been used as retting enzymes for production of natural fibers for paper and for textiles and have been proposed for the production of potato and pulse puree and powder products.[1,21]

Pectate Lyase Enzyme from *Bacillus Subtilis* (continued)
5. Safety (continued)

In the 1999 GRAS affirmation of carboydrase and protease, FDA acknowledged the occupational reports of allergies and irritation from skin contact with enzymes or inhalation of dust from enzymes used in the manufacture of laundry detergents.[2] However, FDA stated that these reports were not relevant to the safety of ingesting such enzymes in food.

Pectate lyase from *B. subtilis* causes random cleavage of α-1,4 glycosidic bonds in pectic acid by transelimination, and has been characterized as an endo-pectate lyase (EC 4.2.2.2).[Appendix 2] The reaction products are Δ4-5 oligogalacturonates with a 4,5-dehydrogalacturonic acid group at the non-reducing end. These products are not considered toxic and are similar to products from commercial pectinase use and from *B. subtilis*-fermented foods.

Japan Cellfoods notes that traditionally fermented foods, such as natto in Japan, are consumed in large quantities and there has been no reported problems of pathogenicity or toxicity with enzyme preparations derived from *B. subtilis* for food. The recent literature search (see 5.4.) has not provided any reference to refute this fact. Three articles reported the use of *B. subtilis* in traditionally fermented food from Asia and Africa (see discussion in 5.1).[8-10] Thus, the fermented food products resulting from the enzymes present in *B. subtilis* are non-toxic. Japan Cellfoods controls and monitors the manufacturing process to prevent possible contamination by other microorganisms that may be pathogenic or produce antibiotics. In addition, the purification process includes filtration with a ceramic filter that filters out the fines and any viable microorganisms.

To confirm the safety of its enzyme preparation, Japan Cellfoods tested its Pectinase XP-534 (the unformulated enzyme from Lot 2028022) in mice for acute oral toxicity in accordance with OECD Guidelines for the Testing of Chemicals 401 (Appendix 4). Ten (10) male and ten (10) female ICR mice per group were allocated to the experimental and control group. The test sample was suspended in purified water and administered orally by stomach tube at a dose of 5,000 mg/kg. Controls were administered with 0.6 ml of purified water by stomach tube. There were no abnormalities nor death in any mice. The Study Director concluded that the LD_{50} value was higher than 5,000 mg/kg b.w.

Japan Cellfoods has concluded that pectate lyase obtained from *B. subtilis* is a safe and suitable enzyme because it is substantially equivalent to the pectate lyase found in commercial pectinase obtained from *A. niger*. Further, it is substantially equivalent to other enzymes from *B. subtilis*, carbohydrase and protease, that FDA affirmed as GRAS.[2] In addition, reaction products and any other degradation products from the enzymatic

reaction are identical or substantially equivalent to products formed during the use of commercial pectinase products on food. Further, the use of a nontoxigenic and nonpathogenic source, controlling the process for contamination with pathogenic microorganisms, and using a ceramic filter that excludes microorganisms ensure the production of a safe enzyme preparation.

5.4. Literature Search

Japan Cellfoods conducted an extensive literature search from 1999 to the present for recent articles on the use and toxicity of the source, *Bacillus subtilis* and the enzyme pectate lyase.

Three articles by Isu and Ofuya,[8] Omafuvbe et al.,[9] and Kiers et al.[10] reported the use of *B. subtilis* in traditionally-fermented food from Asia and Africa. These articles support the safe use of the source, *B. subtilis* in food (see discussion in Section 5.1).

Harbak and Thygesen reported on the safety evaluation of a xylanase enzyme expressed in a self-cloned strain of *B. subtilis*.[22] Based on the test program prescribed by the EU's Scientific Committee for Food, the authors conducted acute and subchronic oral toxicity testing of the enzyme in rat feeding studies and mutagenic studies in microbial and tissue culture in-vitro studies. The authors reported no evidence of toxicity at the highest recommended dose (80,000 total xylanase units/kg body weight/day) nor any evidence of mutagenic potential or chromosomal aberrations. This article supports the safety of enzymes produced from *B. subtilis*.

6. Exposure

6. EXPOSURE

In the 1999 GRAS affirmation of carbohydrase and protease from *Bacillus* species,[2] FDA estimated dietary exposure to enzyme preparations at 200 mg/person/day based on the total consumption of microbial-derived enzyme preparations in food as an approximation for the consumption of enzyme preparations derived from *B. subtilis* or *B. amyloliquefaciens* that are consumed in food. Japan Cellfoods believes that the use of pectate lyase derived from *B. subtilis* can be incorporated in this estimate because FDA stated that this was a very conservative estimate that relied on a worst-case assumption that all microbially-derived enzyme preparations were consumed in food.

Enzymes are naturally occurring proteins that are components of living organisms and are consumed in microbially-fermented food or food that are unprocessed or minimally processed and containing microorganisms. Thus, enzymes derived from microorganisms or as components of the diet have been safely consumed in the diet for many years.

Further, the enzymatic products are identical or substantially equivalent to pectin and pectic acid products resulting from traditional food preparation and digestion of plant products in man. In addition, any enzyme that remains in food will be digested in the same way that other proteins are digested in man.

Conclusion

Japan Cellfoods concludes that the use of pectate lyase from *B. subtilis* for use as a processing aid primarily in fruit and vegetable products is safe based on:

The history of safe use of *B. subtilis* in food and enzymes derived from *B. subilis.*

The use of a standard manufacturing process used for fermenting and processing commercial enzymes for use in food.

The use of safe and suitable ingredients for the fermentation process and the purification of the enzyme.

The enzyme, pectate lyase (EC 4.2.2.2), is substantially equivalent to the pectate lyase present as a component of pectinase from *A. niger* and substantially equivalent to other enzymes from *B. subtilis* that have been affirmed as GRAS.

The components of the enzyme preparation are substantially equivalent to components of other commercial enzyme preparations that have been safely used in the diet for many years.

There have been no confirmed reports of allergies or toxicity in consumers consuming food processed with enzyme preparations.

References

REFERENCES

1. Fogarty, W.M., and Ward, O.P., Pectinases and Pectic Polysaccharides, *Prog. Ind. Microbiol.* **13**:59-119 (1974)

2. FDA, "Carbohydrase and Protease Enzyme Preparations Derived from *Bacillus subti*lis or *Bacillus amyloliqueaciens*; Affirmation of GRAS Status as Direct Food Ingredients," 64 FR 19887-19895; April 23, 1999.

