DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

Date: October 25, 2001

From: Director, Division of Standards and Labeling Regulations, Office of Nutritional Products, Labeling and Dietary Supplements, HFS-820

Subject: 75-Day Premarket Notification for New Dietary Ingredients

To: Dockets Management Branch, HFA-305

2001 OCT 29 P2:55

New Dietary Ingredient:	Kombu Fucoidan
Firm:	Takara Shuzo Co., Ltd.
Date Received by FDA:	July 16, 2001
90-Day Date:	October 14, 2001

We are providing Dockets with the attached copies of both the 75-day premarket notification on the aforementioned new dietary ingredient and related FDA correspondence. In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, please place these documents on public display in docket number 95S-0316. Thank you for your assistance.

Shellee Anderson
for Felicia B. Satchell

Attachments

95S-0316

RPT98

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington, DC 20204

SEP 28 2001

Ikunoshin Kato, Ph.D.
Executive Vice President
President, Biomedical Group
Director, Biotechnological Research Laboratories
Takara Shuzo Co., Ltd.
Seta 3-4-1, Otsu
Shiga 520-2193, Japan

Dear Dr. Kato:

This is in response to your letter, dated July 9, 2001, to the Food and Drug Administration (FDA), concerning your intent to market as a new dietary ingredient "Kombu Fucoidan," which is an extract of the brown seaweed *Kjellmaniella crassifolia*. This letter addresses the requirements for marketing a dietary supplement containing Kombu Fucoidan when accompanied by certain claims.

In accordance with 21 U.S.C. 350b(a)(2) [section 413(a)(2) of the Federal Food, Drug and Cosmetic Act (the Act)], you submitted to FDA a 75-day premarket new dietary ingredient notification that was received and filed by us on July 16, 2001. The law requires that for 75 days after FDA receives this notification (i.e., after September 29, 2001), the manufacturer or distributor cannot introduce or deliver for introduction into interstate commerce a dietary supplement containing the new dietary ingredient.

FDA has determined that your notification meets the minimum requirements specified in 21 CFR §190.6. These requirements include providing FDA information on the basis for which you concluded that a dietary supplement containing Kombu Fucoidan is reasonably expected to be safe when used under the conditions recommended or suggested in the product's labeling.

Other information in your notification implies that you intend to make certain claims about a dietary supplement containing Kombu Fucoidan, such as those cited below:

- "...can suppress the over-production of IgE which causes allergy, through enhancement of the production of IL-12 by lymphocytes."
- "...has activities to increase the production of Interleukin-12 and Interferon-γ and to decrease the production of IgE that possibly cause allergy reaction."

21 U.S.C. 343(r)(6) [section 403(r)(6) of the Act] makes clear that a statement included in the labeling of a dietary supplement may not claim to diagnose, treat, cure, or prevent a specific disease or class of diseases. The two bulleted statements above suggest that a dietary supplement containing Kombu Fucoidan is intended to treat or prevent a specific disease or class of diseases.

If you use these claims, Kombu Fucoidan would be represented as a drug within the meaning of 21 U.S.C. 321(g)(1)(B) [section 201(g)(1) of the Act], it would be subject to regulation under the drug provisions of the Act, and it could not be marketed as a dietary supplement. Therefore, if you intend to make claims of this nature, you should contact the Office of Compliance, HFD-310, Center for Drug Evaluation and Research (CDER), Food and Drug Administration, 7520 Standish Place, Rockville, Maryland 20855.

Your notification includes other statements, such as Kumbo Fucoidan "can control the balance of immune system" and "enhance the immune system in an antigen-specific manner" and "is good for health." If you intend to make claims in the labeling of a dietary supplement containing Kombu Fucoidan that the product may be used to affect the structure, function or well-being of the human body, the law also requires that you must have substantiation for the claims, they must be truthful and not misleading, and you must notify FDA about them no later than 30 days after initial marketing of the product. The following two FDA Internet Web sites provide additional details on the types of claims that are allowed for dietary supplements: http://www.cfsan.fda.gov/~dms/ds-labl.html#structure and http://www.cfsan.fda.gov/~dms/hclaims.html.

21 CFR §101.93 specifies the notification requirements on structure/function claims made for dietary supplements, which include providing FDA with the exact text of the statements you intend to use. You also are required to prominently display the following disclaimer near any such statements made on the labels or in the labeling of products: *This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.*

The 30-day post-marketing dietary supplement claims notification process is separate from the 75-day premarket new dietary ingredient notification process. Please direct any 30-day post-market notifications to the following address: Division of Compliance and Enforcement (HFS-810), Office of Nutritional Products, Labeling and Dietary Supplements, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 200 C Street S.W., Washington, D.C. 20204.

As required by 21 U.S.C. 350b(a)(2) [section 413(a)(2) of the Act], FDA will keep your 75-day premarket new dietary ingredient notification confidential for 90 days after the filing date. After October 14, 2001, the notification will be placed on public display at FDA's

Docket Management Branch in docket number 95S-0316. However, any trade secret or otherwise confidential commercial information will not be disclosed to the public.

Please contact us at (202) 205-4168, if you have any questions concerning this matter.

Sincerely yours,

Felicia B. Satchell

Felicia B. Satchell
Director
Division of Standards
and Labeling Regulations
Office of Nutritional Products, Labeling
and Dietary Supplements
Center for Food Safety
and Applied Nutrition

TAKARA SHUZO CO., LTD.
Biomedical Group
Seta 3-4-1, Otsu, Shiga, 520-2193, Japan
Phone: +81 77-543-7235
Facsimile: +81 77-543-2312

TaKaRa
BIOMEDICALS

COPY

9 July, 2001

RECEIVED
(7-16-01)

Division of Standards and Labeling Regulations
Office of Nutritional Products, Labeling, and
Dietary Supplements (HFS-820)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200C Street, SW
Washington, DC20204

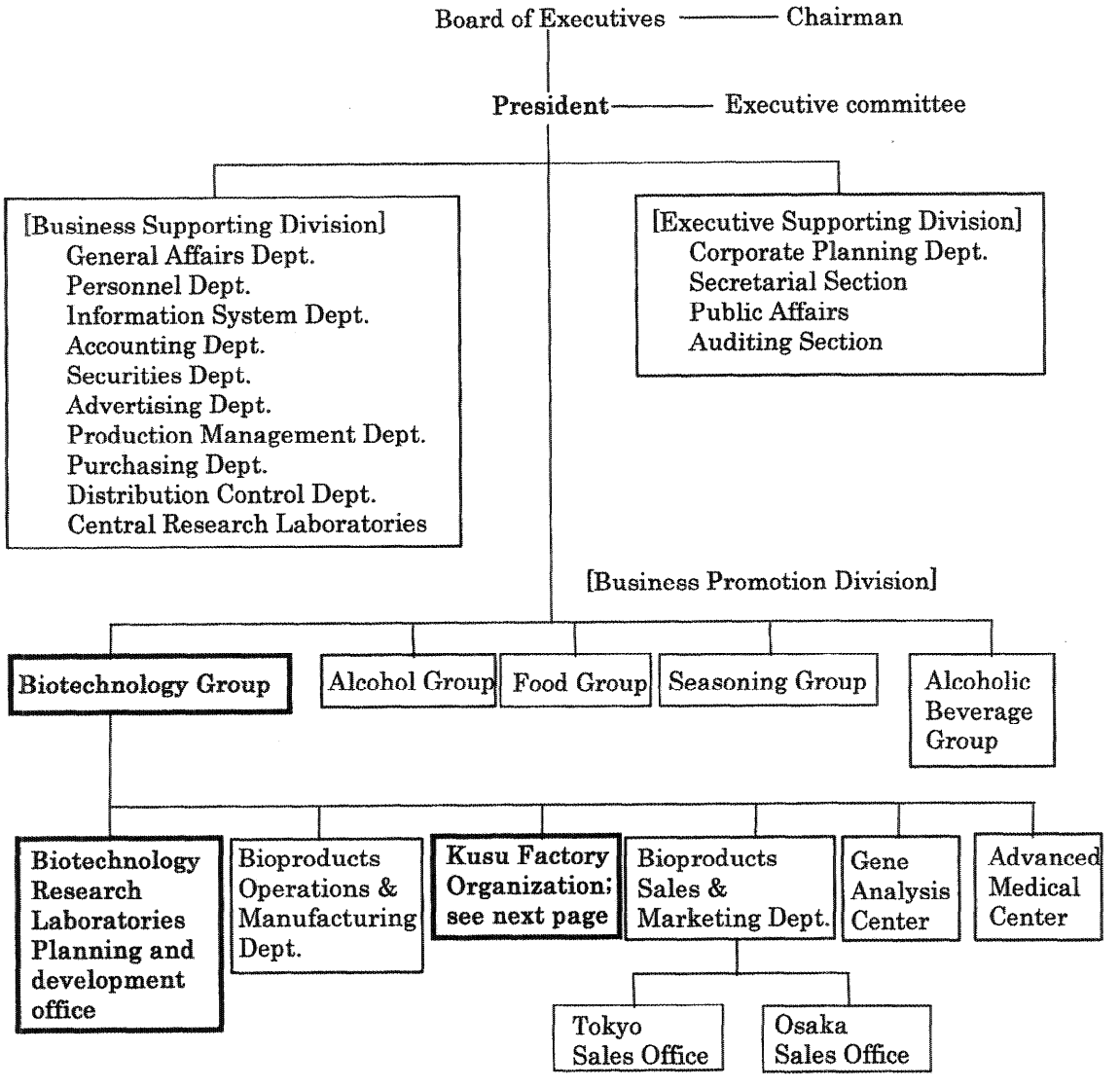
Re: Premarket Notification of New Dietary Ingredient "Kombu Fucoidan"

To Whom It May Concern:

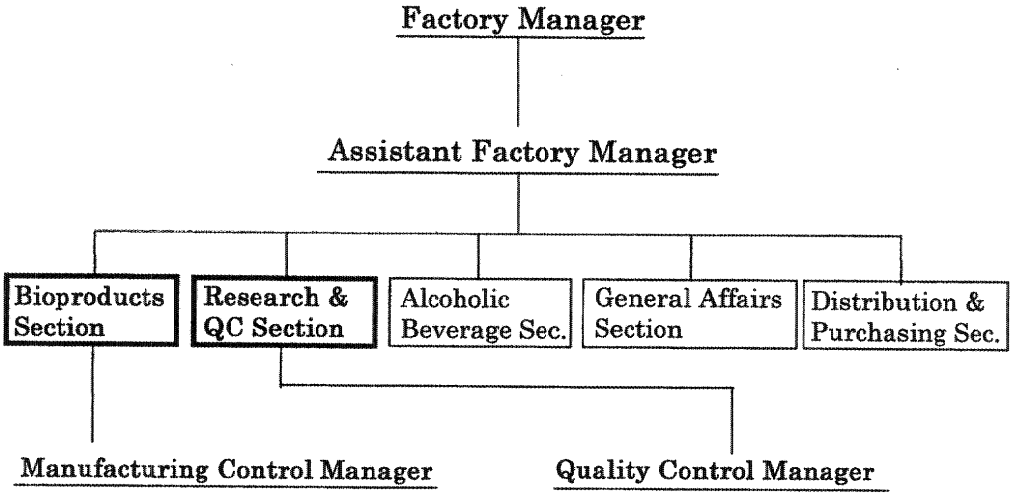
In accordance with 21CFR Section 190.6 and Section 413 of the Federal Food, Drug, and Cosmetic Act, we hereby submit our Premarket Notification for marketing of new dietary ingredient "Kombu Fucoidan".

Ikunoshin Kato, Ph.D.
Executive Vice President
President, Biomedical Group
Director, Biotechnology Research Laboratories

1.2 Organization of Takara Shuzo Co.,Ltd.



1.3 Organization of Kusu Factory of Takara Shuzo Co.,Ltd.



2. Name of New Dietary Ingredient

"Kombu Fucoidan"

3. Identity information

3.1 What is "Kombu Fucoidan"?

Edible seaweed are classified into brown, green and red seaweed. Kombu, Wakame, Hijiki and Mozuku belong to brown seaweed, which are tasty and good for health, and people have consumed such brown seaweed for more than 1,000 years[1)].

Fucoidan is sulfated polysaccharides contained in brown seaweed, and its major component is sulfated fucose. It has been considered that the natural functions of fucoidan are the protection of seaweed from drying and infection of microorganism[2)]. Brown seaweed contains 1~30% of fucoidan as dry weight content, and the Kombu contains about 5% of fucoidan. The structure of fucoidan in brown seaweed differs from species to species, but the structures of fucoidan from seaweed among *Laminariaceae* are similar to each other.

Kombu has been used as a medicine for the treatment of cancer for a long time in China[3)], and researchers have been studied to identify the anti-cancer substance of brown seaweed, especially Kombu.[4-8)].

We have been studying the anti-cancer activity of fucoidan extracted from Kombu, which we named "Kombu Fucoidan", since 1991. For example, we found that "Kombu Fucoidan" has an effect on elongation of survival time of rats carring azoxymethane-induced tumors[9)]. We also found that "Kombu Fucoidan" has activities to increase the production of Interleukin-12 and Interferon-γ and to decrease the production of IgE that possibly cause allergy reaction. From the results, it was elucidated that "Kombu Fucoidan" was a novel substance that control the balance of immuno system[11)].

3.2 Production of "Kombu Fucoidan"

We use Gagome Kombu (*Kjellmaniella crassifolia*), which is harvested more than 2000 t (dry weight) a year in Hokkaido. Gagome Kombu contains larger amount of "Kombu Fucoidan" than other species of Kombu.

In order to produce "Kombu Fucoidan", dried Gagome Kombu was

Finally, "Kombu Fucoidan" was freeze-dried and prepared as uniform powder[1)].

Iodine and sodium chloride, of which excessive uptake is undesirable, were removed

3.3 Physical properties of "Kombu Fucoidan"

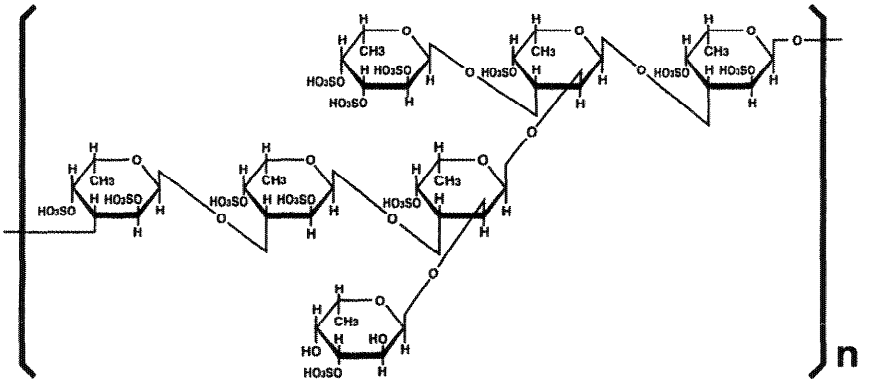
"Kombu Fucoidan" is a dietary fiber and its calorie is almost zero. Since calcium chloride is used for its extraction, calcium ions are contained as the counter ion of sulfuric acid residues of "Kombu Fucoidan". The molecular weight of "Kombu Fucoidan" is about 200,000. It is degraded into small molecules at a high temperature under acidic condition.[1, 10)].

3.4 Structure of "Kombu Fucoidan"

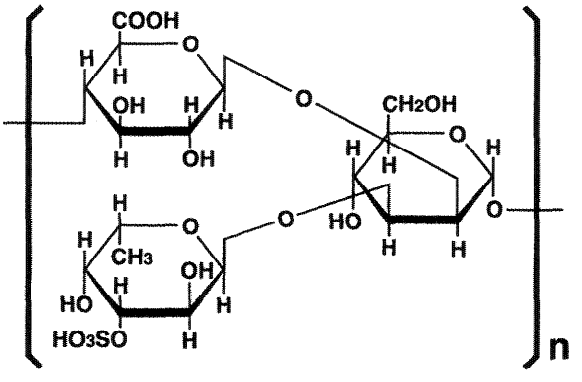
We have discovered several fucoidan-degrading enzymes from marine bacteria. We used these enzymes in the structural analyses of "Kombu Fucoidan". We elucidated the structure of 3 kinds of "Kombu Fucoidan", F-Fucoidan, U-Fucoidan and

G-Fucoidan, by site-specific enzymatic cleavage and several instrumental analyses (NMR, Mass spectrometer etc.) [11] (Fig. 1). As the component, F-Fucoidan has sulfate and fucose, U-Fucoidan has sulfate, fucose, mannose and glucuronic acid, and G-Fucoidan has sulfate, fucose and galactose.

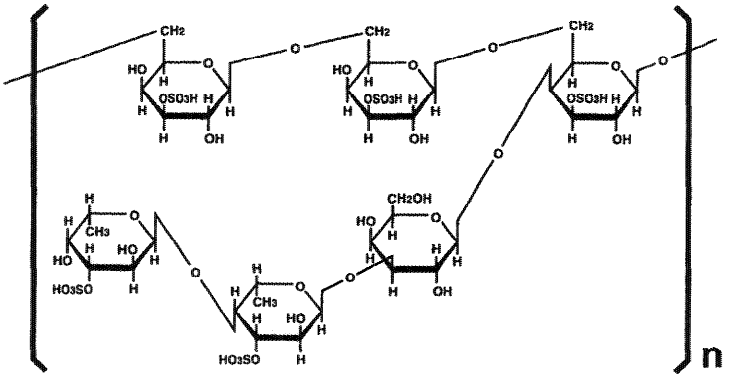
There have not been any studies of absolute structure of fucoidan (including "Kombu Fucoidan") so far. We have elucidated the absolute structure of three molecules of "Kombu Fucoidan" at the first time.



F-Fucoidan



U-Fucoidan



G-Fucoidan

Fig.1 Structures of 3 kinds of Kombu Fucoidan

4. Safety information

4.1 Eating experience of Kombu in Japan

According to the statistics of the General Affairs Agency in 1994, the annual consumption of Kombu of the capital cities in Japan was 265-1,147g per family (national mean was 544 g). Since Kombu is usually taken as processed food such as snacks and side dishes in Japan, an amount of Kombu consumption should be much larger than above statistics.

As about 5% of dried Kombu is "Kombu Fucoidan", the safety of Kombu will directly reflect the safety of "Kombu Fucoidan".

In Japan, Kombu has been considered to be good for health and consumed for more than 1,000 years.

4.2 Marketing experience of the product containing "Kombu Fucoidan"

We have been selling the bottled health drink , named "APOIDAN-U" which contains about 200mg of "Kombu Fucoidan", since 1996. 4,000,000 bottles of "APOIDAN-U" have been sold out so far (for about 4 years), and we have not heard any complaints about this product from consumers. Although we recommend to take one bottle of "APOIDAN-U" a day, some people take more than 3 bottles, and there have not been also any complaints from such consumers.

These results strongly suggest that "Kombu Fucoidan" is a safe food ingredient.

4.3 Bacterial reverse mutation tests of "Kombu Fucoidan"

The mutagenicity of "Kombu Fucoidan" was examined by using histidine requiring tester strains of TA98, TA100, TA1535 and TA1537 (*Salmonella typhimurium)* and the

tryptophan requiring strain of WP2uvrA (*Escherichia coli),* either in the presence or absence of metabolic activation systems.