3. Sakai, T., Protopectinase from Yeasts and a Yeastlike Fungus, *Methods in Enzymology*, **161**:335-350 (1988)

4. Brinton, C.S., Wichmann, H.J., Willaman, J.J., Wilson, C.P., and Dore, W.H., Definitions Written by the Committee on Nomenclature of Pectin of the Agriculture-Food Division, *J. Am. Chem. Soc.*, **49**:37-39 (1927).

5. Fogarty, W.M., and Ward, O.P., Pectic Substances and Pectinolytic Enzymes, *Process Biochemistry*, **7**:13-18 (1972)

6. Voragen, A.G.J., and Pilnik, W., Pectin-degrading Enzymes in Fruit and Vegetable Processing, in Biocatalysis in Agricultural Biotechnology (J.R. Whitaker and P.E. Sonnet, Editors), ACS Symposium Series 389:93 (1989)

7. Miyazaki, Y., Purification and Characterization of Endo-Pectate Lyase from *Bacillus macerans, Agric. Biol Chem.*, **55**:25-30 (1991)

8. Isu N.R. and Ofuya C.O., Improvement of the traditional processing and fermentation of African oil bean (Pentaclethra macrophylla Bentham) into a food snack--'ugba'. *International journal of food microbiology*. **59(3)**:235-9 (2000)

9. Omafuvbe B.O., Shonukan O.O., and Abiose S.H., Microbiological and biochemical changes in the traditional fermentation of soybean for 'soy-daddawa': Nigerian food condiment. *Food Microbiology*, **17(5)**:469-474 (2000)

10. Kiers J.L., Van Laeken A.E., Rombouts F.M., and Nout M.J., In vitro digestibility of bacillus fermented soya bean. *International journal of food microbiology*. **60(2-3)**:163-9 (2000)

11. Committee on Food Chemicals Codex, Enzyme Preparations, *Food Chemicals Codex*, 4th Ed., National Academy Press, Washington, D.C. pp 129-134; 786-788, 1996

References (continued)

12. Macmillan, J.D., Phaff, H.J., and Vaughn, R.H., The Pattern of Action of an Exopolygalacturonic acid-trans-eliminase from *Clostridium multifermentans*, *Biochemistry*, **3**:572-578 (1964)

13. Nasuno, S. and Starr, M.P., Polygalacturonic Acid *trans*-Eliminase of *Xanthomonas campestris*, *Biochem. J.*, **104**:178-185 (1967)

14. Starr, M.P. and Nasuno, S., Pectolytic Activity of Phytopathogenic Xanthomonads, *J. Gen. Microbiol.*, **46**:425-433 (1967)

15. Nasuno, S. and Starr, M.P., Pectic Enzymes of *Pseudomonas marginalis*, *Phytopathology*, **56**:1414-1415 (1966)

16. Hasegawa, S., and Nagel, C.W., A new Pectic Acid Transeliminase Produced Exocellularly by a Bacillus, *J. Food Sci.*, **31**:838-845 (1966)

17. Ward, O.P. and Fogarty, W.M., Polygalacturonate Lyase of a Bacillius Species Associated with Increase in Permeability of Sitka Spruce (*Picea sitchensis*), *J. Gen. Microbiol.*, **73**:439-446 (1972)

18. Sugiura, J., Yasuda, M., Kamimiya, S., Izaki, K., and Takahashi, H., Purification and Properties of Two Pectate Lyases produced by *Erwinia* carotovora, *J. Gen. Appl. Microbiol.*, **30**:167-175 (1984)

19. Starr, M.P. and Moran, F., Eliminative Split of Pectic Substances by Phytopathogenic Soft-rot Bacteria (Erwinia carotovora and B. polymyxa), *Science*, **135**:920-921 (1962)

20. U. S. Food and Drug Administration, "Agency Response Letter to the Enzyme Technical Association GRAS Notice No. GRN 000089," dated April 3, 2002

21. Rombouts, R.M., and Pilnik, W., Pectinases and other Cell-wall Degrading Enzymes of Industrial Importance, *Symbosis* **2**:79-90 (1986)

22. Harbak L. and Thygesen, H.V., Safety evaluation of a xylanase expressed in Bacillus subtilis. *Food and Chemical Toxicology* **40(1)**:1-8 (2002)

Appendices

APPENDICES

Title	Tab
Improvement of the strain for the production of PECTINASE XP-534	1
Sakamoto, Tatsuji, Hours, Roque Alberto, and Sakai, Takuo, Purification, Characterization, and Production of Two Pectic Transeliminases with Protopectinase Activity from *Bacillus subtilis*, *Biosci. Biotech. Biochem.*, 58(2), 353-358, 1994.	2
Technical Information: UniCell Beans®, CEL 0032, Japan Cellfoods Co., Ltd. Takarazuka, Japan, October 2000	3
Test of Pectinase XP-534 Concentration for Acute Oral Toxicity in Mice, June 12, 2000. Japan Food Research Laboratories	4

1

Nagase ChemteX

Improvement of the strain for the production of PECTINASE XP-534

Nagase ChemteX Corporation

Strain : *Bacillus subtilis* IFO 3134

The method of the improvement : Mutation by nitrosoguanidine(NTG)

<Genealogy of *Bacillus subtilis* strain>

Bacillus subtilis IFO 3134 —NTG→ # 01034 —NTG→ # 02094 —NTG→ # 03039 —NTG→ # 04012 —NTG→ # 05180 —NTG→ # 06008 —NTG→ # 07091 —NTG→ # 08007 —NTG→ # 09047 —NTG→ # 10025 —NTG→ # 11096 (Current production strain)

: Internal strain No.

→ : Mutation & selection

2

PAGES 00036-000041 HAVE BEEN REMOVED IN ACCORDANCE WITH COPYRIGHT LAWS
PLEASE SEE APPENDED BIBLIOGRAPHY FOR A LIST OF THE REFERENCES THAT
HAVE BEEN REMOVED FROM THIS REQUEST