The test was conducted at the dose of 312.5, 625, 1,250 and 5,000 μg "Kombu Fucoidan" per plate. As a result, the numbers of revertant colonies in the test substance treated plates of the tester strains were not increased at least twice the concurrent negative controls in a dose-dependent manner. Further, bactericidal effect and precipitation of the test substance were not noted.

The results concluded that "Kombu Fucoidan" had no mutagenicity under this experimental condition. (See attached document 8.1).

4.4 Acute oral toxicity test of "Kombu Fucoidan"

A group of ten SD rats (five males and five females) received a single oral dose of "Kombu Fucoidan" at 0 (control) and 2000mg/kg by using a stomach sonde, in order to observe the signs of toxicity for 14days and estimate LD_{50} value. (See attached document 8.2)

1) Clinical signs and mortality

There was no death and no abnormality in any animal throughout the study.

2) Body weights

No differences in mean body weight on days 2,3,5,8 and 15 after administration were observed between the control and "Kombu Fucoidan" groups.

3) Macroscopic findings

No treatment-related changes was noted.

4) LD_{50} value

The LD_{50} value of "Kombu Fucoidan" was estimated to be greater than 2000mg/kg.

4.5 Chronic oral toxicity test of "Kombu Fucoidan"

A group of ten ICR mice was kept in a plastic cage with free access for "Kombu Fucoidan" (2mg/ml in water) or tap water (control) as the sole drinking water, in order to observe the signs of toxicity for 34 weeks, change of body weight, and abnormality in internal organs.

1) Clinical signs and mortality

There was no death and no abnormality in any animal throughout the study.

2) Body weight

There was no difference between the average body weights of the "Kombu Fucoidan" group and control group throughout the study (Fig.2).

3) Abnormality in internal organs

There was no abnormality in internal organs in any animal of "Kombu Fucoidan" group (Fig.3).

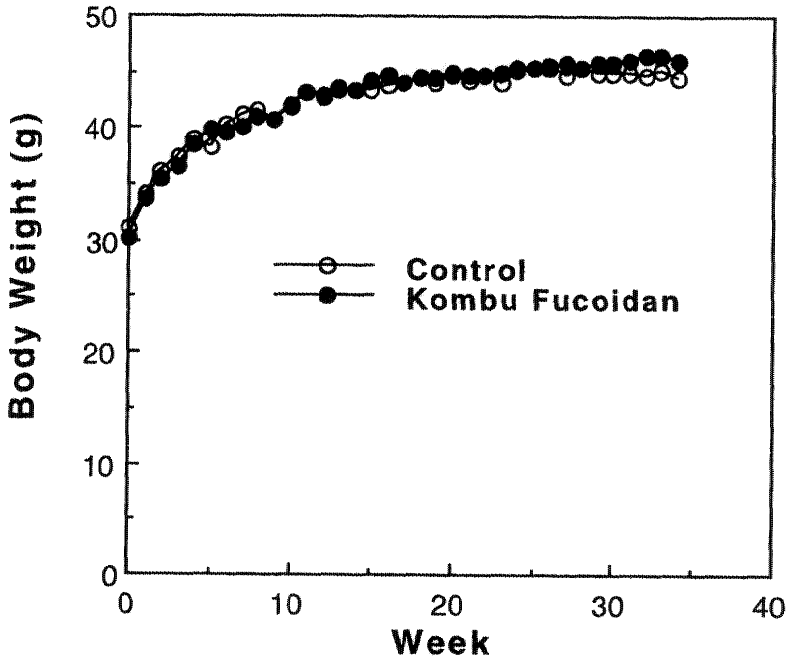


Fig.2 Variation of body weight of mice

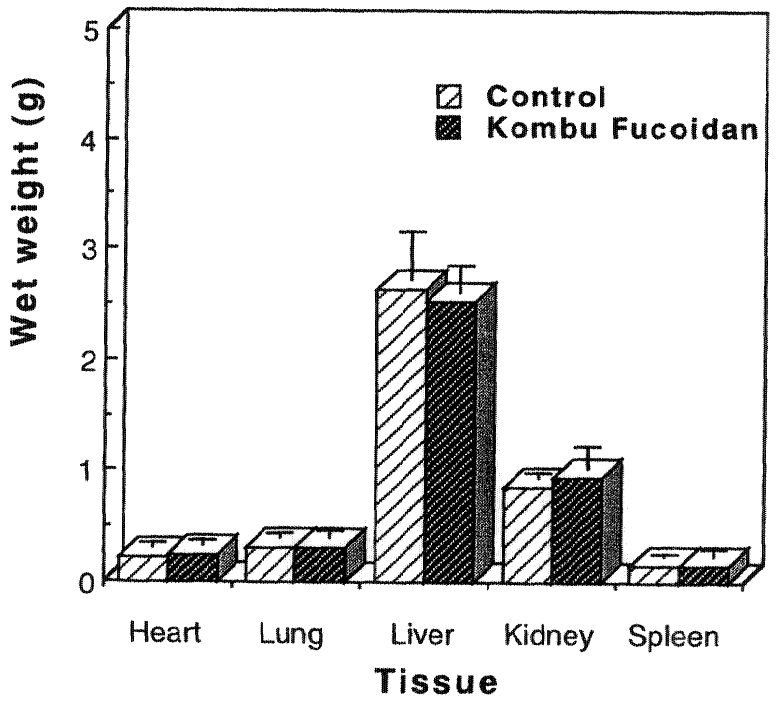


Fig.3 Wet weight of internal organs of mice fed with or without Kombu fucoidan

5. Efficacy information

5.1 Effects of "Kombu Fucoidan"

"Kombu Fucoidan" is a novel substance that can "control the balance of immune system". "Kombu Fucoidan" shows activity to enhance the immune system in an antigen-specific manner. On the other hand, "Kombu Fucoidan" suppresses the over-production of IgE that causes allergic reactions. The details are shown as follows.

5.2 Enhancement of the immune system by "Kombu Fucoidan" ("Kombu Fucoidan" enhances the production of Interleukin-12 and Interferon-γ [11].)

Interleukin-12 (IL-12) was discovered as a cytokine that induces the production of Interferon-γ (IFN-γ). By the recent studies, IL-12 was found to increase the cytotoxicity of cytotoxic T lymphocytes (CTL) and natural killer cells(NK cells). Therefore, it is expected that IL-12 will be effective on the patients with immunodeficiency such as AIDS and the patients with cancer.

IFN-γ was discovered as a lymphokine showing an anti-viral activity. IFN-γ, which is produced by Th1 cells and NK cells, shows various activities such as anti-virus, anti-cancer, activation of macrophage and NK cell. By these activities, IFN-γ is able to play a pivotal role in immune response in patients with cancers and infectious diseases, and is currently used in the clinical treatment for chronic hepatitis C and gastric cancer.

We discovered that the production of such important immunoenhancers, IL-12 and IFN-γ, is induced by "Kombu Fucoidan" in an antigen-specific manner. We prepared mice with tumor of mouse sarcoma Meth-A and used lymphocytes of spleens from these mice to examine the effect of "Kombu Fucoidan" on the production of IL-12

and IFN-γ. "Kombu Fucoidan" induced significantly the production of IL-12 and IFN-γ by the lymphocytes cultured in the presence of Meth-A cells, but not in the absence. This effect is dose-dependent. Among the other fucoidans, fucoidan from Hibamata (*Fucus vesiculosus*) showed the similar effect, but fucoidan from Wakame (*Undaria pinnatifida*) and Okinawamozuku (*Cladosiphon okamuranus*) have less effect and fucoidan from Mozuku (*Nemacystus decipiens*) has no effect. These results demonstrate that only fucoidans with appropriate chemical structures show the induction activity of IL-12 and IFN-γ.

Enhancement of the production of IL-12 and IFN-γ by "Kombu Fucoidan" never occurs in the absence of cancer cells or virus-infected cells in the body. An antibody that inhibits the signal transduction between antigen-presenting cells and T cells suppressed the induction activity of "Kombu Fucoidan", and an antibody to IL-12 reduced the enhancement of IFN-γ production to about 50%. These results indicate that the induction activity of "Kombu Fucoidan" is dependent on the signal transduction between antigen-presenting cells and T cells.

It is recognized that the words of immunopotentiation or immunoenhancement are generally used for the functional ingredients with a non-specific enhancement of immune system. Non-specific enhancement of immune system may cause diseases such as allergy and an autoimmune disease. On the contrary, we show that the production of IL-12 and IFN-γ by "Kombu Fucoidan" is enhanced in an antigen-specific manner.

5.3 Suppression of the immune system by "Kombu Fucoidn" ("Kombu Fucoidan" suppresses the over-production of IgE that causes allergic reaction[11].)

The over-production of IgE occurs via activation of cellular immunity resulting in allergy like pollinosis. IFN-γ and IL-12 suppress the over-production of IgE by inhibition of the cellular immunity. In our experiment, mice were immunized with ovalbumin as an allergen and used to examine the effect of "Kombu Fucoidan" on the allergic state. "Kombu Fucoidan" reduced the IgE level in cases when it was orally administered for prophylactic and therapeutic treatment.

Together with the above results, we demonstrated that "Kombu Fucoidan" can suppress the over-production of IgE which causes allergy, through enhancement of the production of IL-12 by lymphocytes.

5.4 Conclusion

These findings suggested that "Kombu Fucoidan" is a useful novel dietary ingredient that can "control the balance of immune system".

We considered that the enhancement of immune system (induction of IL-12 and IFN-γ) by "Kombu Fucoidan" must be closely related to so-called "Kombu is good for health" in Japan and China.

References

1) T. Sakai & I. Kato, Food Chemical Monthly Dec, pp66-71 (1999)

2) K. Nishizawa & Y. Sugimura, The Book of Seaweed, pp47-52, Kenseisha (1988)

3) Wang Jier Ue, Encyclopedia of Chinese Medicinal Food, Dalian Publishing Company (1992)

4) T. Usui, K. Asari & T. Mizuno, Agric. Biol.Chem., 44. 1965-1966(1980)

5) H. Maruyama & I. Yamamoto, Hydrobiologia, 116／117, 534－536(1984)

6) N. I. Mizui, M. Fujihara, J. Himeno, K. Komiyama, I. Umezawa & T. Nagumo, Kitasato Arch. Exp. Med., 58, 59－71(1985)

7) M. Ellouali, C. Boisson-Vidal, P. Durand & J. Jozefonvicz, Anticancer Res.,13, 2011-2019(1993)

8) C. Zhuang, H. Itoh, T. Mizuno & H. Ito, Biosci. Biotech. Biochem., 59, 563-567 (1995)

9) Fu-gong Yu et al., Proceedings of 57th Annual Meeting of the Japanese Cancer Association, p645 (1998)

10) T. Sakai & I. Kato, Food and Science, No. 6, pp89-93 (1998)

11) I. Kato, T. Sakai & H. Sagawa, Japan Food Science, vol. 39, No. 9, pp43-47 (2000)

12) T. Sakai, H. Kimura, K. Katayama, K. Shimanaka, K. Ikai & I. Kato, Glycoconjugate J., 16, S122(1999)

13) T. Sakai, H. Kimura, K. Kojima, S. Nakayama, K. Katayama, Y. Nakanishi, K. Ikai & I. Kato, Abstracts of XⅧth Jap. Carbohydr. Symp., 159(1996)

14) T. Sakai & I. Kato, New Food Industry, vol. 43, No.2, pp8-12 (2001)

6. Use and application information

6.1 "Kombu Fucoidan" quality standards

Appearance	
Content of dietary fiber	
water	
pH (1%solution)	
Viscosity (1%solution)	
Content of ash	
Arrhenic	
Heavy metals	
General bacterium	
A group of *Esherichia coli*	

6.2 How to use "Kombu Fucoidan"

We recommend to take a product (Drink or Granules) containing 200mg "Kombu Fucoidan" in a day.

6.3 Efficacy of "Kombu Fucoidan"

"Kombu Fucoidan" has activities to increase the production of Interleukin-12 and Interferon-γ and to decrease the over-production of IgE that possibly cause allergy reaction. "Kombu Fucoidan" is a useful novel dietary ingredient that can "control the balance of immune system". We considered that the enhancement of immune system (induction of IL-12 and IFN-γ) by "Kombu Fucoidan" must be closely related to so-called "Kombu is good for health" in Japan and China.

7. Labeling information

Products by use of "Kombu Fucoidan"

Fucoidan Drink "KANPAI ICIBAN"

Net content : 100ml in brown bottle
Packaging : 30 bottles per carton
(One carton holds three cardboard boxes containing 10 bottles)

< Ingredients >

① "Kombu Fucoidan"
②
③
④
⑤
⑥
⑦
⑧

< Nutrition Facts >

Values in one serving size 1 bottle (100ml)

Energy	:	4	kilo calories
Protein	:	0.1	g
Lipids	:	0	g
Saccharides	:	1	g
Vitamin C	:	7	mg
Sodium	:	16	mg
Iodine	:	0	mg

< Description of the product >

This product is a drink containing seaweed dietary fiber "Kombu Fucoidan" extracted from natural Gagome Kombu, which is good for health.
A bottle of the product contains 100mg of "Kombu Fucoidan" .
"Kombu Fucoidan" is a useful novel dietary ingredient that can "control the balance of immue system".

< Instruction >

- We recommend to take two bottles a day .

< Caution >

- It might cause loose bowels in case too much drink .

< Preservation >

- It should not keep in direct sunshine, at a high temperature and frozen temperature.
- It is not a problem to be turbid or precipitated during preservation.

Final Report

Title : Bacterial reverse mutation test of Kombu Fucoidan

PROJECT No. H-00265

Nippon Experimental Medical Research Institute Co., Ltd

3303-58 Ohaza Ohdo, Agatsuma machi, Agatsuma gun, Gunma-ken.

Date of reporting: December 15, 2000.

STATEMENT OF COMPLIANCE

Title : Bacterial reverse mutation test of Kombu Fucoidan

PROJECT No. H-00265

I, the undersigned, hereby declare that this report is the final English version of the original report that has written in Japanese language. Further, I declare that there is no adverse affect on the quality or integrity of the study or the interpretation of the results due to translation of the report.

M. Kashima Date: May. 8, 2001

Masaaki Kashima, D.V.M.
Managing Director
Nippon Experimental Medical Research Institute Co., Ltd.

STATEMENT OF COMPLIANCE

Title : Bacterial reverse mutation test of Kombu Fucoidan

PROJECT No. H-00265

I, the undersigned, hereby declare that this report is the English version of the original report that has written in Japanese language. Further, I declare that the data are exactly reflected in this report and similar to that of the original (Japanese) report.

Date: May 8, 2001

Golam Sarwar, Ph.D.
Study Director (translator)
Nippon Experimental Medical Research Institute Co., Ltd.

PREPARATION OF THE FINAL REPORT

Title : Bacterial reverse mutation test of Kombu Fucoidan

PROJECT No. H-00265

In this study, the Ordinance that describing the Standard for Pre-clinical Safety Studies on Drugs, Ministry of Health and Welfare, Japan (Ministry of Health and Welfare Ordinance No. 21: March 26, 1997) was followed as reference.

Further, this study has been conducted in accordance with the methods describing in this report, and the data are accurately reflected in this report.

Date: December 15, 2000.

Golam Sarwar, Ph. D. (Impression of the seal)

Study director

Nippon Experimental Medical Research Institute Co., Ltd.

Title : Bacterial reverse mutation test of Kombu Fucoidan

PROJECT No. H-00265

1. Purpose

Bacterial reverse mutation test was conducted to clarify whether Kombu Fucoidan had mutagenic potential or not.

2. Compliance with GLP

In this study, the Ordinance that describing the Standard for Pre-clinical Safety Studies on Drugs, Ministry of Health and Welfare, Japan (Ministry of Health and Welfare Ordinance No. 21: March 26, 1997) was followed as reference.

3. Compliance with Guidelines

The present study was conducted in compliance with the Guideline that describing the genotoxicity tests of drugs (Ministry of Health and Welfare Ordinance No. 1604: November 11, 1999) was followed.

4. Sponsor

Name : Takara Shuzo Co., Ltd.
Biomedical Group
Address : Seta 3-4-1, Otsu, Shiga Ken, Japan

5. Contract Laboratory

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

6. Testing Facility

Name : Haruna Laboratory
Nippon Experimental Medical Research Institute Co., Ltd.
Address : 3303-58, Ohaza Ohdo, Agatsuma machi, Agatsuma gun,
Gunma Prefecture, Japan.
Managing Director: Masaaki Kashima, D.V.M.

7. Storage of records and data

(1) Storing period

Stored for a period that described in the Drugs Act, Ministry of Health and Welfare, Japan, Ordinance No. 26-2-3, 26-5-3 and 26-12.

(2) Storing materials and place

① Protocol, records concern to the study, final report in original and raw-data are to be stored in the raw-data archive of Haruna Laboratory, Nippon Experimental Medical Research Institute Co., Ltd.

② Sample is to be stored in the material storage (No. 1) of the same facility.

③ Records are to be stored in the storage room of the same facility.

8. Schedule of the study

Initiation of the Project	:	October 18, 2000
Initiation of experiment	:	October 31, 2000
Completion of experiment	:	November 10, 2000
Draft report	:	November 22, 2000
Final report	:	December 15, 2000
Completion of the Project	:	December 15, 2000

9. Study personnel and work responsibility

Study director, protocol preparation, test substance control, supervising, management and preparation of final reporting

: Golam Sarwar
Division of Mutation Research, Nippon Experimental Medical Research Institute Co., Ltd

Test substance preparation, media & reagents preparation and experiment

: Haruki Inoue, Mutsumi Takano, Kazuko Iiduka

Colony counting : Haruki Inoue, Mutsumi Takano, Kazuko Iiduka

Data processing and judgement: Golam Sarwar, Haruki Inoue, Mutsumi Takano, Kazuko Iiduka

Unpredicted Situation Effecting The Reliability Of The Study And Deviations From The Protocol

Title：Bacterial reverse mutation test of Kombu Fucoidan
PROJECT No. H-00265

There were no unpredicted situation affecting the reliability of the study and no deviations from the protocol.

Contents

	Page
Preparation of the Final Report	1/4
Outline of the Study	2/4
Unpredicted Situation Effecting the Reliability of the Study and Deviation from the Protocol	4/4
Ⅰ. Summary	1
Ⅱ. Purpose of Study	2
Ⅲ. Materials and Methods	2
Ⅳ. Results	8
Ⅴ. Discussion and Conclusion	9
Ⅵ. References	10
Fig. 1	11
Fig. 2	12
Appendix 1	13
Appendix 2	14
Attached sheet 1	15

I. Summary

The mutagenicity of Kombu Fucoidan was examined using histidine requiring tester strains of TA 98, TA 100, TA 1535 and TA 1537 (Salmonella typhimurium) and tryptophane requiring strain of WP2uvrA (Escherichia coli) either in the presence or absence of metabolic activation systems.

The test was conducted at the doses of 312.5, 625, 1250, 2500 and 5000 μg/plate. As a result, the numbers of revertant colonies in the test substance treated plates of the tester strains were not increased at least twice the concurrent negative controls in a dose-dependent manner. Further, bactericidal effect and precipitations of the test substance were not noted.

The findings concluded that Kombu Fucoidan had no mutagenicity under this experimental condition.

Ⅱ. Purpose of Study

Bacterial reverse mutation test was conducted to clarify whether Kombu Fucoidan had mutagenic potential or not.

Ⅲ. Materials and Methods

1. Test substance

(1) Test substance name : Kombu Fucoidan
(2) Lot No. : F-9811-FD
(3) Purity : 95%
(4) Melecular weight : 200000 in average
(5) Stability : Stable in refrigerator
(6) Name of impurities : Water
(7) Appearance at ordinary temperature
: Light greenish brown powder
(8) Solubility : A 20% is soluble in water at 20°C and stable
(9) Storing condition : Under refrigeration
(10) Producing date : May 23, 1998
(11) Validity period : 3 years from the date of production (2001. 5. 22)
(12) Supplier
Name : Takara Shuzo Co., Ltd.
Biomedical Group
Address : Seta 3-4-1, Otsu, Shiga Ken, Japan
(13) Disposal : After completion of the study, the test substance is to be forwarded to the sponsor being preserved a little portion.

2. Controls

(1) Negative control

Pure water (medicinal grade, Lot No. 99H27A, Fuso Pharmaceutical Industries Ltd) was used.

(2) Positive control

The substances of 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2, Lot No.

PAE1151, purity:98.7%), Sodium azide (SA, Lot No. ACK5372, purity:91.5%) and 2-Aminoanthracene (2-AA, Lot No. DSJ3206, purity:96.5%) of Wako Pure Chemical Industries Ltd and 9-Aminoacridine (9-AA, Lot No. 106F06681, purity:98.0%) of Sigma Chemical Co., Ltd were used

3. Preparation of test substance

(1) The solvent and the reason of it's selection

According to the sponsor, 20% of the test substance was found soluble and stable in water. Based on solubility and stability, pure water was used as solvent in the test.

(2) Preparation of test solution

To prepare the highest dosing mixture, 264 mg of test substance was weight and match up in 5 mL of pure water (medicinal grade, Lot No. 99H27A, Fuso Pharmaceutical Industries Ltd). The lower doses were prepared by serial dilution with the solvent. They were prepared just before use.

In soluble condition, the test substance was stable as temperature formation, foaming and coloration were not detected when checked macroscopically. Further, homogeneity was confirmed macroscopically.

4. Preparation of control substance

(1) Negative control

The solvent that used for the preparation of test substance was used as negative control.

(2) Positive control

The substances of AF-2, 9-AA and 2-AA were dissolved separately in DMSO (special grade, Lot No. ELP3545, Wako Pure Chemical Industries Ltd) and SA was dissolved in pure water (medicinal grade, Lot No. 98F22A, Fuso Pharmaceutical Industries Ltd) to prepare the required concentrations, distributed, stored at below -80℃ in a deep freezer and used after thawing.

5. Tester strains

(1) Procurement

The tester strains of TA 98, TA 100, TA1535 and TA 1537 of Salmonella

typhimurium and WP2uvrA of Escherichia coli were obtained from Japan Bioassay Laboratory (2445 Hirasawa, Hadano shi, Kanagawa Ken). Upon receipt, they were subcultured for 8 hr, in each culture DMSO was added (8:0.7), distributed and stored at below -80℃ in a deep freezer until use. The stock cultures that confirmed for genotypes were used.

(2) Subculturing

A 24 μL of frozen stock (being thawed) was transferred aseptically to 12 mL of freshly prepared Nutrient broth No. 2 (Lot No 028 59355, Oxoid) and subcultured at 37℃ with agitation (80 times/min) for 10 hr in a water bath shaker (L-10, Taitec Ltd) with a program unit (PU-9, Taitec). Then, optical density (OD) of each culture was measured at 660 nm using a spectrophotometer (Fuji Kogyo Co., Ltd), confirmed the cell density and used.

6. Media

(1) Nutrient broth (NB)

A 2.5% solution of NB No. 2 (Oxoid) was prepared in pure water, pipetted into L-shape test tube (15×80×180 mm) at a volume of 12 mL per tube and autoclaved.

(2) Minimal glucose agar plates

They were purchased commercially as Vital medium AMT-0 (Lot No. DZA19E01) from Kyokuto Pharmaceutical Co., Ltd and used.

(3) Identification of plates

Each of the plate was identified by writing the serial number in the top of the lid and the lower part.

(4) Top agar

Top agar that used for Salmonella strains was prepared by mixing pre-autoclaved soft agar (consisting of 0.6% Bacto agar, Lot No. 139900XA, Difco Laboratories and 0.5% NaCl) with a solution of 0.5mM L-histidine (Lot No. 11H0197, Sigma Chemical Co. Ltd)-0.5mM D-biotin (120H0305, Sigma Chemical Co. Ltd) at a ratio of 10:1. Similarly, soft agar was mixed with 0.5 mM L-tryptophane (Lot No. DSG 2309, Wako Pure Chemical Industries Ltd) for Escherichia coli.

7. Rat liver homogenate (S9) and S9 mix

(1) Source of S9

The S9 (Lot No. RAA-433) of Sprague-Dawley male rats that induced by Phenobarbital and 5,6-Benzoflavone was purchased from Kikkoman Corporation, Japan, stored at below -80℃ in a deep freezer and used after thawing.

(2) Preparation of S9 mix

The mixture of each mL consisting of 0.1 mL S9, 4 μmol NADPH (Lot No.050006), 4 μmol NADH (Lot No. 010034), 5 μmol glucose-6-phosphate (Lot No. 115001) of Oriental Yeast Co., Ltd., 33 μmol KCl, 8 μmol $MgCl_2$, 100 μmol sodium phosphate buffer (pH 7.4) and adjusted to 1 mL by adding pure water. The mixture was prepared prior to use and kept in ice-cold water bath during use.

8. Doses

The doses of 5, 10, 50, 100, 500, 1000 and 5000 μg/plate were used in dose determination test. Whereas the doses of 312.5, 625, 1250, 2500 and 5000 μg/plate were used in mutagenicity test.

9. Experimental procedure

The dose determination and mutagenicity tests were carried out in pre-incubation method[1,2] either in the presence or absence of metabolic activation system.

10. Experimental condition

(1) Treatment

A 0.5 mL of S9 mix or phosphate buffer(pH 7.4), 0.1 mL of each concentration of test substance or negative or positive control, 0.1 mL of each tester strain were taken into heat sterilized glass tube (13×100 mm), mixed and agitated for 20 min at 37℃ in water bath shaker (Iwaki Glass). After that, 2 ml of molten top agar of 45℃ was taken into each tube, mixed and plated by pouring into pre-numbered minimal glucose agar plate. Duplicate plates were used for each dose in dose determination and mutagenicity tests except for the negative control of mutagenicity test in which triplicate plates were used.

(2) Used amount of positive control in each tester strain

Tester strains	Without metabolic activation (-S9)		With metabolic activation (+S9)	
	Substances	Dose(μg/plate)	Substances	Dose(μg/plate)
Salmonella Typhimurium				
TA 98	AF-2	0.1	2-AA	0.5
TA 100	AF-2	0.01	2-AA	1.0
TA 1535	SA	0.5	2-AA	2.0
TA 1537	9-AA	80.0	2-AA	2.0
Escherichia coli				
WP2uvrA	AF-2	0.01	2-AA	10.0

(3) Sterility test

The test substance of highest dose and S9 mix were plated for sterility test.

(4) Incubation

After solidification of top agar, plates were inverted and incubated at 37℃ for 48 hr in an incubator (MFR-116S, Isuzu Industries Ltd).

11. Colony count and judgment

(1) Colony count

After completion of incubation, the colonies of all plates except for positive control plates were counted manually. Each of the positive control plate was counted three times (by rotating at an angle of 120°) using an auto colony counter (Olympus OL-502A, Yoshikawa Industries Ltd) and average of such counts was expressed as revertant colonies per plate. The mean number of colonies in duplicate or triplicate plates was expressed as revertant colonies per dose.

(2) Observation of background

During colony count, bacterial growth inhibition (bactericidal effect) of the test substance was determined from the background lawn under stereozoom microscope (CSZ, Uchida-Yoko Ltd) and at the same time precipitates of the test substance was also determined.

(3) Judgment of the result

The test substance was judged positive (+) when the number of revertant colonies in the test substance treated plates increased dose dependently and became 2-fold compared to that of the negative control and this effect was reasonably reproducible and the others were judged negative (-). Bacterial growth inhibition (bactericidal effect) was judged when the lawn of a test plate was sparse or thin compared to that of the negative control plate.

12. Statistical evaluation

The statistical analysis was not done for the judgment.

IV. Results

A dose selection test was conducted at the doses of 5, 10, 50, 100, 500, 1000 and 5000 μg/plate either in the presence or absence of metabolic activation systems to find out a dose at which the test substance inhibited bacterial growth and caused precipitations. In the test, bacterial growth inhibition (bactericidal effect) and precipitations of the test substance were not noted (Appendix 1).

Due to such results, a total of 5 doses considering 5000 μg/plate as highest and four more lower doses of 2500, 1250, 625 and 312.5 at a nominal ration of 2 were selected and used in the mutagenicity test in either systems.

As a result, the number of revertant colonies in the test substance treated plates of all tester strains were not increased dose dependently and not became 2-fold compared to that of the negative control of each tester strain. Further, bactericidal effect and precipitations of the test substance were not noted (Fig 1&2, Appendix 2).

The positive controls of all tester strains showed marked increase in the number of revertant colonies compared to that of the corresponding negative control of each tester strain.

V. Discussion and Conclusion

The mutagenicity of Kombu Fucoidan was examined either in the presence or absence of metabolic activation systems using the tester strains of TA 98, TA 100, TA 1535, TA 1537 of Salmonella typhimurium and the strain of WP2uvrA of Escherichia coli.

At first, a dose selection test was conducted at the doses of 5～5000 μg/plate. As a result of the test, bactericidal effect and precipitations of the test substance were not noted. Due to such findings, a dose of 5000 μg/plate as highest and four more lower doses at a nominal ratio of 2 (total: 5 doses) were selected and used in the mutagenicity test.

In the test, the numbers of revertant colonies in the test substance treated plates of all tester strains were not increased dose dependently and not became 2-fold compared to that of the negative control of each tester strain. Further, bactericidal effect and precipitations of the test substance were not noted.

The numbers of revertant colonies in the negative and positive controls of all tester strains were within mean ± 2SD of the background data (Attached sheet 1) which demonstrated that the study was conducted appropriately.

Contamination was not noted in the sterility tests that conducted during dose selection and mutagenicity tests.

According to the findings of this experimental condition Kombu Fucoidan was concluded as non-mutagen.

VI. References

1) Maron, D.M., and Ames B.N. : Revised methods for the Salmonella mutagenicity test, Mutation Res., 113, 173 - 215, 1983

2) Yahagi, T., Nagano, M., Seino, Y., Matsushima, T., and Okada, M.,: Mutagenicities of N-nitosamines on Salmonella, Mutation Res., 48, 121 – 130, 1977.

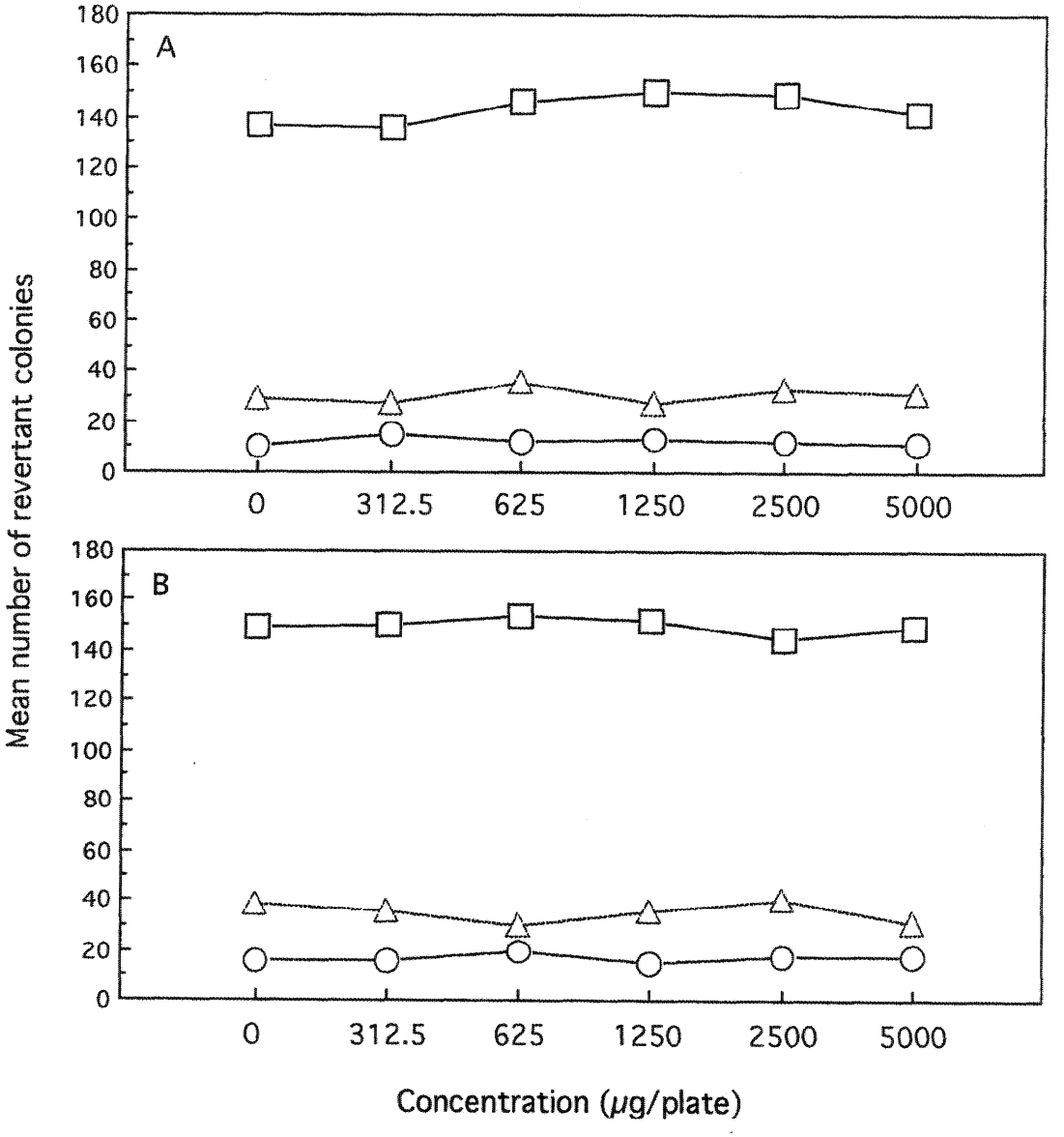


Fig. 1. Dose depending curve of Kombu Fucoidan (Mutagenicity test)

A : Without metabolic activation system (-S9)
B : With metabolic activation system (+S9)

□ : TA100 ; ○ : TA1535; △ : WP2uvrA

PROJECT No. H-00265

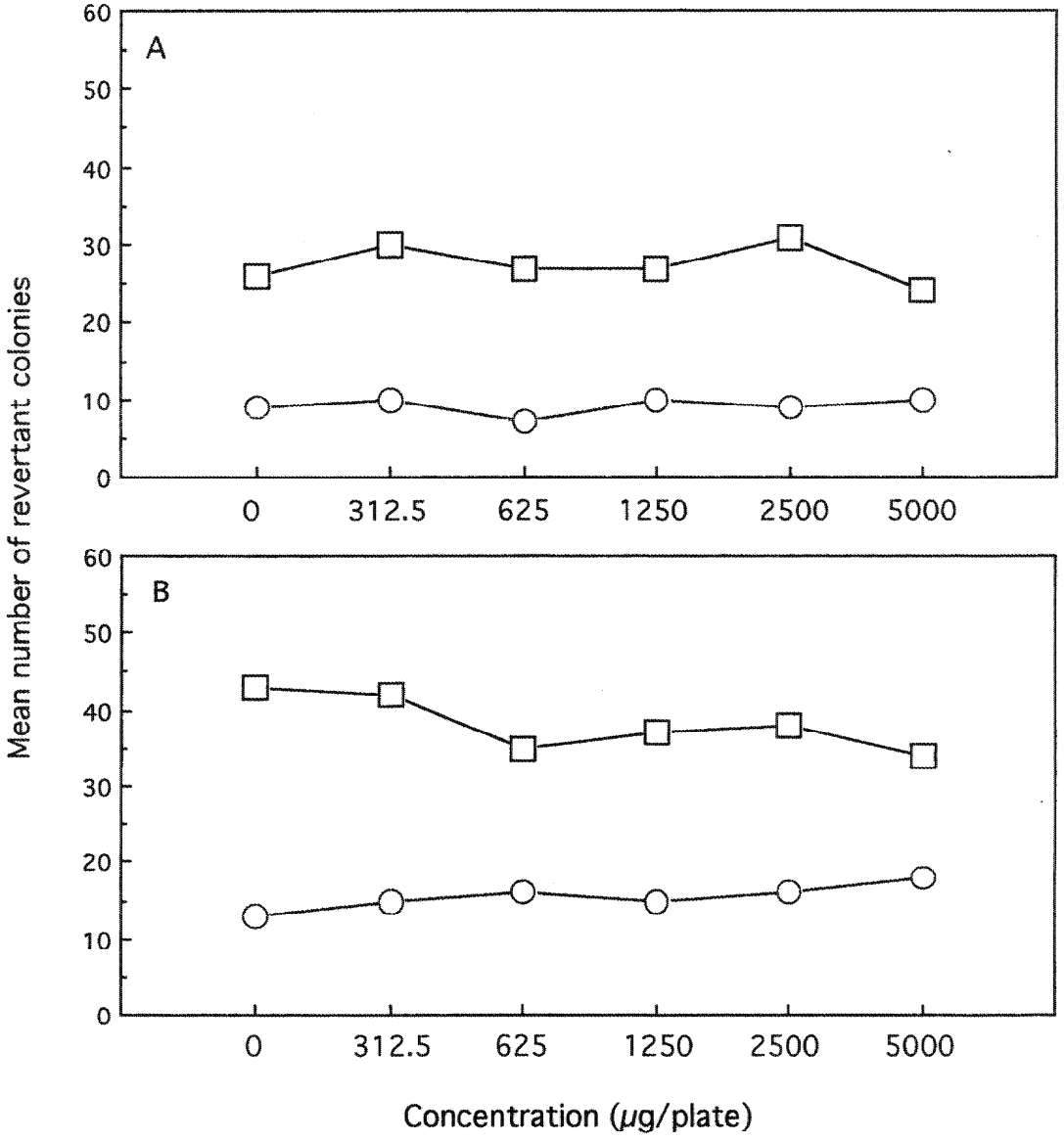


Fig. 2. Dose depending curve of Kombu Fucoidan (Mutagenicity test)

A : Without metabolic activation system (-S9)
B : With metabolic activation system (+S9)
□ : TA98 ; ○ : TA1537

PROJECT No. H-00265

Appendix 1. Reverse mutation test of Kombu Fucoidan in *S. typhimurium* and *E. coli* (Dose determination test)

With (+) or Without (-) S9 mix	Test substance concentration (μg/plate)	Number of revertants (number of colonies/plate)[a]				
		Base-pair substitution type			Frameshift type	
		TA 100	TA 1535	WP2 uvrA	TA 98	TA 1537
S9 mix (−)	Solvent control	146 124 (135)	13 9 (11)	28 34 (31)	24 28 (26)	8 8 (8)
	5	122 136 (129)	6 7 (7)	23 23 (23)	19 21 (20)	4 10 (7)
	10	135 132 (134)	10 17 (14)	23 23 (23)	25 27 (26)	3 3 (3)
	50	137 144 (141)	9 10 (10)	24 38 (31)	24 24 (24)	7 3 (5)
	100	134 138 (136)	11 4 (8)	31 30 (31)	18 27 (23)	5 3 (4)
	500	146 147 (147)	10 6 (8)	33 26 (30)	18 23 (21)	5 4 (5)
	1000	132 141 (137)	6 11 (9)	37 41 (39)	20 19 (20)	4 9 (7)
	5000	125 156 (141)	11 12 (12)	25 28 (27)	18 20 (19)	5 5 (5)
S9 mix (+)	Solvent control	154 151 (153)	14 12 (13)	35 40 (38)	35 33 (34)	11 11 (11)
	5	141 139 (140)	10 15 (13)	39 26 (33)	41 37 (39)	8 9 (9)
	10	152 143 (148)	11 14 (13)	35 40 (38)	29 31 (30)	6 10 (8)
	50	144 147 (146)	10 11 (11)	28 31 (30)	25 34 (30)	7 6 (7)
	100	155 147 (151)	11 17 (14)	32 30 (31)	37 34 (36)	8 6 (7)
	500	134 145 (140)	10 9 (10)	40 39 (40)	31 37 (34)	8 8 (8)
	1000	152 131 (142)	9 8 (9)	40 31 (36)	33 30 (32)	7 10 (9)
	5000	144 136 (140)	12 15 (14)	42 35 (39)	26 29 (28)	10 13 (12)
Positive control not requiring S9 mix	Name	AF−2	SA	AF−2	AF−2	9−AA
	Concentration (μg/plate)	0.01	0.5	0.01	0.1	80
	Number of colonies/plate	560 576 (568)	448 479 (464)	173 181 (177)	522 544 (533)	585 600 (593)
Positive control requiring S9 mix	Name	2−AA	2−AA	2−AA	2−AA	2−AA
	Concentration (μg/plate)	1	2	10	0.5	2
	Number of colonies/plate	841 926 (884)	224 216 (220)	1054 998 (1026)	486 492 (489)	223 245 (234)

[a] : The average number of colonies in each concentration.