3

CEL0032
October 2000

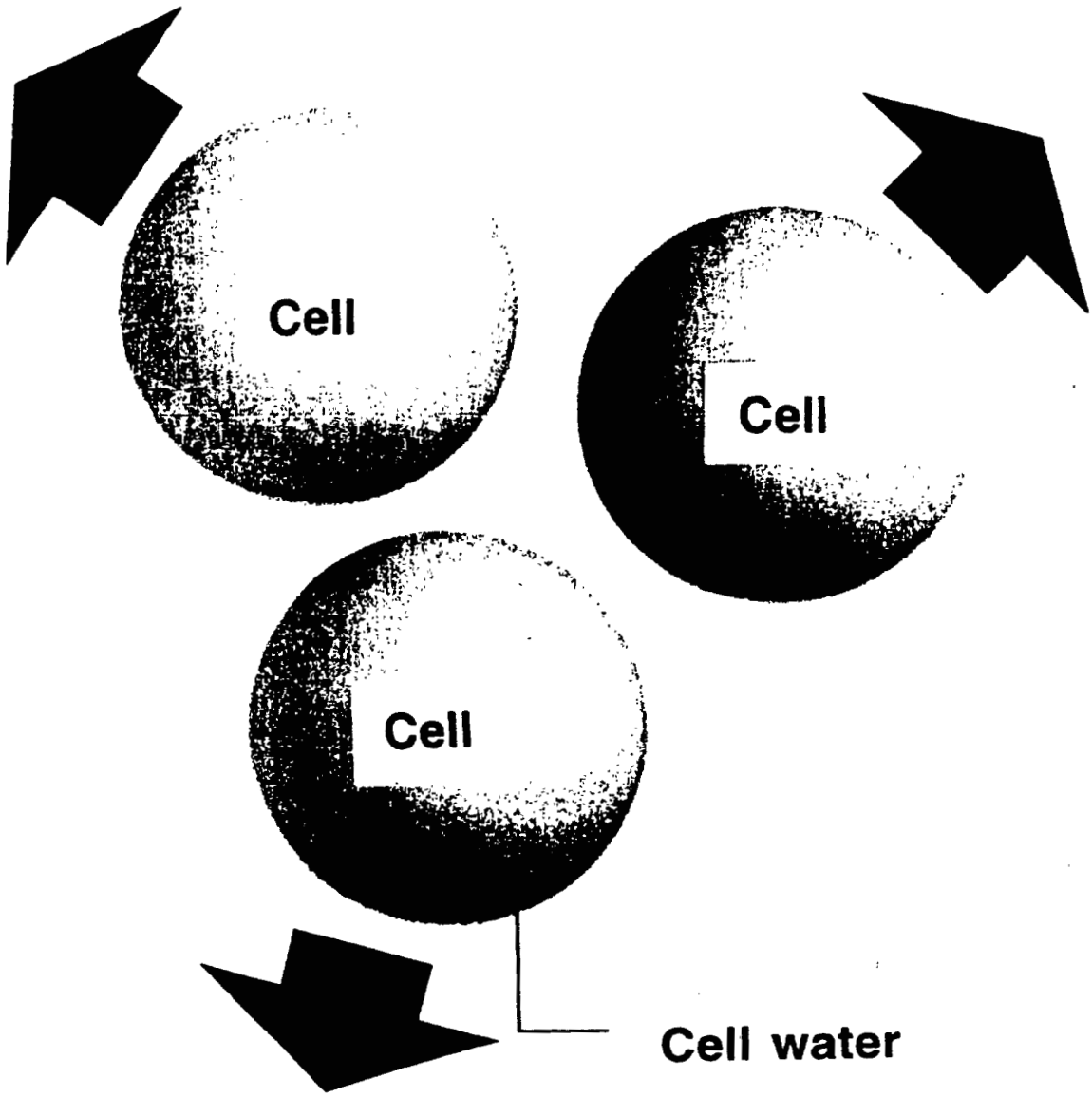
Technical Information

UniCell Beans®

Japan Cellfoods Co., Ltd.
Takarazuka, Japan

What is UniCell Beans?

The UniCell Method breaks the soy down into single cell units, enabling digestion of the whole bean. When the soy is mixed in bread, it gives a better flavor and aroma without the normal soy aftertaste: an altogether tastier way to consume isoflavone.



CONTENTS

Page

Introduction 1

Soy as a valuable food ingredient 1

Why We Developed UniCell Beans? 1

Food shortages caused by population growth 1

Turning this infinite resource into food 2

Nutrition Problems 2

What is the P/S ratio? 3

What is UniCell Beans? 5

Main Features of UniCell Beans 6

What is a cell? 6

Intracellular Water Content 9

Potential Applications of UniCell Beans 9

Making Bread with UniCell Beans 10

Effects of UniCell Beans on Bread-Making 10

Using UniCell Beans in Bread-Making 11

Considerations 12

Conclusion 12

Finally 12

Introduction

Soy as a valuable food ingredient

About 100 million tons of soybeans are produced worldwide each year. In Japan they have long been known as "the meat of the field" and regarded as a source of life in much the same way as eggs. The significance of soybeans as a food source can be related to their high nutritional value.

The balance of essential amino acids in soy protein, superior amongst vegetable proteins, has long been recognized. In recent years the basis of tests has shifted from animals to humans, and it has become clear that the nutritional value of soy protein for adults is comparable to that of beef, and for infants to that of dairy milk.

Already known as the "miracle crop" in the West, soybeans are expected to play an important role in providing properly balanced diets and in relieving global food shortages.

At present, however, most processing methods destroy bean cells and use proteins for soybean milk, tofu etc. Cooking oil is also extracted from soybeans. The leftover insoluble residue of processed soybeans (okara) and oil cake are used as animal feeds and fertilizers, since turning them into human food products is practically unfeasible.

Primarily, it is possible to achieve well-balanced nutrition if each foodstuff is consumed entirely. In this respect, soybeans differ from other pulses. Since their tissue is hard and processing is difficult, only limited portions of their constituents are used and the remainder is dealt with as a residue in most cases. Furthermore, the soybean has a characteristic smell, which can affect the taste of other foods, severely limiting the scope of its application and the amounts added as a food ingredient.

Why We Developed UniCell Beans®?

Food shortages caused by population growth

The population of the world currently stands at 5,800 million, of which 1,500 million earn less than one dollar a day and 800 million are unsure of where their next meal is coming from.

It is estimated that the world population will break the 10 billion mark in 2030. Food supply must increase with this growth in population. However, agricultural land necessary for food production has decreased by 15% throughout the world. Considering the future of world food supply, the solution of vast famines and the introduction of new technology are one and the same.

In this sense the answer to food shortages can only lie in biotechnology, which allows utilization of inherent biological features.

Table 1 Nutritional Composition of Japanese, American and Chinese Soybeans (including black and green soybeans)

(per 100 grams)

		Japanese soy	American soy	Chinese soy
Energy	(kcal)	417	433	422
	(kJ)	1,745	1,812	1,766
Water	(g)	12.5	11.7	12.5
Protein	(g)	35.3	33.0	32.8
Sugars	(g)	19.0	21.7	19.5
Carbohydrates				
of which sugars	(g)	23.7	24.6	26.2
of which fiber	(g)	4.5	4.2	4.6
ash content	(g)	5.0	4.8	4.4
Minerals				
Calcium	(mg)	240	230	170
Phosphorous	(mg)	580	480	460
Iron	(mg)	9.4	8.6	8.9
Sodium	(mg)	1	1	1
Potassium	(mg)	1,900	1,800	1,800
Vitamins				
A-retinol	(µg)	0	0	0
A-carotene	(µg)	12	8	15
A effect	(IU)	0	0	0
B1	(mg)	0.83	0.88	0.84
B2	(mg)	0.30	0.30	0.30
Niacin	(mg)	2.2	2.1	2.2
C	(mg)	0	0	0
Salt equivalent	(g)	-	-	-
Disposal ratio	(%)	0	0	0

Source: Japanese Food Composition Databook, 4th ed. 1987, Watanabe & Saio

Turning this infinite resource into food

UniCell Beans is based on innovative technology which permits us to use the whole of the soybean for food. This technology can also be applied to other agricultural products.

For example, vegetables that do not meet the standard market criteria (size, shape, color, and pest damage) are all thrown away in today's marketplaces. Fruit and vegetable juices are produced by extracting intracellular fluids and the rest is disposed of as waste.

Employing the "UniCell" technology makes it possible to utilize all edible portions of food products without producing waste products.

Nutrition Problems

In recent years, the diet of people in the so-called developed countries including Japan and other Western countries has become richer and more diversified. This has led the people to consume excessive animal fat and proteins, causing younger

people to contract geriatric diseases, and school children to become more obese and liable to have diabetes, etc.

To overcome these problems, Low-Fat and Non-Fat dairy products containing extremely low-level or substantially no animal fat and additional calcium and fiber have become increasingly popular in the U.S. today. This reflects health-oriented mind of the American consumers.

In this situation, it is quite understandable that there is a keen interest today in the soybean, which is rich in vegetable fats and proteins and is a highly efficient source of nutrition.

What is the P/S ratio?

Generally, for reducing the blood-cholesterol level, it is necessary to reduce saturated fatty acids and increase fats containing unsaturated fatty acids that are taken into a human body.

The ratio of the amount of polyunsaturated fatty acids (P) to that of saturated fatty acids (S) is used as a criterion for evaluating the blood-cholesterol level. According to a recent investigation of today's average Japanese diet, the P/S ratio is estimated to have dropped to about 0.7 or less (about 0.3 in other Western countries).

To prevent diseases caused by extravagant lifestyles unique to developed nations, however, it is believed to be necessary to keep the P/S ratio at a level of 1.0 to 1.4 (ideally 2.0).

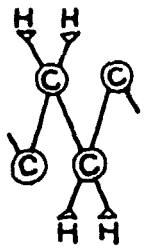
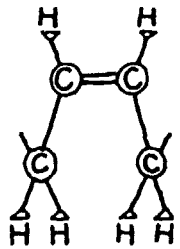
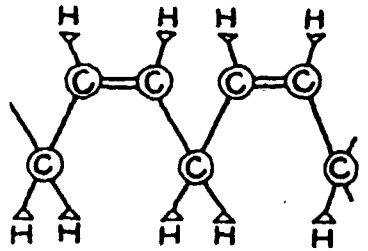
When we ask ourselves how we should go about closing this difference, it is needless to say that the choice of food we eat is important. Problems arise, however, with the fats in natural foods and those contained in processed foods.

If we eat processed foods that include meat, eggs or milk, for example, then we cannot avoid eating a certain amount of saturated fatty acids.

Even if we replace all the fats in our diet with vegetable fats, we would only achieve at the most a P/S ratio of 0.8, far below the recommended range of 1.0 to 1.4. This is where soy comes into its own.

Soybean products composed of protein-rich amino acid tissues and soy oil containing approximately 60% polyunsaturated fatty acids are excellent substitutes for meat, eggs and milk.

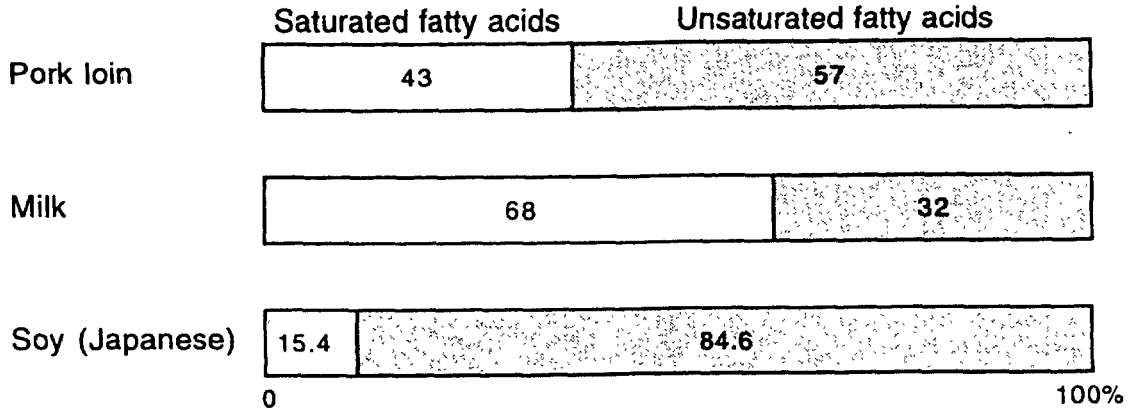
Table 2 Fatty Acids Affecting Cholesterol Levels

Fat	Saturated fatty acids	Unsaturated fatty acids	Polyunsaturated fatty acids
Chemical formula			
Composition	Palmitic acid Stearic acid	Oleic acid	Linolic acid Linolenic acid (2 double bonds) Arachidonic acid (3 double bonds)
Type	Meat Shortening Butter Palm oil Beef tallow Lard		Safflower oil Sunflower oil Soybean oil Cottonseed oil Sesame oil
Properties	• Often found in animal fats. • Palmitic acid causes blood-cholesterol levels to rise considerably.	• Oleic acid often causes blood-cholesterol levels to change.	• Linolic acid and linolenic acid cannot be formed in the human body. Arachidonic acid is a fatty acid induced from linolic acid in the body. These three acids are therefore called the essential fatty acids. • Linolic acid reduces blood-cholesterol levels. • Improve lipid metabolism, especially cholesterol metabolism, in bloodstreams. • Due to many double bonds, polyunsaturated fatty acids are easily oxidized. Special care is needed.

000049

Table 3 Proportions of Fatty Acids Influential on the Metabolism of Cholesterol

Unsaturated fatty acid	Food	Saturated fatty acid	
Linolic acid		Palmitic acid	Myristic acid
5.6%	Beef	27.6%	3.7%
20.1	Pork	20.7	2.0
18.8	Chicken	26.8	0.4
2.5	Mutton	22.5	2.5
0.5	Butter	32.5	12.9
51	Soybean oil	10	0.3
75	Safflower oil	6.4	--
29	Rapeseed oil	3	--
2.5	Sardine oil	15.5	6.6



Source: Japanese Food Composition Databook, 4th ed. 1987, Watanabe & Saio

Fig. 1 Difference in Fat Composition

What is UniCell Beans®?