Solvent : Distilled water for injection

PROJECT No. H-00265

Appendix 2. Reverse mutation test of Kombu Fucoidan in *S. typhimurium* and *E. coli* (Mutagenicity test)

With (+) or Without (-) S9mix	Test substance concentration (μg/plate)	Number of revertants (number of colonies/plate)[a]				
		Base-pair substitution type			Frameshift type	
		TA 100	TA 1535	WP2 uvrA	TA 98	TA 1537
S9 mix (−)	Solvent control	138 141 (137) 133	8 7 (10) 15	29 36 (29) 21	27 27 (26) 25	6 10 (9) 11
	312.5	138 134 (136)	15 14 (15)	27 26 (27)	28 31 (30)	8 12 (10)
	625	144 147 (146)	12 11 (12)	27 44 (36)	28 26 (27)	7 7 (7)
	1250	152 147 (150)	15 10 (13)	27 26 (27)	20 34 (27)	6 13 (10)
	2500	149 148 (149)	14 10 (12)	34 31 (33)	36 25 (31)	8 9 (9)
	5000	143 141 (142)	10 12 (11)	29 32 (31)	28 19 (24)	10 10 (10)
S9 mix (+)	Solvent control	144 158 (149) 144	15 13 (16) 20	37 44 (38) 32	42 43 (43) 45	12 14 (13) 12
	312.5	157 143 (150)	18 14 (16)	36 34 (35)	40 44 (42)	18 12 (15)
	625	153 155 (154)	19 20 (20)	30 30 (30)	35 34 (35)	16 15 (16)
	1250	160 144 (152)	13 16 (15)	36 34 (35)	35 39 (37)	15 14 (15)
	2500	151 139 (145)	16 20 (18)	39 40 (40)	45 30 (38)	15 17 (16)
	5000	151 146 (149)	15 20 (18)	30 31 (31)	26 41 (34)	19 16 (18)
Positive control not requiring S9 mix	Name	AF−2	SA	AF−2	AF−2	9−AA
	Concentration (μg/plate)	0.01	0.5	0.01	0.1	80
	Number of colonies/plate	580 505 (543)	451 475 (463)	251 274 (263)	494 516 (505)	624 584 (604)
Positive control requiring S9 mix	Name	2−AA	2−AA	2−AA	2−AA	2−AA
	Concentration (μg/plate)	1	2	10	0.5	2
	Number of colonies/plate	973 948 (961)	255 267 (261)	1100 1051 (1076)	532 523 (528)	241 260 (251)

[a] : The average number of colonies in each concentration.
Solvent : Distilled water for injection.

PROJECT No. H-00265

Attached sheet 1 Historical background data (Preincubation method)

Negative control	Distilled water for injection									
S 9 mix	−					+				
Tester strain	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
N	33	29	29	29	29	33	33	29	29	29
Mean	136	11	35	24	9	142	14	42	34	12
S.D.	9	2	6	4	3	8	3	6	5	3
2S.D.	18	4	12	8	6	16	6	12	10	6
Mean-2S.D.	118	7	23	16	3	126	8	30	24	6
Mean+2S.D.	154	15	47	32	15	158	20	54	44	18

Positive control	AF-2	SA	AF-2	AF-2	9-AA	2-AA	2-AA	2-AA	2-AA	2-AA
S 9 mix	−					+				
Tester strain	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
N	259	231	217	231	259	253	231	213	227	249
Mean	510	527	220	529	603	986	221	935	562	216
S.D.	37	56	28	34	54	95	31	85	43	32
2S.D.	74	112	56	68	108	190	62	170	86	64
Mean-2S.D.	436	415	164	461	495	796	159	765	476	152
Mean+2S.D.	584	639	276	597	711	1176	283	1105	648	280

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide、SA : Sodium azide 、9-AA : 9-Aminoacridine、
2-AA : 2-Aminoanthracene
Data collection period : October 1,1999～September 30,2000

PROJECT No. H-00265

QC STATEMENT OF COMPLIANCE

Title : Bacterial reverse mutation test of Kombu Fucoidan

PROJECT No. H-00265

Items of audit and inspection	Date of inspection	Date of reporting	
		Study director	Managing director
Protocol			
Draft	16. 10. 2000		
	17. 10. 2000	17. 10. 2000	17. 10. 2000
Final	19. 10. 2000	19. 10. 2000	19.10. 2000
Dose selection test			
Test substance preparation and administration	31. 10. 2000	01. 11. 2000	01. 11. 2000
Colony counting	02. 11. 2000	06. 11. 2000	06. 11. 2000
Mutagenicity test			
Test substance preparation and administration	07. 11. 2000	07. 11. 2000	07. 11. 2000
Colony counting	10. 11. 2000	13. 11. 2000	13. 11. 2000
Raw data	07. 12. 2000		
	11. 12. 2000	12. 12 .2000	11. 12 .2000
Final report			
Draft	07.12. 2000		
	11. 12. 2000	12. 12 .2000	11. 12 .2000
Final	15. 12. 2000	15. 12. 2000	15. 12. 2000

According to inspection, I, the undersigned, hereby confirm that this study has been conducted as per the protocol (GLP as reference) and the data are accurately reflected in this report.

Date: December 15, 2000.

In charge of QC

Satoru Sakamoto, D.V.M. (Impression of the seal)

Quality Assurance Unit

Nippon Experimental Medical Research Institute Co., Ltd.

Final Report

Title : Bacterial reverse mutation test of Kombu Fucoidan

PROJECT No. H-00265

I the undersigned, hereby declare that the contents of this final report is confirmed by me.

Date: December 15, 2000.

Masaaki Kashima, D.V.M. (impression of the seal)

Managing director

Nippon Experimental Medical Research Institute Co., Ltd.

Contract Laboratory

FINAL REPORT

Acute Oral Toxicity Study of Kombu Fucoidan in Rats

PROJECT No. H-00288

December 8, 2000

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

STATEMENT

Study Title: Acute Oral Toxicity Study of Kombu Fucoidan in Rats

PROJECT No. H-00288

I, the undersigned, hereby declare that this report is the exact English version of the original report that written in Japanese language. Further, declare there is no difference in the contents of this report to that of the original (Japanese) report.

Study director: Iwao Kaneko.
Translated by

Iwao Kaneko Date: 4/23/01

Iwao Kaneko
Nippon Experimental Medical Research Institute Co., Ltd.

APPROVAL OF FINAL REPORT

Study Title: Acute Oral Toxicity Study of Kombu Fucoidan in Rats

PROJECT No. H-00288

I, the undersigned, hereby declare that this study has been conducted in reference to the Japanese GLP Standards for Safety Studies on Drugs (Ministry of Health and Welfare of Japan, Ordinance No. 21: March 26, 1997).

Further, this study has been conducted in accordance with the methods stated herein, and the data has been obtained from the study are accurately reflected in this final report.

Study Director: Iwao Kaneko <impression of seal>
Date: December 8, 2000
Nippon Experimental Medical Research Institute Co., Ltd.

INTRODUCTION

Study Title: Acute Oral Toxicity Study of Kombu Fucoidan in Rats

PROJECT No. H-00288

Objective

The study designed to assess the toxicity of Kombu Fucoidan following a single oral dose to rats.

GLP standards

This study was conducted in reference to the Japanese GLP Standards for Safety Studies on Drugs (Ministry of Health and Welfare of Japan, Ordinance No. 21: March 26, 1997).

Test guideline

This study was conducted in reference to the Amendments to the Single and Repeated Dose Toxicity Studies (YakuShinYaku No. 88: August 10, 1993)

Sponsor

Takara Shuzo Co., Ltd.
3-4-1 Seta, Otsu-shi, Siga-ken, Japan

Contract Laboratory

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

Testing Facility

Haruna Laboratory
Nippon Experimental Medical Research Institute Co., Ltd.
3303-58 Ohdo, Agatsuma-machi, Agatsuma-gun, Gunma-ken, Japan
Management: Masaaki Kashima

Archives

Retention period:

A period specified in the provisions of the Article 26-2-3, Article 26-5(3),c, and Article 26-12, the Enforcement Regulations of the Pharmaceutical Affairs Law.

Items and location:

Protocol, raw data and final report (original) will be stored in raw data archive at Haruna Laboratory, Nippon Experimental Medical Research Institute Co., Ltd.
Recods will be retained in record archive at Haruna Laboratory, Nippon Experi-

mental Medical Research Institute Co., Ltd.

Study time schedule

Study initiation:	October 10, 2000
Animal reciept:	October 10, 2000
Grouping of animals:	October 11, 2000
Experiment initiation:	October 11, 2000
Administration:	October 12, 2000
Macroscopic examination:	October 26, 2000
Experiment termination:	October 26, 2000
Draft report:	November 17, 2000
Final report:	December 8, 2000
Study completion:	December 8, 2000

Study personnel

Study direction, protocol preparation, work instructions and managment, final report preparation : Iwao Kaneko *

Animal health assessment :	Michiko Takahashi
Test substance management :	Akira Tomisawa
Test substance preparation :	Akira Tomisawa
Dosing, clinical observation and body weight measurement :	Akira Tomisawa, Tasaburo Hashizume, Yukihisa Karasawa
Macroscopic examination :	Akira Fukutome
Statistical analysis :	Yukio Tanaka, Masahiro Kasumi, Wataru Koike, Yukiko Takefuchi

* : Safety Research Department, Nippon Experimental Medical Research Institute Co., Ltd.

UNPREDICTED HAPPENINGS CONSIDERED TO HAVE AFFECTED THE RELIABILITY OF THE STUDYAND DEVIATIONS FROM THE PROTOCOL

Study Title: Acute Oral Toxicity Study of Kombu Fucoidan in Rats

PROJECT No. H-00288

There were no unpredicted happenings considered to have affected the reliability of the study and no deviations from the protocol.

CONTENTS

Page

STATEMENT 1/5
APPROVAL OF FINAL REPORT 2/5
INTRODUCTION 3/5
UNPREDICTED HAPPENINGS CONSIDERED TO HAVE AFFECTED THE RELIABILITY OF THE STUDYAND DEVIATIONS FROM THE PROTOCOL 5/5
1. Summary 1
   1.1 Clinical signs and mortality 1
   1.2 Body weights 1
   1.3 Macroscopic findings 1
   1.4 LD50 value 1
2. Materials and Methods 1
   2.1 Test materials 1
   2.2 Test substance preparation 2
   2.3 Animals and Environmental conditions 2
   2.4 Constitution of the groups, dose levels and rationale for dose selection 3
   2.5 Administration 4
   2.6 Observation, measurement and examination 4
   2.7 Statistical analysis 5
3. Results 5
   3.1 Mortality and clinical signs 5
   3.2 Body weights 5
   3.3 Macroscopic findings 5
4. Discussion and Conclusion 5

Figures
1. Body weight changes (male) 6
2. Body weight changes (female) 7

Tables
1. Mortality (male) 8
2. Mortality (female) 9
3. Clinical signs (male) 10
4. Clinical signs (female) 11
5. Body weights (male) 12
6. Body weights (female) 13
7. Necropsy (male) 14
8. Necropsy (female) 15

Appendices
1. Clinical signs (male) 16
2. Clinical signs (female) 17
3. Body weights (male) 18
4. Body weights (female) 19
5. Necropsy (male) 20
6. Necropsy (female) 21

1. Summary

A group of ten SD rats [Crj:CD(SD)IGS] (five males and five females) received a single oral dose of Kombu Fucoidan at 0 (control) and 2000 mg/kg by gavage, in order to observe the signs of toxicity for 14 days and estimate LD50 value.

1.1 Clinical signs and mortality

There were no death and was no systemic response in any animal throughout the study.

1.2 Body weights

No defferences in mean bodyweight on days 2, 3, 5, 8 and 15 after treament were present between the control and treatment group.

1.3 Macroscopic findings

No treatment-related change was noted.

1.4 LD50 value

The LD50 value to either male or female rats of the test substance was demonstrated to be greater than 2000mg/kg bodyweight.

2. Materials and Methods

2.1 Test materials

2.1.1 Test substance

Identity :	Kombu Fucoidan
Lot No. :	F-9811-FD
Purity :	95%
Molecular weight :	Mean molecular weight of 200000
Physical State :	Pale greenish-brawn powder
Stability :	Stable under refrigeration
Date of manufacture :	May 23, 1998
Expiration date :	May 22, 2001
Storage conditions :	Under refrigeration, away from light, sealed
Sponsor :	Takara Shuzo Co., Ltd. 3-4-1 Seta, Otsu-shi, Siga-ken, Japan
Residual test substance :	All the remaining test substance was returned to the sponsor after completion of treatment.

2.1.2 Vehicle

Water for injection J.P. (Fuso Pharmaceutical Industries, Ltd., Lot No. 90901D; hereinafter referred to as water for injection)

2.2 Test substance preparation

2.2.1 Preparation method

A required amount of the test substance was weighed (adjusted for purity) and solubled in water for injection, at a concentration of 100mg/mL (w/v). The homogeneity of the test substance in the vehicle was visually checked and complete dissolution was confirmed. The stability in the vehicle was visually checked at preparation and just after administration, where no heat, coloration or foam was observed. Content analysis was not performed for the test substance formulation.

2.2.2 Time of preparation

The test substance was prepared on the day of doseing.

2.3 Animals and Environmental conditions

2.3.1 Animals

Pooled SD rats [Crj:CD(SD)IGS], 18 animals of each sex (body weights at delivery: 111-127g for males; 99-111g for females), were delivered at 5 weeks of age on September 29, 2000 from Atsugi Breeding Center, Charles River Japan Inc. (795 Shimofurusawa, Atsugi-shi, Kanagawa-ken). Then they were transferred on October 10, 2000 and acclimatized for 5 days prior to use. These rats were quarantined as pooled animals from September 29 to October 5, 2000. The animals were weighed during the quarantine period and assessed for health state on the last day of quarantine. They were acclimatized for 5 days, from the end of quarantine to the previous day of administration.

On the day of grouping, healthy 10 animals of each sex were selected and allocated to groups with computer system by stratified randomization based on the body weights taken on the day of grouping. The animals were 6 weeks old, and their body weight range was 188-200g for males and 140-154g for females on the day of administration.

2.3.2 Environmental conditions

Animals were individually housed in stainless bracket cages for rats (260W×380D×180Hmm) in an animal room (Room No.1 of Building E) where the environmental conditions were set as follows: temperature at 22±3℃; humidity at 50±20% (actual values: temperature within the range of 19-25℃; humidity within the range of 30-70%); ventilation frequency of at least 10 times per hour (all-fresh-air system); and lighting of 12 hours per day (from 6:00 a.m. to 6:00 p.m., 150-300 lux). Animals were allowed free access to pellet feed for experimental animals (CE-2, Lot No. E2080,

Clea Japan Inc.) and drinking water (household tap water). Cages, feeders, trays and watering bottles were autoclaved (121℃ for 30 min) prior to use. Watering bottles and trays were changed at least twice weekly. The animal room was cleaned after work every day, and the floor was sterilized by wiping with 400-fold dilution of benzethonium chloride (Hyamine, Sankyo Co., Ltd.).

Identification was made for animals by writing abbreviated animal numbers on the root of the tail with an oil marker, and for cages by attaching colored labels showing study No., administration route, dose levels, etc.

2.3.3 Analysis of impurities and contaminants in diet and water

For impurities and contaminants in the diet, we obtained a copy of the results of the analysis that was conducted by Tokyo Kenbikyo-in (44-1, Hakozaki-cho, Nihonbashi, Chuo-ku, Tokyo) at the request of the manufacturer. Checked before the diet was served, the analytical results were confirmed to be within the range of acceptable limits specified by our facility. For impurities and contaminants in the drinking water, the analysis was conducted by the Environmental Hygiene Laboratory Center of Gunma Pharmaceutical Association (5-18-36 Nishikatagai-cho, Maebashi-shi, Gunma-ken) based on the Ministerial Ordinance Concerning Water Quality Standards (Ministry of Health and Welfare of Japan, Ordinance No. 69, 1992) with the water samples periodically collected by our facility. The types of analyses included clean water testing on standard parameters (August 3, 1999) trihalomethan test (August 8, 2000), and the tests pursuant to the building management law on all parameters (February 15, 2000) and with omitted parameters (September 6 and October 3, 2000). All the analytical results were within the above water quality standards, there being no abnormal values considered to have affected the study.

2.4 Constitution of the groups, dose levels and rationale for dose selection

2.4.1 Constitution of the groups and dose levels

The constitution of the groups and the treatment regime are indicated in the following table.