In our regular diet we do not press the food and drink the juice. Instead, we eat the food as whole cells. If we were to press our meat, fish, rice and vegetables, we would lose real pleasure of eating these foodstuffs and there would be little need for cooking; in short, our food culture would not have developed.

Eating food cells in their entirety gives us not only an appetite but also a rich source of nutrition necessary for our health, due to the intercellular substance and so on they contain.

If that is the case, then why do we press soybeans and not eat them whole? Soy milk and tofu mainly use the soluble proteins and emulsified oil of the soybean; the insoluble residue, known as okara, is simply thrown away. Almost 1 million tons of okara is discarded annually.

Soy oil only accounts for 20% by weight of soybeans. Of the remaining 80% waste, roughly one tenth is used in processed food production for human consumption, and the rest for animal feeds, and agricultural fertilizer.

Our UniCell Beans has been developed to make it possible to eat the whole of the individual soybeans, our valuable source of vegetable protein. In developing it, strenuous efforts were made to reduce the unpleasant smell of the soybean as much as possible.

To achieve this objective, we stabilized protein bodies and lipid bodies in the cells and separated the individual soybean cells without destroying them. Then, we eluted the cells to produce soybean powder so that the fats would not oxidize or create enzyme breakdown with the proteins.

The thin soybean cells are separated from each other as can be seen in the microphotograph (Fig. 5). The protein bodies and lipid bodies can also be seen within each cell.

Most of the individual cells, or *unicells*, have solid rodlike form. The result is that the characteristic soy smell has all but disappeared and the particle size has become uniform, thus enabling the beans to be used in food production and as a nutritional supplement.

Our next objective is to introduce the soybean as a new food material to those parts of the world where it is not commonly eaten and to develop a wide range of uses for it as part of a healthy diet.

Main Features of UniCell Beans®

1. Minimized soy smell due to unbroken cell walls and membranes.
2. Changes of acids and other constituents are avoided since proteins and fats are stabilized (as if in a sac).
3. Possible to utilize 100% of all soybean constituents.
4. Applicable to all kinds of food products requiring water retention capability since the intracellular water content does not easily evaporate.
5. Zero emission of waste material in soybean processing.

What is a cell?

At birth a human body has about 30 trillion cells, and as adults we have 60 trillion. Everyday we lose 10,000 brain cells.

Although animal cells and plant cells are similar to each other on the whole, the plant cells have a double wall and a single membrane. In each of these plant cells, a nucleus is surrounded by cytoplasm. The nucleus itself contains DNA, vacuoles and pigment.

When producing fruit or vegetable, every cell is destroyed and the juice comes from the vacuoles. Then, the residue is thrown away. For instance, carrots produce 50% juice and 50% processing residues.

When soy oil is extracted from soybeans, 20% is oil, 80% residue. In making

soybean milk and tofu, the cells are crushed and ground to extract the proteins and fats. These extracted portions only reach 50%, however, as measured as a solid product equivalent. Some of the solid residue is used for human consumption as okara, but the majority of the solid residue and the supernatant solution are thrown away.

Soybean cells differ greatly from most other plant cells. The soybeans are rich in protein and fats due to the intracellular protein bodies, which hold the reserve proteins, and the lipid bodies, which hold the fats. The proteins and fats can be found separately in sacs.

UniCell Beans is produced without destroying the bean cells. Instead, by using enzymes we dissolve pectin that joins the individual cells. Therefore, the soybean constituents, such as the protein bodies and lipid bodies, are confined within every cell, and this makes it possible to use UniCell Beans as an ingredient in food production with hardly any unpleasant smell.

One gram of carrot contains approximately 30 million cells or more. The same amount of soybean also contains a countless number of cells.

Everyone must have recently heard about genetically modified soybeans, where the DNA in the cells has been modified. This and other studies into cells, such as animal cloning, form part of the latest biotechnology.

UniCell Beans, with every cell separated, is based on a completely new concept in soybean processing.

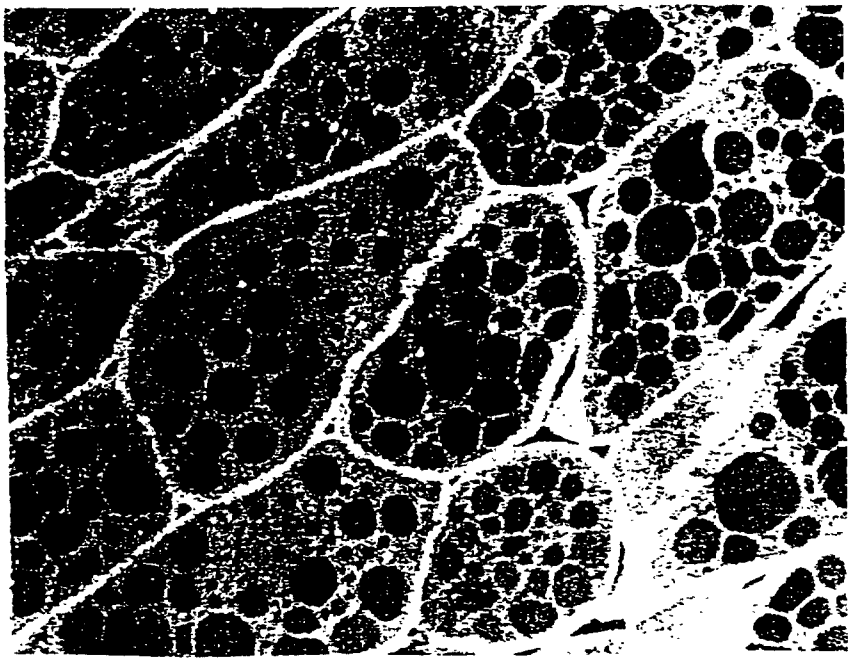


Fig. 2 Soybean Cells Photographed by Transmission Electron Microscope—*Bean cells are visibly full of protein bodies (black sacs) with their reserve protein and lipid bodies (small white particles), which hold the fats. The value of soy fat and protein has now been recognized.*

000052

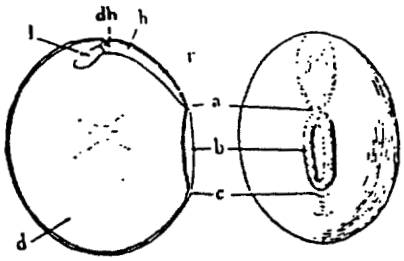


Fig. 3 Soybean Seed

a: micropyle (will become the germ pore)
b: hilum c: raphe r: radicle
h: hypocotyl l: primary leaf
d: cotyledon db: cotyledon base

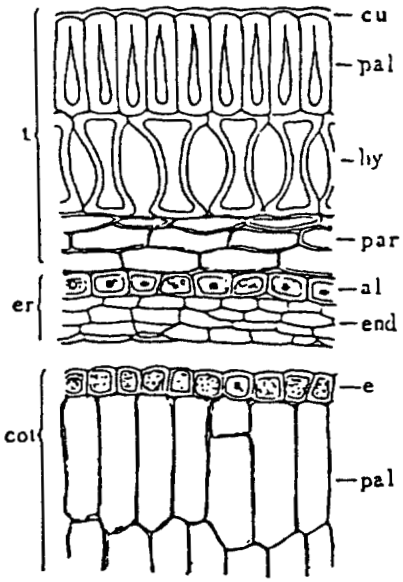


Fig. 4 Internal Structure of Soybean Seed

t: testa er: remaining tissue of endosperm
cot: cotyledon cu: cuticle pal: palisade tissue hy: Hourglass type cells in hypodermis
par: cavernosus al: aleurone
end: endosperm e: epidermis of cotyledon
(Source: Food Crops, Kiyochika Hoshikawa)

Fig. 5 Microphotograph of UniCell Beans (200x)

Intracellular Water Content

The water content of food material exists in the form of bound water, which is combined with surrounding intracellular molecules, and free water, which is found between the molecules. UniCell Beans provides an enhanced capability of retaining such water content within the individual cells.

This means that, by using the UniCell Beans in bread making, an extra water content can be introduced and held in a dough and finished bread in the form of intracellular water in addition to the usually required amount of water for making the dough. This intracellular water content and moisture-retaining capability of the UniCell Beans serve to produce soft bread and preserve its softness for a prolonged period of time. This, of course, is good news for the bread-making industry. Potential application of the "UniCell" technology is not limited to the bread-making industry, however. The UniCell's outstanding moisture-retaining capability will find its applications in a wide range of food industries.

In our example of bread making, if 3% by weigh of UniCell Beans is added to a bread dough, the amount of added water increases by about 6%. If a finished loaf is cut into 5 slices, we add 5 beans-worth of Ohio soy to one piece of toast. There are 150 million cells, each containing intracellular water as if "capsulated" in the cells. This prolongs the softness and moistness of the bread.

Potential Applications of UniCell Beans®

1. Bread
2. Noodles (fresh noodles, dried noodles, pasta)
3. Confectionery (Japanese, Western, candies)
4. Fish paste products
5. Tofu
6. Yogurt
7. Processed meat products (pastes)

Making Bread with UniCell Beans® (Patent pending)

We have developed a new bread making method using UniCell Beans to produce better tasting bread having moisture-retaining capability.

The secret to making good bread lies in enhancing the flavor of the main ingredient, flour. This is achieved by increasing the amount of β flour starch that is turned into α flour starch. What is necessary for achieving this objective would be as follows:

1. increasing the water ratio in the dough
2. increasing the temperature at the center of the bread

The water content of bread is classified into

1. bound water, and
2. free water

Since the bound water is combined with gluten in the wheat flour, it will not evaporate easily during the baking process even in a hot oven and helps to transfer heat, increasing the temperature at the central part of the bread.

In contrast, the free water evaporates easily when heated in the oven, allowing the heat to escape from the dough. Thus, it cannot be expected to increase the temperature at the central part of the bread during the baking process.

The UniCell Beans is separated into individual cells. Each cell is surrounded by a double cell wall and a cell membrane; inside the cell, the protein bodies and lipid bodies are separately collected in sacs. The intracellular water content of soybeans is different from the earlier-mentioned bound water or free water, or from the water required in making the dough. Since the intracellular water capsulated in the cell walls and cell membranes of the soybean cells is mixed in the flour starch and gluten, it does not evaporate easily and acts as a heat transfer medium during the baking process. Thus, the intracellular water of the UniCell Beans exists as a new form of water content in a finished product and serves to increase the water content by 5 to 6% compared to the conventional product.

Effects of UniCell Beans® on Bread-Making

1. Increases water content by 5-6%.
2. Intracellular water remains in individual beans cells, without making the dough sticky.
3. Accelerates temperature increase at the central part of the bread, causing it to brown well.
4. Provides improved softness and moisture-retaining capability (plus prolonged shelf-life).
5. Eliminates the need for additives, such as yeast food and emulsifiers.
6. Good flavor of soybeans replaces powdered skim milk flavor.
7. Make is possible to produce 100% vegetarian products.

Using UniCell Beans® in Bread-Making

1. Add 3% UniCell Beans to the flour, then you have the following effects:

    1) Water content is increased by 6%.
    2) The soy flavor adds to the flavor of the bread.
    3) Extra softness and moistness prolong the shelf life.

2. There is no need for additives (yeast food and emulsifiers), and only vegetable oils are used to produce such 100% vegetarian products as follows:

    1) Water content is increased by 6%.
    2) The bread serves as a healthy alternative.
    3) The soy flavor is comparable to that of skimmed milk powder.
    4) Possible to prolong the shelf life and the softness of the product.

3. UniCell Beans can be adapted for use in other products where moistness is a criterion, such as cakes and Japanese confectionery.

000056

Considerations

In our recent testing, adding 3% UniCell Beans to flour increased the water content of bread by 6%. The dough came together better. The 6% increase in water content is supposed to have come entirely from the intracellular water of individual cells of the UniCell Beans and not from water added for making the dough.

In controlled bread-making tests, finished loaves of bread baked from the same weight of dough pieces with and without UniCell Beans were compared. The loaves containing the UniCell Beans exhibited a 1% higher yield than the loaves without the UniCell Beans in terms of finished product weight. This suggests that the intracellular water of the UniCell Beans still remained in the bread after the baking process.

Compared to ordinary bread, the bread with UniCell Beans retained its intracellular water weighing 6% of the weight of the flour, and this helped to produce softer bread with higher moisture-retaining property compared to the conventional bread-making method.

Conclusion

By adding UniCell Beans to bread we have ascertained such advantages as

1. Water content is increased.
2. Viscoelasticity of dough improves.
3. Bread browns well.
4. Flavor and taste of bread improves.

The following results were also observed. The UniCell Beans significantly enhances the emulsifying effect of lecithin in the soy. From the Brabender test, the moistness over hourly periods is noticeable, giving good data as to the softness of the bread. Without using additives, such as emulsifiers and yeast food, and considering the vegetable content and the nutritious value of soy-added bread, it is possible to make more "natural" bread with the UniCell Beans.