Group No.	Test materials	Dose level (mg/kg)	Dose volume (mL/kg)	Concentration (mg/mL)	Sex	No. of animals	Animal No.
00	Vehicle (Control[a])	0	20	0	male	5	00M01~00M05
					female	5	00F01~00F05
01	Kombu Fucoidan	2000	20	100	male	5	01M01~01M05
					female	5	01F01~01F05

a : The controls received water for injection.

2.4.2 Rationale for dose selection

The dose level for the main study was chosen on the basis of a preliminary study (PROJECT No.H-00328). In the preliminary study, a group of four SD rats [Crj:CD(SD)IGS] (two males and two females) received a single oral dose of Kombu Fucoidan at 500, 1000 and 2000 mg/kg by gavage. As there were no deaths even at the highest dose of 2000 mg/kg, in compliance with the manual of the Guideline for Medical Devices (1997), the limit dose of 2000 mg/kg was selected for the main study

2.5 Administration

2.5.1 Route of administration and rationale for the selection

Oral administration was selected because oral intake is expected as a route of exposure in humans.

2.5.2 Method of administration

Dose volume was adjusted to the body weights taken just before administration, and administered with a 5mL disposable syringe connected to a gavage.

2.5.3 Administration frequency and rationale for the selection

A single administration was selected in accordance with the Guideline for Medical Devices (Yaku-ShinYaku No.88). Animals were fasted from about 18 hours before to about 3 hours after treatment.

2.6 Observation, measurement and examination

The day of treatment was designated as day 0, and the following observation, measurement and examination were performed on all animals up to day 15 after treatment.

2.6.1 Clinical signs

Animals were observed for clinical signs and mortality at 15, 30 minutes, 1, 3, and 6 hours after treatment on day 0 and thereafter once daily up to day 15.

2.6.2 Body weights

Animals were weighed with an electronic balance (Sartrius Co., Ltd.) on day 0 (just before treatment), and on days 2, 3, 5, 8 and 15 after treatment.

2.6.3 Macroscopic examination

All animals were killed by transecting the abdominal aorta under ether anesthesia at the end of the observation period (day 15 after treatment) and subjected to a macroscopic examination that consisted of opening the cranial, thoracic and abdonial cavities. The macroscopic appearance of all examined orgas was recorded.

2.6.4 Microscopic examination

Microscopic examination was not performed because no abnormal organs were found in macroscopic examination.

2.7 Statistical analysis

Means and standard diviations of the body weights were calculated for the test substance group and the vehicle control. The F-test for homogeneity of variances was performed on the data. If the F-test indicated homogeneous variances, the group means were compared to the vehicle control means using Student's t-test. If the F-test indicated heterohomogeneous variances, Aspin-Welch's t-test was used to compare group to the vehicle control.

3. Results

3.1 Mortality and clinical signs (Table 1~4, Appendices 1 and 2)

There were no deaths and was no systemic response in any animal throughtout the study.

3.2 Body weights (Fig. 1 and 2, Tables 5 and 6, Appendices 3 and 4)

A decrease in body weights was recorded for one female of the test substance group on day 5 after treatment, but the other animals demonstrated satisfactory body weight gains. No defferences in mean bodyweight on days 2, 3, 5, 8 and 15 after treament were present between the control and treatment group.

3.3 Macroscopic findings (Tables 7 and 8, Appendices 5 and 6)

No treatment-related change was noted

4. Discussion and Conclusion

There were no deaths and was no systemic response in any animal throughtout the 14-day observation period. No defferences in mean bodyweight on days 2, 3, 5, 8 and 15 after treament were present between the control and treatment group. No abnomarities were recorded at the macroscopic examination. Therefore、Kombu Fucoidan at a dose of 2000mg/kg did not produce toxic effect in SD rats.

The LD50 value to either male or female SD rats of Kombu Fucoidan was demonstrated to be greater than 2000mg/kg bodyweight.

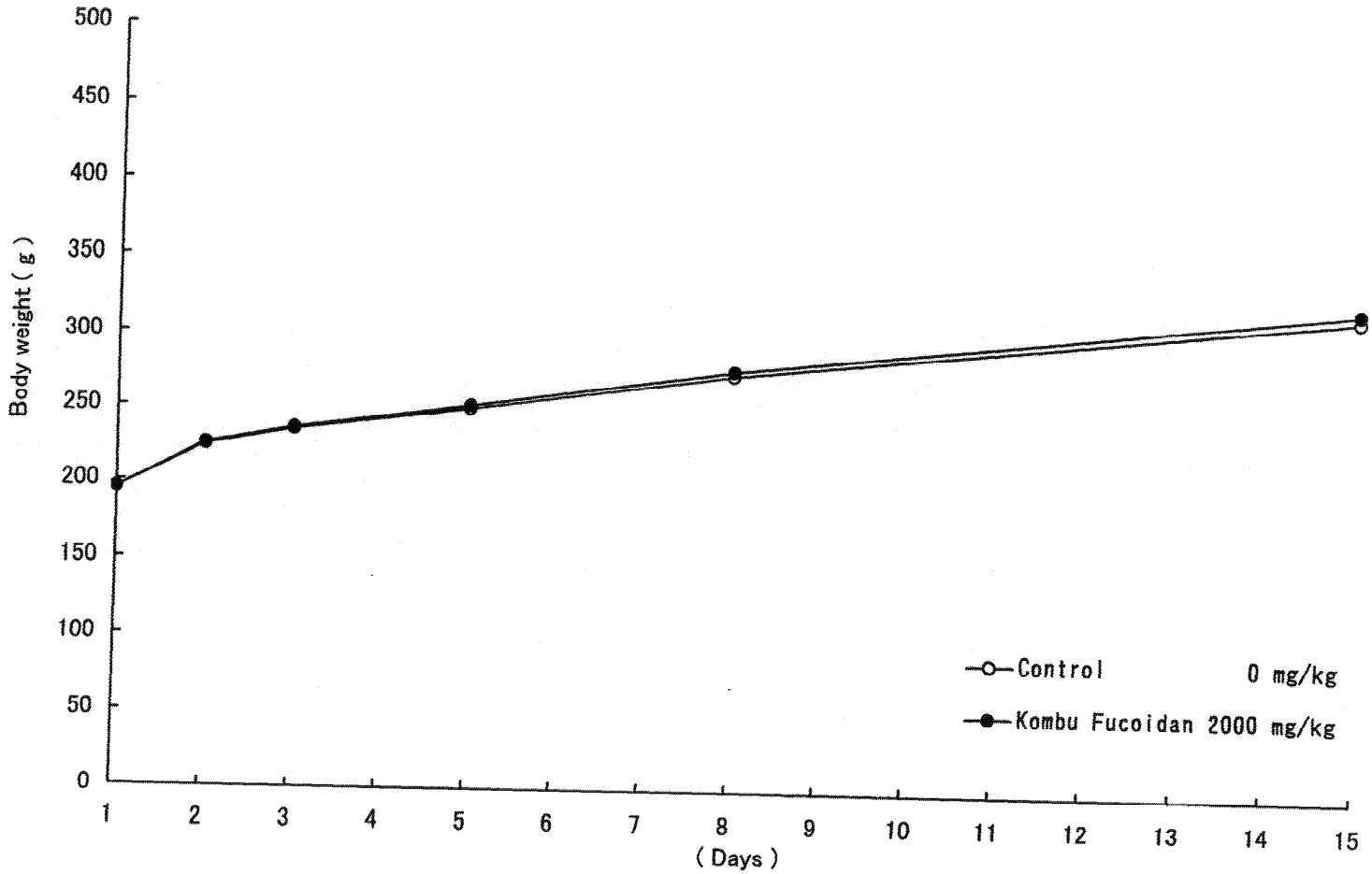


Fig.1 Body weight changes of male rats treated orally with Kombu Fucoidan

PROJECT No.H-00288

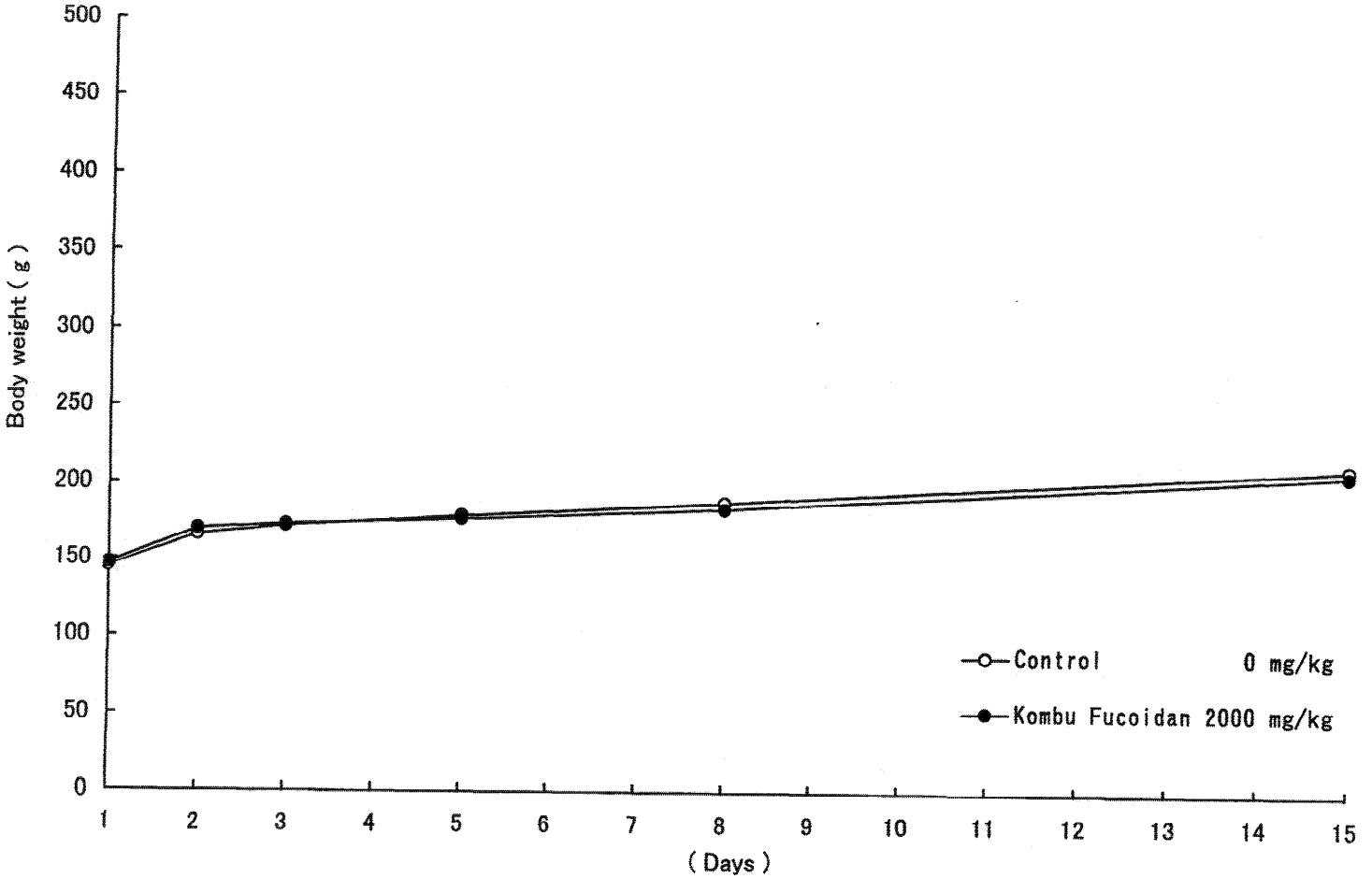


Fig.2 Body weight changes of female rats treated orally with Kombu Fucoidan

PROJECT No.H-00288

Table 1 Mortality of male rats treated orally with Kombu Fucoidan

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	Kombu Fucoidan																					
	2000	No. of death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5

PROJECT No.H-00288

Table 2 Mortality of female rats treated orally with Kombu Fucoidan

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
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	Kombu Fucoidan																					
	2000	No. of death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5

PROJECT No.H-00288

Table 3 Clinical signs of male rats treated orally with Kombu Fucoidan

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 Kombu Fucoidan	No.of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
2000	no abnormality	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

PROJECT No.H-00288

Table 4 Clinical signs of female rats treated orally with Kombu Fucoidan

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 Kombu Fucoidan	No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
2000	no abnormality	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

PROJECT No. H-00288

Table 5 Body weights of male rats treated orally with Kombu Fucoidan

Group-No. Dose (mg/kg)		Days 1	2	3	5	8	15
00	N	5	5	5	5	5	5
Control	Mean	195	224	235	250	274	318
0	S.D.	4	5	6	6	10	20
01	N	5	5	5	5	5	5
Kombu Fucoidan	Mean	195	225	236	252	277	323
2000	S.D.	5	6	8	10	12	20

Unit : g N : No.of animals

PROJECT No.H-00288

Table 6 Body weights of female rats treated orally with Kombu Fucoidan

Group-No. Dose (mg/kg)		Days 1	2	3	5	8	15
00	N	5	5	5	5	5	5
Control	Mean	145	166	172	179	188	212
0	S.D.	4	4	4	3	5	6
01	N	5	5	5	5	5	5
Kombu Fucoidan	Mean	147	170	173	177	184	208
2000	S.D.	5	6	5	7	5	10

Unit : g N : No.of animals

PROJECT No.H-00288

Table 7 Necropsy of male rats treated orally with Kombu Fucoidan

Organs / Findings	Group No. Dose (mg/kg)	00 Control 0	01 Kombu Fucoidan 2000
	No. of animals	5	5
abnormality		0	0

PROJECT No.H-00288

Table 8 Necropsy of female rats treated orally with Kombu Fucoidan

Organs / Findings	Group No. Dose (mg/kg)	00 Control 0	01 Kombu Fucoidan 2000
	No. of animals	5	5
abnormality		0	0

PROJECT No.H-00288

Appendix 1 Clinical signs of male rats treated orally with Kombu Fucoidan

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	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Kombu Fucoidan	01M02	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2000	01M03	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	01M04	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	01M05	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

— : no abnormality

PROJECT No.H-00288

Appendix 2 Clinical signs of female rats treated orally with Kombu Fucoidan

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	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Kombu Fucoidan	01F02	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2000	01F03	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	01F04	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	01F05	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

— : no abnormality

PROJECT No.H-00288

Appendix 3 Body weights of male rats treated orally with Kombu Fucoidan

Group-No. Dose (mg/kg)	Animal No.	1	2	3	5	8	15
		Days					
00 Control 0	00M01	192	220	231	249	269	316
	00M02	198	224	238	252	271	307
	00M03	193	224	231	242	264	296
	00M04	199	232	244	259	290	348
	00M05	191	222	231	248	276	322
	N	5	5	5	5	5	5
	Mean	195	224	235	250	274	318
	S.D.	4	5	6	6	10	20
01 Kombu Fucoidan 2000	01M01	192	219	232	247	268	315
	01M02	200	232	242	258	281	324
	01M03	200	231	246	267	296	356
	01M04	194	225	233	246	271	314
	01M05	188	219	225	244	268	306
	N	5	5	5	5	5	5
	Mean	195	225	236	252	277	323
	S.D.	5	6	8	10	12	20

Unit : g N : No.of animals

PROJECT No.H-00288

Appendix 4 Body weights of female rats treated orally with Kombu Fucoidan

Group-No. Dose (mg/kg)	Animal No.	1	2	3	5	8	15
		Days					
00 Control 0	00F01	143	166	172	180	189	207
	00F02	143	168	174	181	184	209
	00F03	144	159	169	174	187	206
	00F04	152	171	177	181	196	218
	00F05	144	167	167	181	186	218
	N	5	5	5	5	5	5
	Mean	145	166	172	179	188	212
	S.D.	4	4	4	3	5	6
01 Kombu Fucoidan 2000	01F01	154	177	180	188	193	224
	01F02	140	162	166	170	181	204
	01F03	147	168	171	178	183	207
	01F04	146	168	175	170	183	207
	01F05	150	173	174	177	182	198
	N	5	5	5	5	5	5
	Mean	147	170	173	177	184	208
	S.D.	5	6	5	7	5	10

Unit : g N : No.of animals

PROJECT No.H-00288

Appendix 5 Necropsy of male rats treated orally with Kombu Fucoidan

Group No.	00					01				
	Control					Kombu Fucoidan				
Dose (mg/kg)	0					2000				
Animal No.	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	1	1	1	1	1
	M	M	M	M	M	M	M	M	M	M
	0	0	0	0	0	0	0	0	0	0
Organs / Findings	1	2	3	4	5	1	2	3	4	5
abnormality	-	-	-	-	-	-	-	-	-	-

- : Negative

PROJECT No.H-00288

Appendix 6 Necropsy of female rats treated orally with Kombu Fucoidan

Group No.	00					01				
	Control					Kombu Fucoidan				
Dose (mg/kg)	0					2000				
Animal No.	00F01	00F02	00F03	00F04	00F05	01F01	01F02	01F03	01F04	01F05
Organs / Findings										
abnormality	-	-	-	-	-	-	-	-	-	-

- : Negative

PROJECT No.H-00288

QC CONFIRMATION

Study Title: Acute Oral Toxicity Study of Kombu Fucoidan in Rats
PROJECT No. H-00288

Study Phase	Date of inspection	Date of Reporting	
		Study Director	Management
Protocol (draft)	September 22, 2000	September 26, 2000	Septembet 25, 2000
Protocol (final)	October 11, 2000	October 11, 2000	October 11, 2000
Process Base Inspections			
Weights of animals, test substance preparation, administration, and clinical observation			
	October 12, 2000	October 16, 2000	October 13, 2000
Macroscopic finding	October 26, 2000	October 31, 2000	October 30, 2000
Raw data	November 24, 27, 2000	November 28, 2000	November 27, 2000
Report (draft)	November 24, 27, 2000	November 28, 2000	November 27, 2000
Report (final)	December 8, 2000	December 8, 2000	December 8, 2000

I, the undersigned, have confirmed the concordance of study operations with the protocol, and the consistency of the final report with the raw data.

Satoru Sakamoto <impression of seal>
QC inspector (Quality Assurance Unit Manager)
Date: December 8, 2000
Nippon Experimental Medical Research Institute Co., Ltd.

CONFIRMATION

Study Title: Acute Oral Toxicity Study of Kombu Fucoidan in Rats
PROJECT No. H-00288

I, the undersigned, have confirmed the contents of the final report of the above study.

Management: Masaaki Kashima <impression of seal>
Date: December 8, 2000
Nippon Experimental Medical Research Institute Co., Ltd.

Japanese Journal of Cancer Research, Vol.91, 548 (2000)

Antigen presenting cell (APC)-dependent IFN-γ-inducing effect of Fucoidan from *Kjellmaniella crassifolia* Miyabe(Gagome Kombu) in splenic lymphocyte from tumor sensitized mice

Takanari Tominaga[1], Eiji Nishiyama[1], Takeshi Sakai[2], Hiroaki Sagawa[1], Ikunoshin Kato[1]

[1]*Biotech Res. Lab., Takara Shuzo Co., Ltd. (Seta 3-4-1, Otsu, Shiga 520-2193,Japan)*
[2]*Hirosaki Biotech Res. Lab., Takara Shuzo Co., Ltd. (Zaifu-cho 82-4, Hirosaki, Aomori 036-8216, Japan)*

Introduction

Interleukin-12 (IL-12) is produced by antigen-presenting cells (APC), induces type 1 helper-T cells (Th1) to produce interferon γ (IFN-γ), and enhances the cytotoxic activity of cytotoxic T lymphocytes (CTL) and natural killer cells (NK)[1)].

IFN-γ is produced by Th1 and activates cellular immunity mediated by CTL, NK, macrophages, and others, but suppresses cellular immunity mediated by type 2 helper T cells (Th2) involved in allergic diseases[2)].

We know that IFN-γ production is directly induced by T-cell mitogens such as Concanavalin A or by the interaction of APC with T cells during antigenic recognition. The former induction involves the direct binding of mitogens to T cell receptors, and the latter induction involves the stimulation of the respective receptors due to the APC-T cell interaction and the induction of production by IL-12 produced by APC[3)4)].

For the induction of IL-12 production, the T cell receptor (TCR)-major histocompatibility complex (MHC) reaction and costimulatory molecules such as T cell CD40L-APC CD40 and T cell CD28-APC B7 reactions are known[5)6)].

It is generally accepted that allergic diseases develop when the balance between Th1

and Th2 activity is disrupted in favor of Th2 activity. Th2-derived cytokines induce the differentiation of B cells into IgE antibody-producing cells. The produced IgE binds to the receptor on the mast cell and causes it to release chemical mediators. These inflammatory mediators directly produce allergic symptoms[7].

IFN-γ produced by Th1 act to suppress the activation of Th2 and, in the treatment of allergic diseases in which Th2 is predominantly activated, it is important to induce the production of IFN-γ by activating Th1[2].

In this study, using lymphocytes derived from normal or antigen-sensitized mice, we investigated the IFN-γ-inducing activity of fucoidan from Gagome Kombu, and also the effect of its oral administration in the rat model of ovalbumin (OA)-elicited anti-IgE production, an allergic disease model.

Materials and Methods

1. Induction of the production of IFN-γ and IL-12 in splenic lymphocytes

IFN-γ inducting activity in lymphocytes derived from non-sensitized mice

ICR mice (female, 7 weeks old, weight about 2 5 g) and C57BL/6 mice (female, 6 weeks old, weight about 20 g) were obtained from Japan SLC and used for experiments after preliminary feeding for 1 week. The spleen was removed from ICR mice, minced, and suspended in RPMI-1640 medium (Gibco, Inc.) containing 10% fetal calf serum (FCS) (Hyclone, Inc.) to obtain a suspension of single cells. Adherent cells were removed by allowing them to adhere to a plastic dish, and nonadherent cells were used as splenic lymphocytes. Nonadherent cells were suspended in 10% FCS-containing RPM-1640 medium to 2×10^6 cells/ml, and 200 μl/well of the cell suspension was dispensed in 96-well microtiter plates. To each well except those for controls, Gagame Kombu-derived fucoidan of various concentrations or 2 μg of Concanavalin A (ConA; Nakalai Tesq, Inc.) was added, and

the cells were cultured at 37¡C in a CO_2 (5%) incubator for 4 days. After culture, the culture supernatant was recovered, and the concentration of IFN-γ was determined using an ELISA Kit (Genzyme, Inc.).

IFN-γ inducing activity in lymphocytes derived from tumor antigen-sensitized mice

C57BL/6 mice (female, 6 weeks old, weight about 20 g) were purchased from Japan SLC and used for experiments after preliminary feeding for 1 week. Mice were immunized by intra peritoneal inoculation with 1×10^6 Meth-A sarcoma cells. The spleen was removed 15 days later. Splenic lymphocytes were obtained as described above, suspended to 2×10^6 cells/ml, and 100 μl/well of the cell suspension was dispensed in 96-well microtiter plates. To prepare stimulating cells, Meth-A sarcoma cells were suspended to 2×10^6 cells/ml in RPMI-1640 medium containing 50 μg/ml of mitomycin C (Kyowa Hakko Kogyo Co., Ltd.), and the cell suspension was incubated at 37¡C for 30 min. The sarcoma cells were washed twice and suspended to 2×10^6 cells/ml in RPMI-1640 medium containing 10% FCS. The layer of splenic lymphocytes in each well was overlaid with 100 μl of this stimulating cell suspension. To each well except those for controls, Gagome Kombu-derived fucoidan of various concentrations or 2 μg of ConA was added, and the cells were cultured at 37¡C in a CO_2 (5%) incubator for 4 days. After culture, the supernatant was recovered, and the concentrations of IFN-γ and IL-12 were determined separately, using the appropriate ELISA Kit for each (Genzyme, Inc. and ENDOGEN, Inc., respectively).

Effect of anti-IL-12 antibody and costimulatory molecule (CD28, CD40) antibody on the IFN-γ-inducing activity of Gagome kombu-derived fucoidan

A mixture of the above prepared C57BL/6 mouse-derived splenic lymphocytes and Meth-A sarcoma cells was dispensed in 96-well microtiter plates, and Gagome

Kombu-derived fucoidan was added to all wells to a final concentration of 100 μg/ml. In addition, to the wells except for controls, anti-IL-12 antibody (R & D, Inc.), anti-CD28 antibody, or anti-CD40 antibody was added to final concentrations of 1 μg/ml, 10 μg/ml, and 10 μg/ml, respectively, and the cells were incubated at 37¡C in a CO_2 (5%) incubator for 4 days. After culture, the supernatant was recovered and the concentration of IFN-γ and IL-12 was determined by ELISA.

Comparison of the IFN-γ production-inducing activity of various fucoidans

C57BL/6 mice were immunized by inoculation with Meth-A mouse sarcoma cells, and the spleen was removed 24 days after inoculation. C57BL/6 mice-derived splenic lymphocytes and Meth-A mouse sarcoma cells, both of which were prepared as described above, were mixed, and the mixture was dispensed in 96-well microtiter plates. As test substances, each fucoidan prepared from Gagome Kombu, Okinawa Mozuku, Hibamata, and Sporophyl of Wakame was added to each well to a final concentration of 10-500 μg/ml, and the cells were cultured at 37¡C in a CO_2 (5%) incubator for 4 days. After culture the supernatant was recovered, and the concentration of IFN-γ was determined using an ELISA Kit.

2. IgE antibody production suppressive activity

Effect of prophylactic administration of fucoidan

Groups of four to five 5-week-old male Wistar rats (Japan SLC, Inc.) were sensitized by peritoneal injection of 100 μl of 0.01% OA (Sigma, Inc.) in normal saline and 100 μl of Inject Alum (Pierce, Inc.), and blood was collected from the abdominal portion of the vena cava 14 days later. The blood specimen was centrifuged at 2,000 rpm for 5 min, the serum was separated, and the level of the antigen-specific IgE antibody was measured by passive

cutaneous anaphylaxis (PCA) in rats. Specifically, 0.1 ml of serial 2-fold (2- to 64-fold) dilutions of plasma in normal saline was injected intracutaneously into the shaved back of 7-week-old male Wistar rats. After 48 h, 1 ml of a mixture of 0.05% OA and 0.5% Evans Blue (Nakarai Tesq, Inc.) was injected into the caudal vein and, 30 min later, the rat was sacrificed by decapitation-exsanguination and observed for blue spots on the back. Spots more than 5 mm in diameter were regarded as positive, and the highest dilution was used to express the titer of IgE antibody.

The group administered Gagome kombu-derived fucoidan was given access adlibitum to drinking water containing 0.1% or 1% solution of fucoidan from 7 days before antigen sensitization to the day of blood collection. The control group was given tap water adlibitum.

Effect of therapeutic administration of fucoidan

Rats that had been sensitized as described above received a booster immunization at 19 days after the first sensitization under the conditions indicated above, and blood was collected from the abdominal portion of the vena cava at 14 days after the last immunization. The blood specimen was tested by PCA to determine the level of antigen-specific IgE antibody.

The group administered Gagame Kombu-derived fucoidan was given access adlibitum to drinking water containing 0.1% or 1% solution of fucoidan from the day of booster immunization to the day of blood collection. The control group was given tap water adlibitum.

Results

1. Induction of the production of IFN-γ and IL-12 in splenic lymphocytes

Induction of IFN-γ production in normal mouse-derived splenic lymphocytes

Gagome Kombu-derived fucoidan at doses of 10-500 μg/ml had no IFN-γ production-inducing effect. However, the T cell mitogen ConA at a dose of 10 μg/ml showed a marked production-inducing effect (Fig. 1).

IFN-γ and IL-12 induction by antigen stimulation of sensitized lymphocytes

The addition of Gagome Kombu-derived fucoidan at doses of 1-100 μg/ml at the time of antigen stimulation of splenic lymphocytes derived from Meth-A sarcoma cell-immunized mice induced the production of IFN-γ and IL-12 in a dose-dependent manner (Figs. 2 and 3).

Effects of anti-IL-12 antibody and anti-costimulatory molecule(CD28, CD40) antibody on the induction of IFN-γ and IL-12 production by Gagome kombu-derived fucoidan

The induction of IFN-γ production by Gagome kombu-derived fucoidan was 50% suppressed by anti-IL-12 antibody, and completely suppressed by anti-CD40 or anti-CD28 antibody. The production of IL-12 was suppressed below the detection limit by treatment with either antibody (Figs. 4 and 5).

Comparison of the IFN-γ production-inducing activity of various fucoidans

At the tested doses of 10-500 μg/ml, Gagome Kombu-and Hibamata-derived fucoidans equally induced IFN-γ production in sensitized lymphocytes, and Okinawa Mozuku- and Sporophyl of Wakame-derived fucoidans more weakly, but Mozuku did not induce IFN-γ (Fig. 6).

2. IgE antibody production suppressing activity

Effect of prophylactic administration of Gagome Kombu-derived fucoidan (administration before antigen sensitization)

The sensitization of rats by OA antigen and alum resulted in the elevation of IgE titer in serum 14 days after sensitization. The production of IgE antibody was markedly suppressed in rats fed with 1% Gagome Kombu-derived fucoidan-containing drinking water from 7 days before antigen sensitization (Fig. 7a).

Effect of therapeutic administration of Gagome Kombu-derived fucoidan (administration after antigen sensitization)

The production of IgE antibody was also markedly suppressed in rats fed with 1% Gagome Kombu-derived fucoidan-containing drinking water from the day of booster immunization by the same antigen at 19 days after the first sensitization (Fig. 7b).

Discussion

The IFN-γ production-inducing activity of Gagome Kombu-derived fucoidan was not observed in lymphocytes derived from normal mice; it was observed, together with IL-12 production, only in lymphocytes derived from antigen-sensitized mice when the fucoidan was added under stimulation by the same antigen. These results suggest that Gagome Kombu-derived fucoidan does not directly stimulate naive T cells in the interphase to produce IFN-γ, but stimulate sensitized T cells to produce it at the time of antigen presentation reaction.

The induction of IFN-γ by Gagome Kombu-derived fucoidan was about 50% suppressed by anti-IL-12 antibody and completely suppressed when the APC-T cell interaction was blocked by anti-costimulatory molecule antibody. These findings suggest that during antigen recognition Gagome Kombu-derived fucoidan may enhance the IL-12

production by APC and induce IFN-γ production by memory T cells that have been activated by APC-T cell interaction. The intensity of IFN-γ induction varied according to origins of fucoidan, and Gagome Kombu-derived fucoidan showed strong induction.

Since in the rat model of OA-elicited IgE production, antigen-specific IgE antibody was elevated, its suggested that Th2 is predominantly activated, that is, allergic reactions are more prone to develop. However, IgE antibody production was suppressed in Gagome Kombu-drived fucoidan-administered rats, indicating alleviation of the allergic state. We consider that the administration of the fucoidan induces the production of IFN-γ, suppresses the Th2 activation by antigen sensitization, and corrects the Th1/Th2 activity balance. Clinical trials with IFN-γ have been conducted for allergic diseases[8], and some antiallergenic drugs have been reported to induce IFN-γ as a mechanism of action[9]. Since the suppressive effect of Gagome Kombu-derived fucoidan on IgE antibody production was effective in oral administration even after antigen sensitization for therapeutic purposes, we consider that it is highly effective for the relief of symptoms of allergic diseases represented by asthma, pollenosis, and atopic dermatitis.

In addition to indications for the treatment of allergic diseases, IFN-γ has been clinically used as an anticancer agent. Clinical trials with IL-12 have also been conducted in patients with cancer[10] or hepatitisC[11]. We consider that IFN-γ and IL-12 are effective for these diseases.

We consider that Gagome Kombu-derived fucoidan does not induce cytokine production in the normal state, but exerts a cytokine-inducing effect when an exogenous antigen enters the body and activates the immune system. In other words, we speculate that Gagome Kombu-derived fucoidan acts on the body without causing excessive inflammatory reactions by specifically inducing only cytokines that are necessary for immunoregulation to maintain homeostasis.

Reference

1)Okumura H et al. (1995) Cloning of a new cytokine that induces IFN-γ production by T cells. Nature. 378, 88.

2)Fiorentino D et al. (1989) Two type of mouse T helper cell.Ⅳ. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. 170, 2081.

3)Hsieh CS et al. (1993) Development of Th1 CD4+ T cells through IL-12 production by Listeria-induced macrophages. Science. 260, 547.

4)Seder RA et al. (1993) Interleukin 12 acts directly on CD4+ Tcells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming. Proc. Natl. Acad. Sci. USA. 90, 10188.

5)Stuber E et al. (1996) Bloking the CD40L-CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. J. Exp. Med. 183, 693.

6) Lenschow DJ et al. (1996) CD28/B7 system of T cell costimulation. Annu. Rev. Immunol. 14, 233.

7) Price JF et al. (1982) Antigen provocation to the skin, nose and lung, in children with asthma ; immediate and dual hypersensitivity reactions. Clin. Exp. Immunol. 47, 587.

8) Schneider LC et al. (1998) Long-term therapy with recombinant interferon-γ (rIFN-γ) for atopic dermatitis. Ann. Allergy Asthma Immunol. 80, 263.

9) Kou K et al. (1997) Immunomodulation by TYB-2285 of Dermatophagoides farinae (DF) antigen-induced IFN-γ and IL-4 production in lymphocytes from children with bronchial asthma. Allergi. 46, 502.

10) Atkins MB et al. (1997) Phase I evaluation of intervenous recombinant human interleukin-12 in patients with advanced malignancies. Clin. Cancer Res.3, 409.

11) Zeuzem S et al. (1999) A phase I/II study of recombinant human interleukin-12 in patients with chronic hepatitis C. Hepatology. 29, 1280.

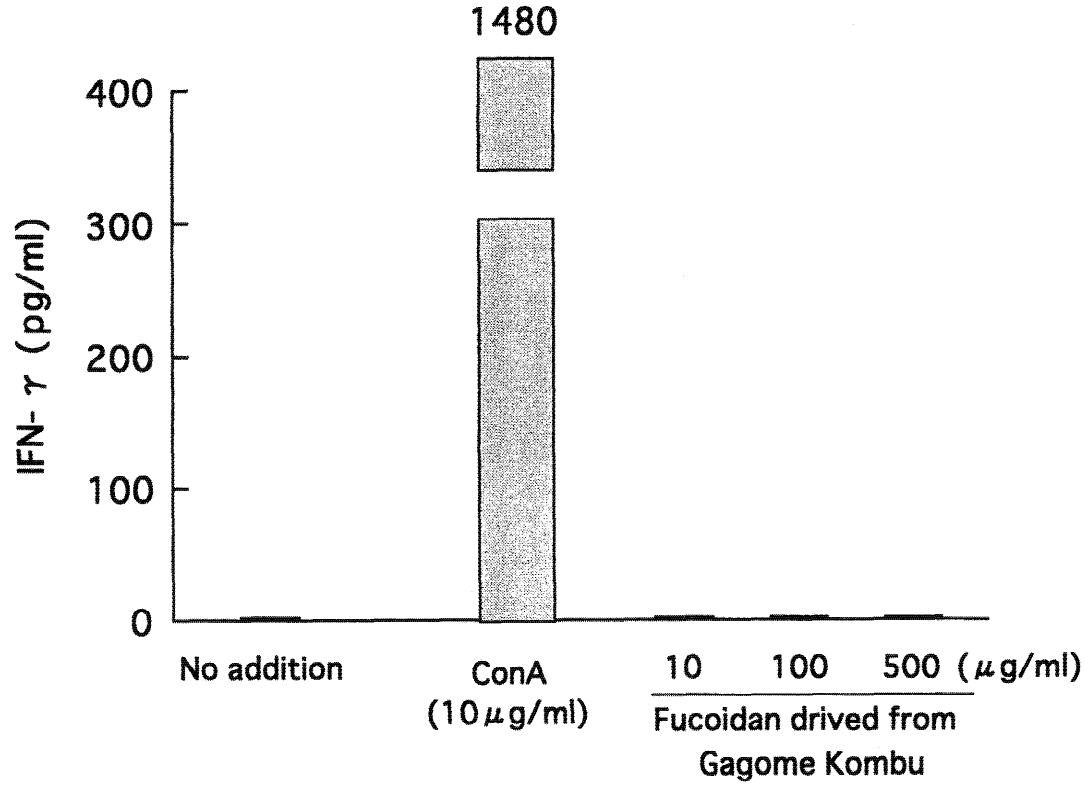


Fig. 1 Induction of IFN-γ production in splenic lymphocytes of normal mice by fucoidan from Gagome Kombu