Finally

The soy protein that can be taken from 1 acre (4 hectares) is enough for one adult for 6 years. The equivalent area of wheat or maize supplies 2 to 2.5 years of protein, or 2 months as cattle fodder. The current issue is how soy could be used more effectively as a countermeasure for the predicted future world food shortages.

At present in order to meet beef and pork consumption volumes, soy residues are used as fodder, however the recirculation rate drops to 1/4 to 1/8 when consumed as meat.

The energy consumption conversion is often used when talking about global food shortages. This involves not only the calories we take directly from food but also indirectly from cattle and pork fodder. These calories together are known as original calories.

Based upon this calculation, if we assume that the world population would take the same calorie intake as the average American, where the meat based diet gives

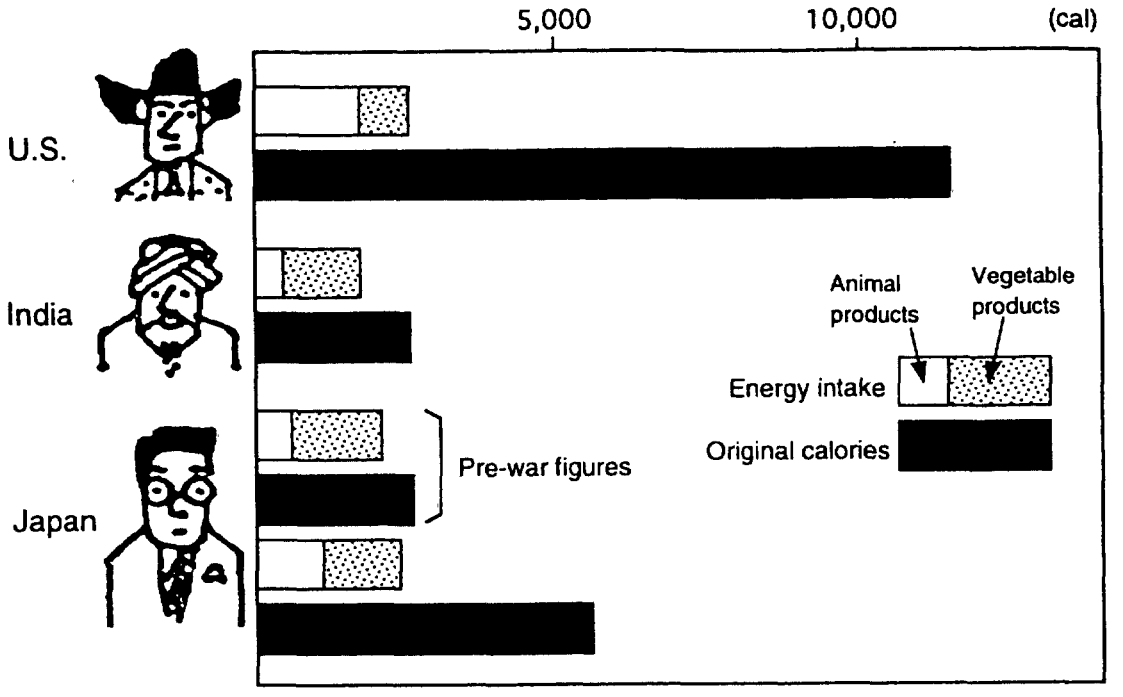


Fig. 6 Energy Used Daily per Person in Developed and Developing Countries

10,000 kilocalories a day, the world could only support 1.7 billion people.

In contrast, Indians, who have a diet based on pulses, consume only 2,500 to 3,000 kilocalories a day. The Japanese, who eat both meat and pulses, have a daily intake of 5,000 kilocalories, which is exactly the world average. Such a daily intake could support a world population of 6 billion.

One of the main reasons why the highly nutritious soy has not played a larger role before is the characteristic unpleasant smell and unpleasant taste and the fact that it cannot be used easily in food production, unless as soy protein in limited products.

The UniCell Beans uses a new manufacturing method; each of the soy cells is separated.

The double walls and membranes of the soy cells are mainly left intact, resulting in no unpleasant smell and taste and enabling the soy cells to be used in food production.

The animal and vegetable protein ratios and animal and vegetable fat ratios (P/S ratio) can improve nutrition. The intracellular water content of soy increases moistness of finished products. These two factors will enable the UniCell Beans to improve new food products and contribute to the diets of those who eat healthily. We also hope that we can play a role in the solution of world food shortages.

We believe that food is not just nutrition, but what gives people the power of life.

4

Japan
Food
Research
Laboratories

Japan Food Research Laboratories
Authorized by the Japanese Government

HEAD OFFICE : 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
OSAKA BRANCH : 3-1 Toyotsu-cho, Suita-shi, Osaka 564-0051
NAGOYA BRANCH : 5-13 Oosu 4-chome, Naka-ku, Nagoya 460-0011
KYUSHU BRANCH : 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka 812-0034
TAMA LABORATORY : 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025

REPORT

No. 200060696-002 1/4
January 8, 2002

Test of PECTINASE XP-534 Concentration for Acute Oral Toxicity in Mice

Requested by: NAGASE BIOCHEMICALS, LTD.
1-52 Osadano-cho, Fukuchiyama-shi
Kyoto 620-0853
Japan

Received: June 12, 2000

I, the undersigned, hereby declare that the work described in this report was performed under my supervision, as Study Director, in accordance with OECD Guidelines for the Testing of Chemicals 401 (1981), and that the report provides a true and accurate record of the results obtained.

This is a translation of the original report, No. 200060696-001 (July 19, 2000), written in Japanese. The translation was done as faithfully as possible to our knowledge.

Study Director

Hidetaka Sato D.V.M., Ph.D.
Department of Biological Safety Research
Japan Food Research Laboratories
Tama Laboratory

Date

Other Contributors:
Shin-ichi Katsuda D.V.M.
Sumi Fukai
Tomoko Shimazaki
Sachiko Fukuda
Miki Nishimori
Chiyomi Kubo D.V.M.

Test of PECTINASE XP-534 Concentration for Acute Oral Toxicity in Mice

1. Abstract

The test sample, PECTINASE XP-534 Concentration, was tested for the acute oral toxicity in mice in accordance with OECD Guidelines for the Testing of Chemicals 401 (1981).

Oral administration of PECTINASE XP-534 Concentration at a dose of 5,000 mg/kg b.w. caused neither abnormalities nor death in any mice.

Consequently, we concluded that the LD50 value of PECTINASE XP-534 Concentration was higher than 5,000 mg/kg b.w. in both male and female mice.

2. Test sample

PECTINASE XP-534 Concentration

Character: pale brown powder

3. Test period

From June 22 to July 19, 2000

4. Preparation of test suspension

The test sample was suspended in purified water to make a 250 mg/mL test suspension.