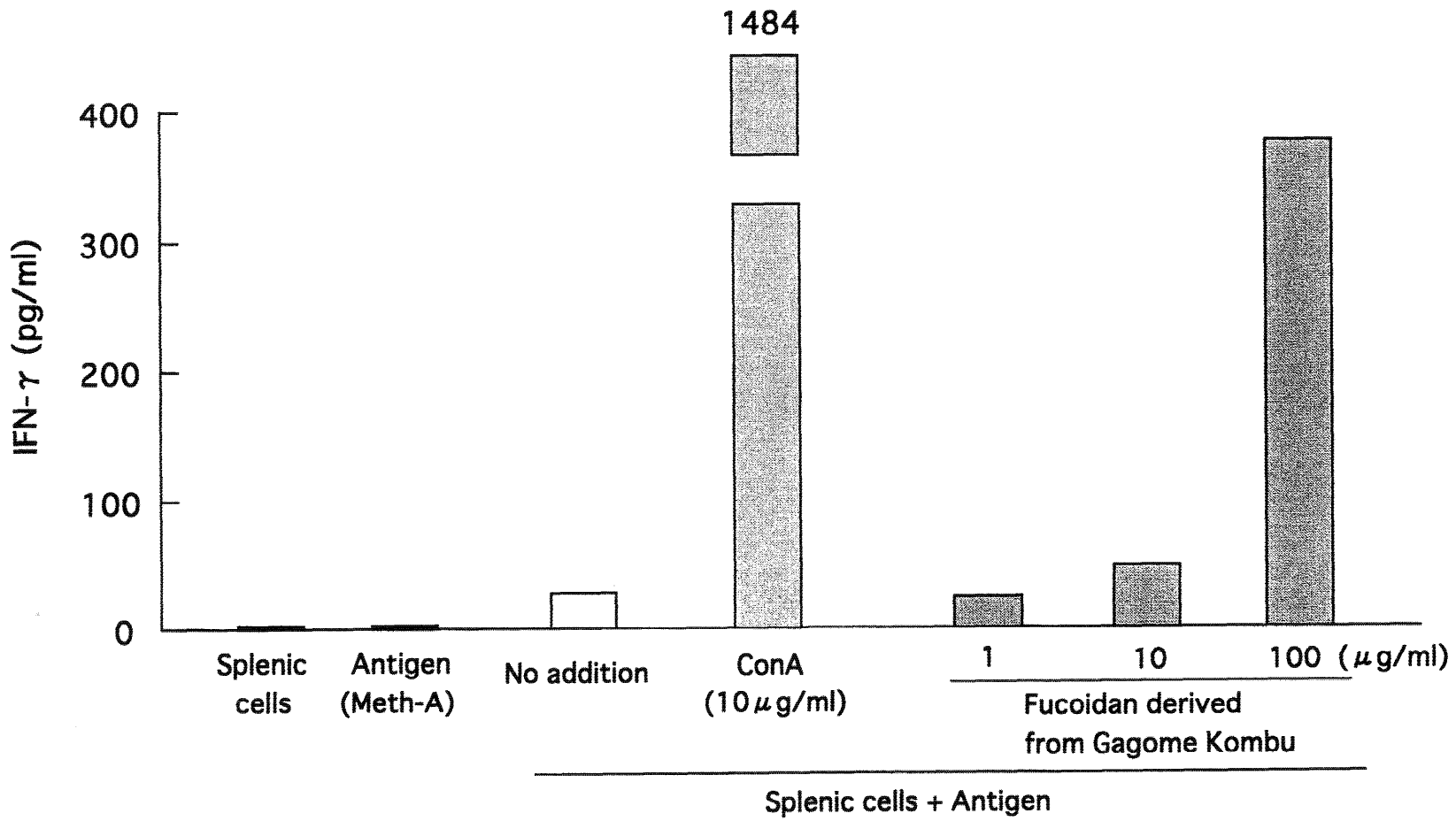


Fig. 2 Induction of IFN-γ production by fucoidan from Gagome Kombu in splenic lymphocytes of antigen-sensitized mice under condition of antigen stimulation

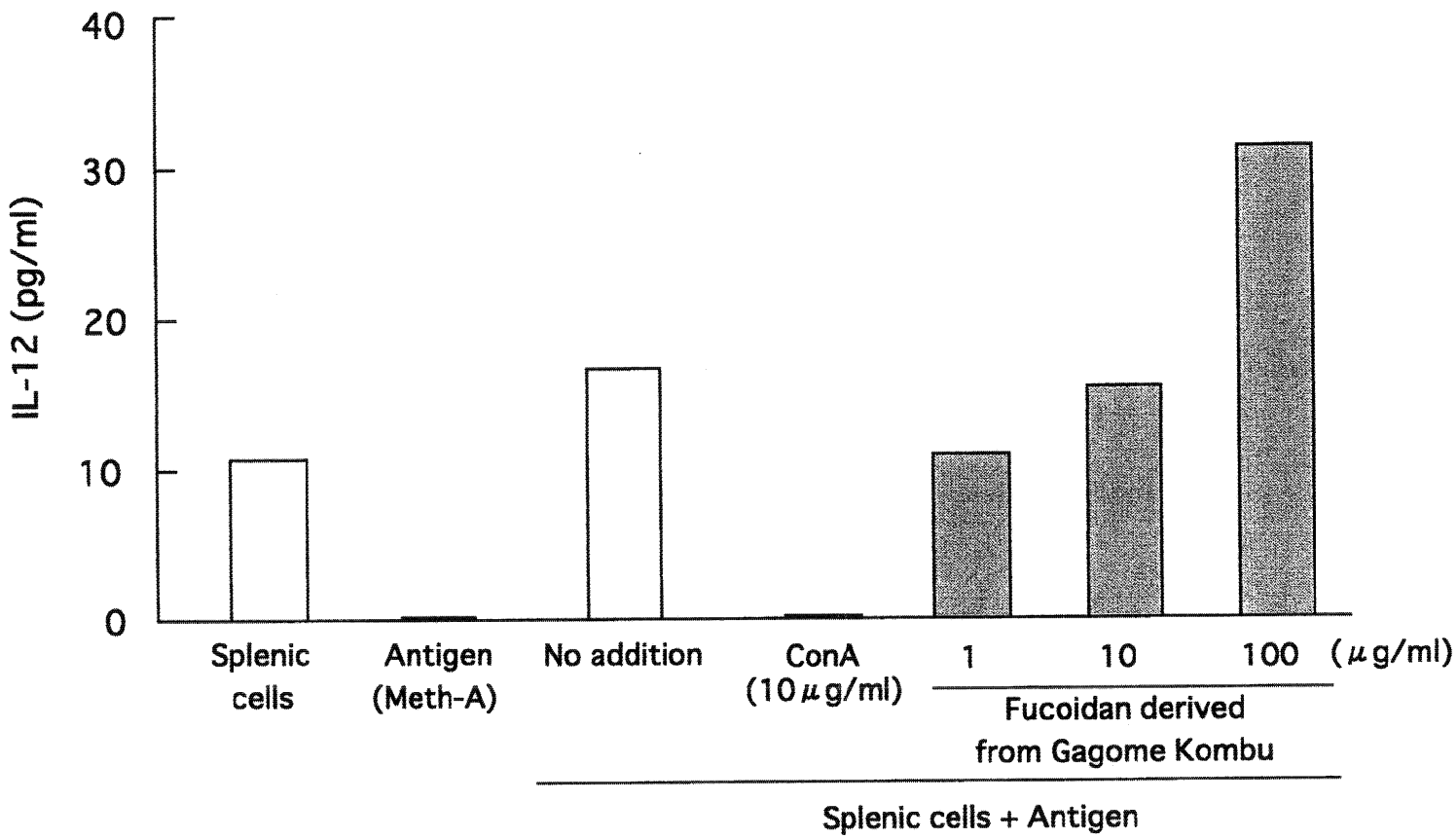


Fig.3 Induction of IL-12 (p70) production by fucoidan from Gagome Kombu in splenic lymphocytes of antigen-sensitized mice under conditions of antigen stimulation

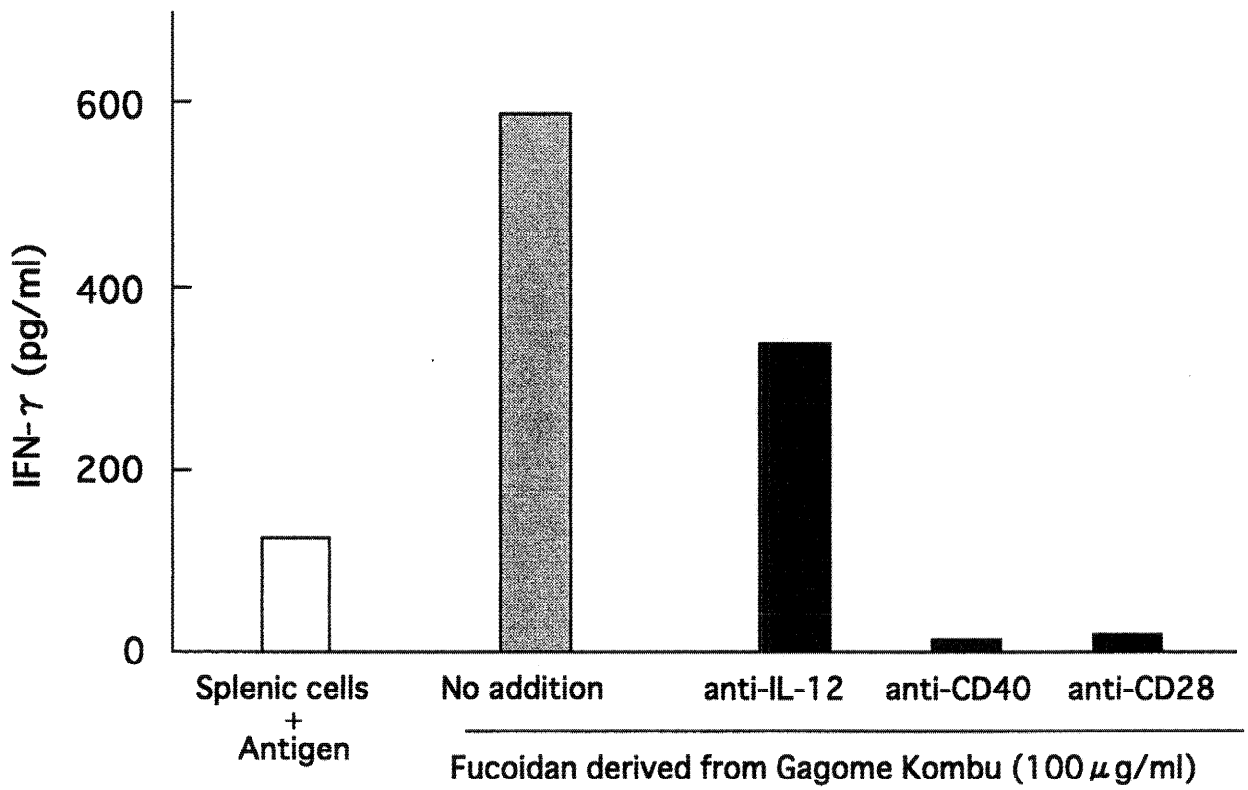


Fig. 4 Effects of various antibodies to induction of IFN-γ production by fucodan from Gagome Kombu

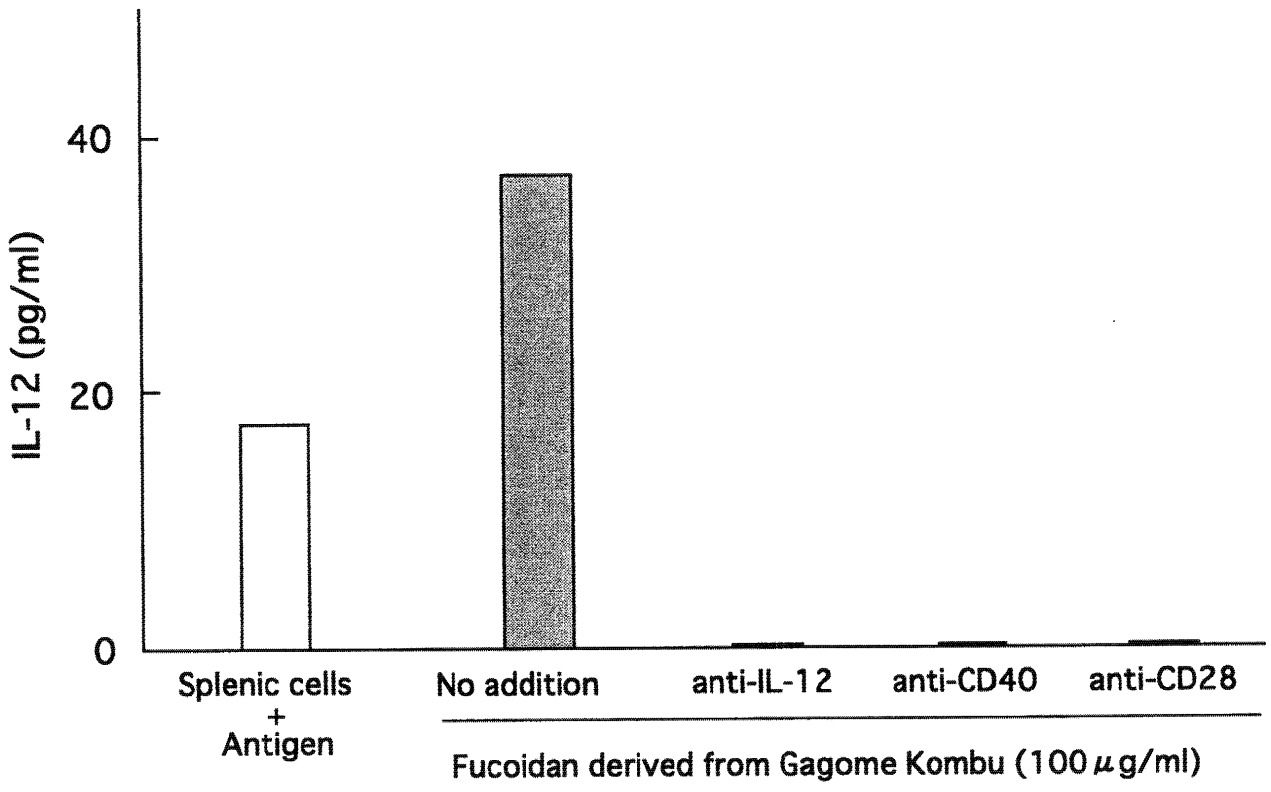


Fig. 5 Effects of various antibodies to induction of IL12 (p70) production by fucoidan from Gagome Kombu

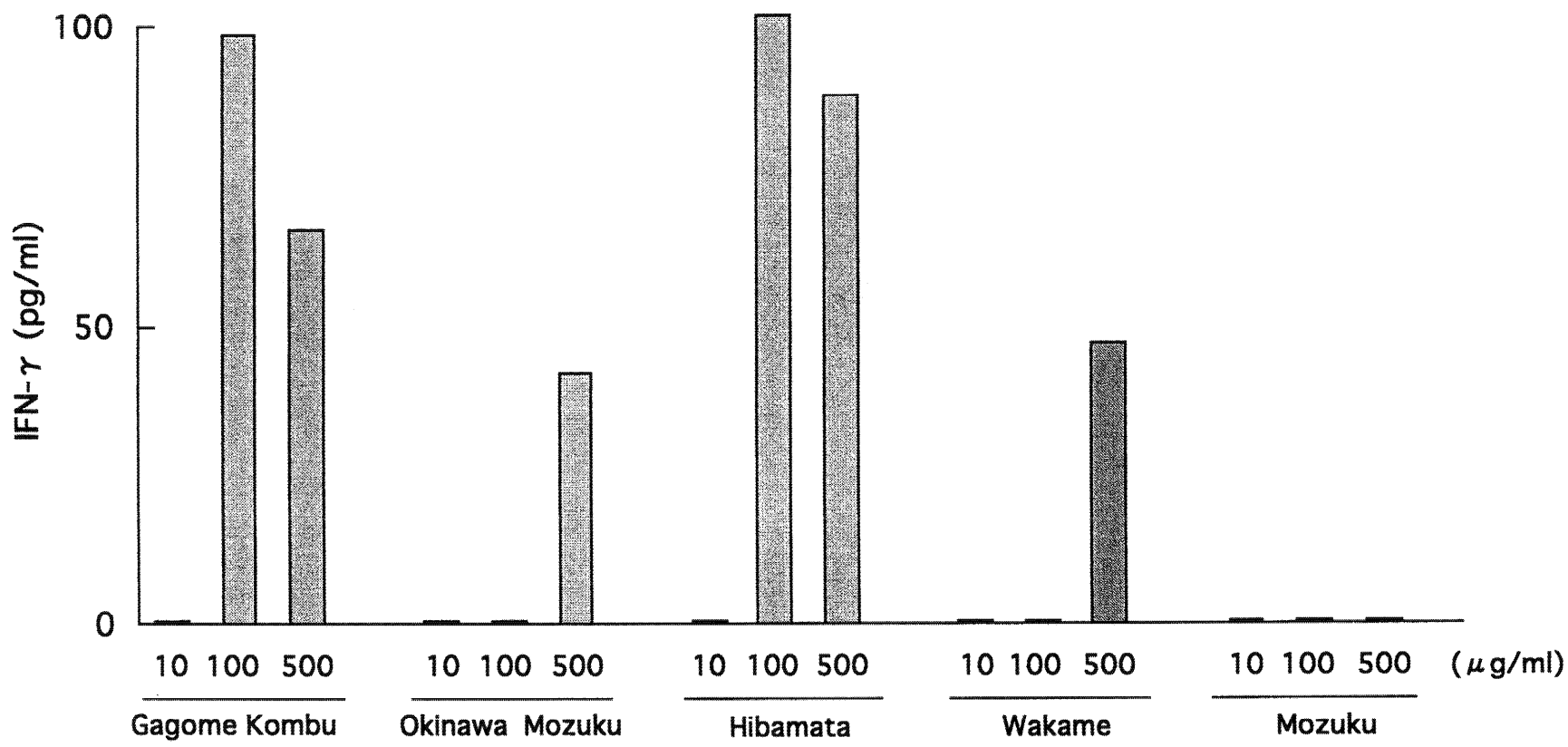


Fig. 6 Induction of IFN-γ production by various kinds of fucoidan

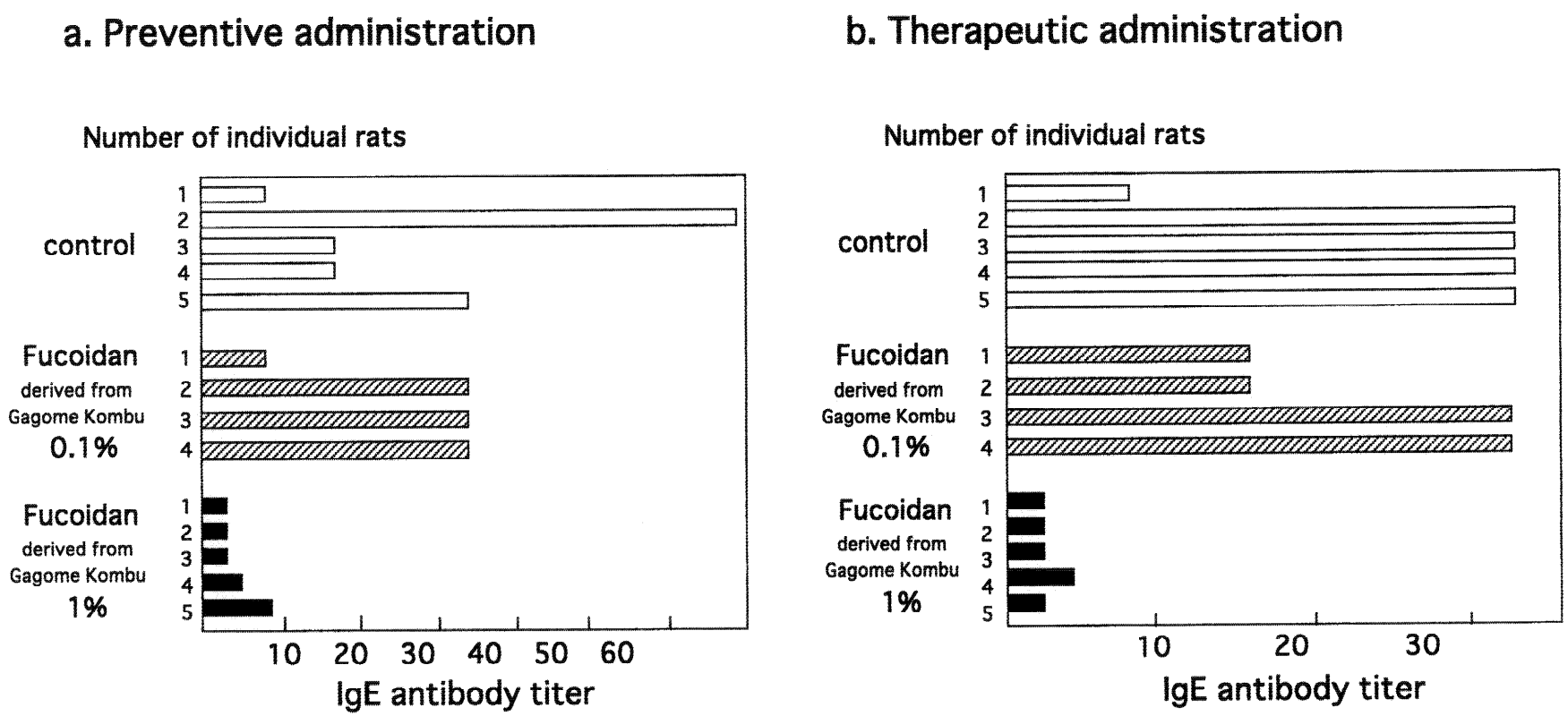


Fig.7 Suppression of antibody production by oral administration of fucoidan derived from Gagome Kombu in the model of IgE antibody production induced by rat OA antigen

Japan Food Science, vol.39, No.9 (2000)

Functionality of fucoidan and its effects

Ikunoshin Kato[1], Takeshi Sakai[2] and Hiroaki Sagawa[1]

[1] *Biotech Res. Lab. Takara Shuzo Co.,Ltd. (Seta 3-4-1, Otsu, Shiga 520-2193, Japan,* [2] *Hirosaki Biotech. Res. Lab. Takara Shuzo Co.,Ltd. (Zaifu-cho 82-4, Hirosaki, Aomori 036-8216, Japan)*

1. Kinds and chemical structures of fucoidan

There are at least 3 kinds of fucoidan derived from the family of *Laminariaceae* (Kombu) in blown seaweed; F-fucoidan consisting of fucose alone, U-fucoidan consisting of glucuronic acid, mannose and fucose, and G-fucoidan consisting of galactose and fucose[1)](Fig. 1). In all types of fucoidan, fucose is sulfated. There is another type of fucoidan derived from *Cladosiphon okamuranus* (Okinawa Mozuku) belonging to the family of *Chordariaceae* in blown seaweed, which has been studied in Japan. A study group of Yakuruto Inc. has reported the putative chemical structure of the fucoidan derived from Okinawa Mozuku[2)]. We also determined the absolute chemical structure of fucoidan derived from Okinawa Mozuku using an enzyme[3)] that specifically degrades this fucoidan(Fig.1).