5. Experimental animals

Male and female mice of the ICR strain, purchased from Japan SLC, Inc., were used. The mice were obtained at an age of four weeks and acclimated to the laboratory conditions for a week. They were housed in plastic cages (five animals per cage) under the standard laboratory conditions (temperature: 23℃±2℃, light-dark cycle: 12/12 hours) and given Labo MR Stock diet [Nihon Nosankogyo K.K.] and tap water *ad libitum*.

6. Procedures

Ten each of male and female mice were allocated to each group. The mice were not fed for about four hours prior to the administration, and then each was weighed. To the mice of the experimental group, the test sample suspended in purified water was administered orally with a stomach tube at a dose of 5,000 mg/kg. To the control group, purified water alone was administered at a volume of 0.6 mL each in males and females in the same manner as described above.

Clinical observations were made frequently on the day of administration and once a day during the following period. The mice were each weighed weekly and the mean body weight values of the experimental and the control groups were statistically analyzed by the t-test (p=0.05). At the end of the test period (14 days), all mice were sacrificed for necropsy.

7. Results

1) Deaths of animals

No mice of either males or females died throughout the experimental period.

2) Clinical observations

No abnormalities were observed in either males or females throughout the experimental period.

3) Body-weight changes (Tables 1 and 2)

No significant difference in the body weight gain between the experimental and the control groups was detected in either males or females.

4) Necropsy

No remarkable changes were found in any organ of either males or females.

8. Discussion

The acute oral toxicity of PECTINASE XP-534 Concentration in mice was tested in accordance with OECD Guidelines for the Testing of Chemicals 401 (1981).

The guidelines recommend that if compound-related mortality is produced at a dose of 5,000 mg/kg, a full study may need to be considered. In the present test, oral administration of 5,000 mg/kg of the test sample caused neither death nor abnormalities at necropsy in any mice.

Consequently, we concluded that the LD50 value of the test sample was higher than 5,000 mg/kg.

Table 1. Body-weight changes after oral administration of the test sample (Male)

Group	Body weight (g)		
	Pre-administration	7 days	14 days
Experimental group	28.0±0.6 (10)	32.8±1.5 (10)	37.9±2.0 (10)
Control group	27.7±0.7 (10)	32.8±1.3 (10)	37.9±1.3 (10)

Values are mean ± SD.
Values in parentheses show the number of animals.

Table 2. Body-weight changes after oral administration of the test sample (Female)

Group	Body weight (g)		
	Pre-administration	7 days	14 days
Experimental group	25.5±1.2 (10)	28.3±2.4 (10)	31.6±2.7 (10)
Control group	25.4±1.1 (10)	27.9±1.1 (10)	31.1±2.1 (10)

Values are mean ± SD.
Values in parentheses show the number of animals.

Nagase ChemteX

January 18, 2002.

To whom it may concern

Dear Sir,

We herewith inform you that our company name has been changed from NAGASE BIOCHEMICALS, LTD. to Nagase ChemteX Corporation since April 2, 2001.

Hitoshi Morimoto
Manager
Sales & Development Dept.1
Nagase ChemteX Corporation

SUBMISSION END

000065

Reference List for Industry Submission, GRN 000114

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB Info*
00036-000041	Sakamoto, Tatsuji, Hours, Roque Alberto, Sakai, Takuo	Purification, Characterization, and Production of Two Pectic Transeliminases with Protopectinase Activity from Bacillus Subtilis	1994	Biosci. Biotech. Biochem.	Vol. 58, No. 2, pp. 353-358

NA- Not applicable

AM

Japan Cellfoods Co.,Ltd.
SORIO 3-5F,2-2-1 Sakaemachi,Takarazuka,
Hyogo 665-0845,Japan
Phone:+81-797-84-0014 Fax:+81-797-84-9446

September 4, 2002

Linda S. Kahl, Ph.D.
Office of Food Additive Safety, (HFS 255)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

VIA: Facsimile
Original by Mail

Re: Clarification Amendment
GRAS Notification for Pectate Lyase Enzyme Preparation from *Bacillus subtilis*

Dear Dr. Kahl:

Japan Cellfoods Co., Ltd is submitting this letter that clarifies our original GRAS Notification dated August 16, 2002 and amends the original notice.

Japan Cellfoods Co., Ltd originally agreed that the references and other information that are the basis for this determination are available for review at our facilities in Japan, or alternatively, at the office of Phoenix Regulatory Associates, Ltd., 21525 Ridgetop Circle, Suite 240, Sterling, VA 20166. If requested, Japan Cellfoods Co., Ltd also agrees to send the underlying data and information directly to FDA.

The original signed letter and two copies will follow.

Sincerely,

Toru Akazawa, President
Japan Cellfoods Co., Ltd.

AM

Nagase ChemteX

October 4, 2002

Penelope Rice
Office of Food Additive Safety, (HFS 255)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

VIA: Facsimile
Original by Mail

Re: Clarification Amendment 02, GRN 114
GRAS Notification for Pectate Lyase Enzyme Preparation from *Bacillus subtilis*

Dear Ms. Rice:

Nagase ChemteX Corporation is submitting this letter that clarifies the GRAS Notification No. 114 originally submitted by Japan Cellfoods Co., Ltd and amends the original notice.

This letter confirms that the pectate lyase enzyme preparations derived from *Bacillus subtilis* are manufactured by Nagase ChemteX Corporation in accordance with good manufacturing practice and meet the general and additional requirements listed for enzyme preparations in Food Chemicals Codex, Fourth Edition (pp. 129-134).

The original signed letter and two copies will follow.

Sincerely,

Hiroji Ohshima
Quality Control Section
Nagase ChemteX Corporation

cc: Japan Cellfoods Co. Ltd.

000069

AM

PHOENIX
REGULATORY ASSOCIATES, LTD.
Washington DC Headquarters
21525 Ridgetop Circle
Suite 240
Sterling, Virginia 20166
European Office: Dorset, England

December 4, 2002

Penelope Rice
Office of Food Additive Safety, (HFS 255)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

VIA: Facsimile
Original by Mail

Re: GRN 114, Pectate Lyase Enzyme Preparation from *Bacillus subtilis*

Dear Ms. Rice:

On November 26, 2002, you requested typical use levels (wt%) for pectate lyase enzyme by telephone.

The typical use level for uses other than for soy beans is 0.5-1.0%. This use level includes the use of pectate lyase enzyme in combination with other enzymes.

Sincerely,

Clyde A. Takeguchi, Ph.D.
Phoenix Regulatory Associates, Ltd.

000071

Phone 703-406-0906 • Facsimile 703-406-9513
http://www.phoenixrising.com • email: phoenix@phoenixrising.com