2. Functionality of fucoidan

2-1. Fucoidan induces the production of Hepatocyte Growth Factor (HGF).

HGF is a cytokine found as a potent inducer of liver regeneration by T. Nakamura et al. of Osaka University in 1984, which is a relatively large heparin-binding polypeptide (85 kDa)[4]. A very wide range of physiological action of HGF has been reported, such as the effectiveness of treatment for hepatic cirrhosis and alcoholic hepatitis[5,6] and ischemic diseases caused by diabetes[7]. Gene therapy using HGF genes for ischemic diseases, in which the peripheral blood vessels are deteriorated by diseases such as diabetes and amputation of the lower limbs is required, has been attempted. In other words, HGF restores blood vessels. HGF is regarded as one of the biological restoration factors showing treatment effects of various disorders.

Our group found that F-fucoidan and G-fucoidan enhance in vivo and in vitro production of HGF[8]. This indicates that oral uptake of these fucoidans, which are the main constituents of brown seaweed, enhances in vivo production of HGF. Recently, we learned by chance that it is tradition in Korea to greet women soon after giving birth by saying "Have you had Wakame soup?" (Wakame, Undaria pinnatifida). This would indicate that Koreans had known by their long experiences the fact that oral uptake of fucoidan enhances the production of HGF.

F-fucoidan from *Kjellmaniella crassifolia* (Gagome Kombu), enzymatic degradated product of F-fucoidan (7-12S Fd-F) and G-fucoidan enhanced HGF production as same as heparin, but enzymatic degradated product of U-fucoidan (3-1S Fd-U) did not (Fig. 2). Fucoidan from Kombu, fucoidan derived from Wakame, Ressonia and *Nemacystus decipiens* (Mozuku gathered in the Sea of Japan) enhanced HGF production, but fucoidan from Okinawa Mozuku did not (Fig. 3). These results suggested that the degree of sulfation of fucoidan is related with the enhancement activity of HGF production.

2-2. Fucoidan induces production of Interleukin-12 and Interferon-γ.

Interleukin-12 (IL-12) was found as a cytokine that induces production of interferon-γ (IFN-γ). IL-12 induces proliferation of T cells and natural killer cells (NK cells), and induces not only cytotoxic activity, which is effective in attacking cancer cells, but also IFN-γ. Therefore, it is expected that IL-12 is effective in treating patients with immunodeficiency such as AIDS and patients with cancer.

IFN-γ was found as a lymphokine showing anti-viral activity that is produced in lymphocytes. IFN-γ, which is produced in T cells and NK cells as described above, has been reported to be a multifunctional factor showing various physiological activities such as anti-viral activity, suppression of cell growth, anti-tumor effects and enhancement of the activity of macrophages and NK cells, and is clinically used for treatment of chronic viral hepatitis type C and renal cancer.

We reported last year that the production of such important immunoenhancers, IL-12 and IFN-γ, is induced by fucoidan[9]. In spleen cells of mice with a cancer cell line (Meth-A), production of IL-12 and IFN-γ was induced by fucoidan mixtures (F, G, U) derived from Gagome Kombu in proportion to its concentration (Fig. 4). It was also found that fucoidan derived from Gagome Kombu and *Fucaceae* (Hibamata) among brown seaweed has very strong induction activity of IFN-γ production. The induction activity of IFN-γ in fucoidan derived from Okinawa Mozuku and Wakame was about half of that in fucoidan derived from Gagome Konbu and Hibamata. On the other hand, fucoidan derived from Mozuku in the Sea of Japan had no induction activity of IFN-γ.

Such cytokines are induced by fucoidan only in spleen cells of mice with cancer cells, i.e., indicating that IL-12 and IFN-γ are induced by fucoidan only when cancer antigens are presented to T cells by antigen-presenting cells (APC). Therefore, antibodies against CD28, the surface antigen of T cells, and CD40, the surface antigen

of APC, both of which are required for interaction between T cells and APC, completely block the induction of IL-12 and IFN-γ by fucoidan. Th0 cells that are activated by antigen proliferate and differentiate into either Th1 or Th2 cells, which are capable of specific immune responses. Th1 cells mediate cell-mediated immunity, and Th2 cells mediate humoral immunity. For example, if viral infection occurs, IL-12 is initially produced by macrophages, and the surrounding helper T cells differentiate into Th1 cells, then Th1 cells attack the infected cells. In other words, IL-12 is the cytokines that induce Th0 cells to differentiate into Th1 cells, and these cells produce cytokines such as IL-2, IL-3 and IFN-γ.

It was clear that fucoidan is a potent inducer of IL-12 and IFN-γ and causes Th0 cells to differentiate into Th1 cells. A similar situation has been reported[10]. OK-432 prepared from inactivated Streptococcus by penicillin G, which is commercially available as an adjuvant for treatment of cancer (Picibanil, Chugai Pharmaceuticals Inc.), is a potent inducer of IL-12, and induces Th0 cells to differentiate into Th1 cells.

2-3. Fucoidan suppresses the production of immunoglobulin antibody that causes allergic reaction.

The level of immunoglobulin E (IgE) is high in patients with allergosis and in patients with vermination. Basophils, mast cells and eosinophils sensitized by binding of IgE to FCε receptors on their surfaces release histamine, serotonin and various cytokines upon stimulation of allergens.

As described above, fucoidan induces IL-12 production, and IL-12, with which the Th-responsiveness is switched to the Th1 state, induces IFN-γ and also reduces the production of IL-4, which is involved in production of IgE. Administration of ovalbumin and alam into the abdominal cavity of rats elevated the antibody titer of IgE.

We examined the antibody titer of IgE in such rats, which received preventive oral administration of a fucoidan solution over about 3 weeks from 1 week before administration of these allergens or therapeutic oral administration of a fucoidan solution over 2 weeks from about 3 weeks after administration of the allergens . There were no changes in the antibody titer of IgE in the rats by the administration of 0.1% fucoidan solution, while in the rats received 1% fucoidan solution, the antibody titer of IgE was markedly reduced (Table 1). These results suggested that allergy is prevented and treated by oral uptake of fucoidan.

Conclusions

It was clarified that Kombu, Wakame and Mozuku, which have been very common constituents of traditional oriental diet, are potent inducer of Th1 cells. As described above, such immunopotentiation activity is generally observed in sulfated saccharides, but the mechanism of induction of Th1 cells remains to be elucidated.

References

1) Sakai, T., Kimura, H., Katayama, K., Shimanaka, K., Ikai, K. and Kato, I., Three kinds of enzymes that degrade sulfated fucose-containing polysaccharide from brown seaweed, fucoidanase, sulfated fucoglucuronomannanlyase, and sulfated fucogalactanase, *Glycoconjugate J.*, **16**, S122 (1999)

2) Nagaoka, M., Shibata, H., Kimura-Takagi, I., et al., Structural study of fucoidan from *Cladosiphon okamuranus* TOKIDA., *Glycoconjugate J.*, **16**, 19-26 (1999)

3) Sakai, T., Ishizuka, K., Kojima, K., Shimanaka, K., Ikai, K., Kato, I., Two novel enzymes derived from oceanic bacteria, a-D-glucuronidase and endo-a-L-fucosidase, and their use for analysis of the structure of fucoidan (sulfated glucuronofukan) derived from Okinawa mozuku, Abstract of the 21st Annual Meeting of the Japan Carbohydrate Society, **64**, Nagoya (2000)

4) Nakamura, T., Nawa, K. and Ichihara, A., Partial purification and characterization of Hepatocyte Growth Factor from serum of hepatectomized rats, *Biochem. Biophys. Res. Commun.*, **122**(3), 1450-1459 (1984)

5) Ueki, T., Kaneda, Y., Tsutsui, H., Nakanishi, K., Sawa, Y., Morishita, R., Matsumoto, K., Nakamura, T., Takahashi, H., Okamoto, E. and Fujimoto, J., Hepatocyte growth factor gene therapy of liver Cirrhosis in rats, *Nature Medicine*, **5**, 226-230 (1999)

6) Tahara, M., Matsumoto, K., Nukiwa, T. and Nakamura, T., Hepatocyte growth factor leads to recovery from alcohol-induced fatty liver in rats, *J. Clinical Investigation*, **103**, 313-320 (1999)

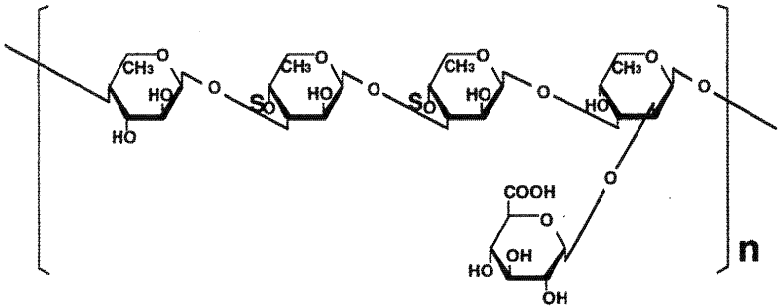
7) Morishita, R., Nakamura, S., Hayashi, S., Taniyama, Y., Moriguchi, A., Nagano, T., Taiji, M., Noguchi H., Takeshita S., Matsumoto, K., Nakamura, T., Higaki, J., and

Ogihara, T., Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy, *Hypertension*, 33(6), 1379-1384 (1999)

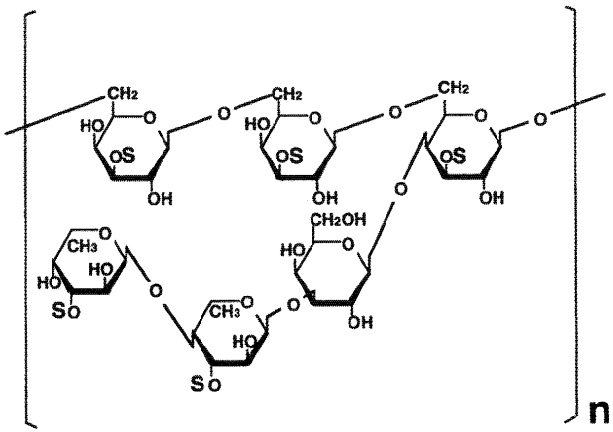
8) Sagawa, H., Akiyama, K., Ohno, M., Sakai, T., Kato, I., Induction of hepatocyte growth factor by fucoidan derived from brown algae, Abstract of the 72nd Meeting of the Japan Biochemical Society, 2P-002, Yokohama (1999)

9) Tominaga, T., Mizutani, S., Sakai, T., Sagawa, H., Kato, I., Induction of IL-12-dependent IFN-γ by fucoidan derived from gagome konbu under conditions of stimulation by antigens, Abstract of the Annual Meeting of the Japan Agricultural Chemistry Society in 2000, 74, 77, Tokyo (2000)

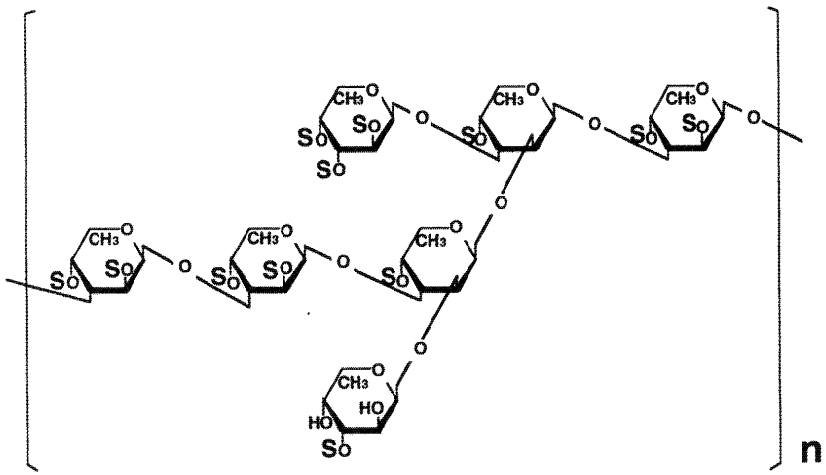
10) Fujimoto, T., Duda, RB., Szilvasi, A., Chen, X., Mai, M. and O'Donnell, MA., Streptococcal preparation OK-432 is a potent inducer of IL-12 and a T helper cell 1 dominant state, *J. Immunol.*, 158, 5619-5626 (1997)



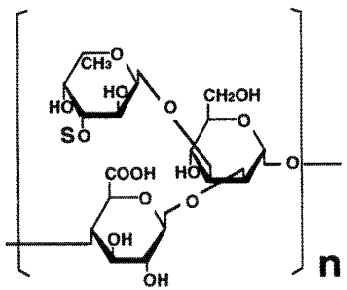
Fucoidan derived from Okinawa mozuku



G-fucoidan derived from Gagome kombu

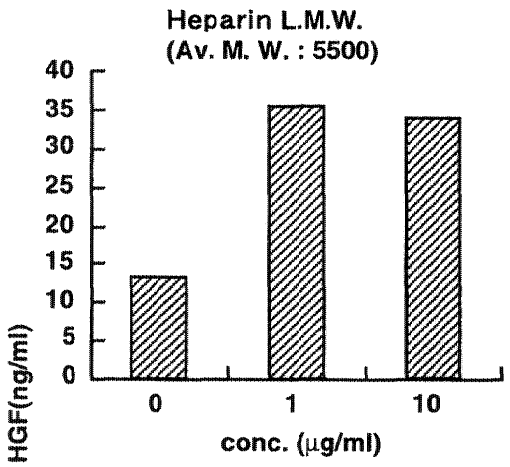


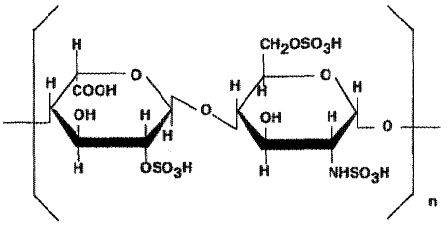
F-fucoidan derived from Gagome kombu



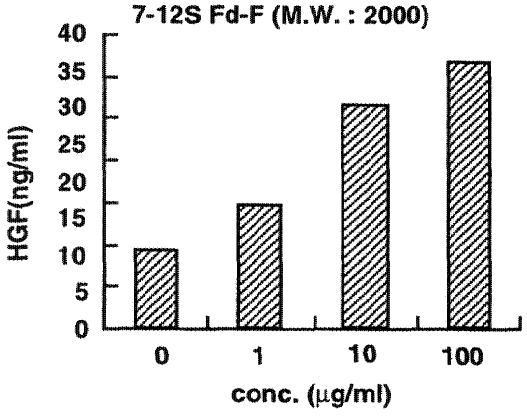
U-fucoidan derived from Gagome kombu
(S = SO_3H)

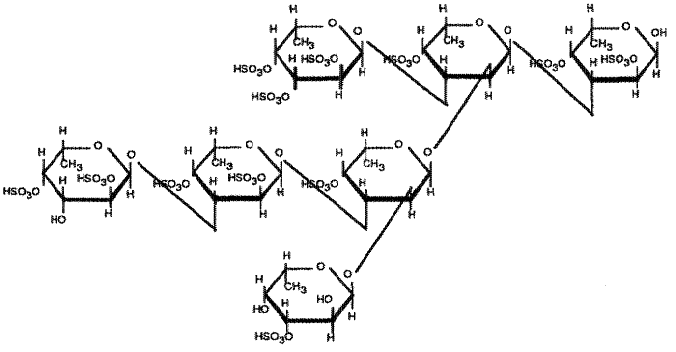
Fig.1



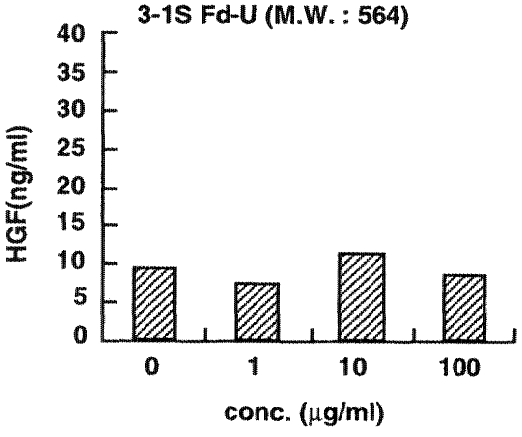


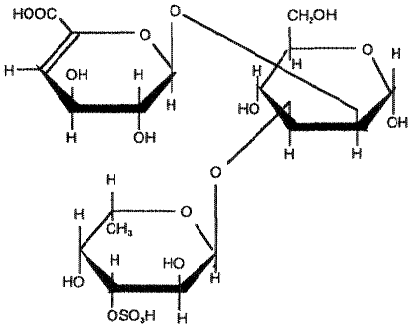
Heparin





F-Fd emzyme degradation product
(7-12S Fd-F)





U-Fd emzyme degradation product
(3-1S Fd-U)

Fig.2

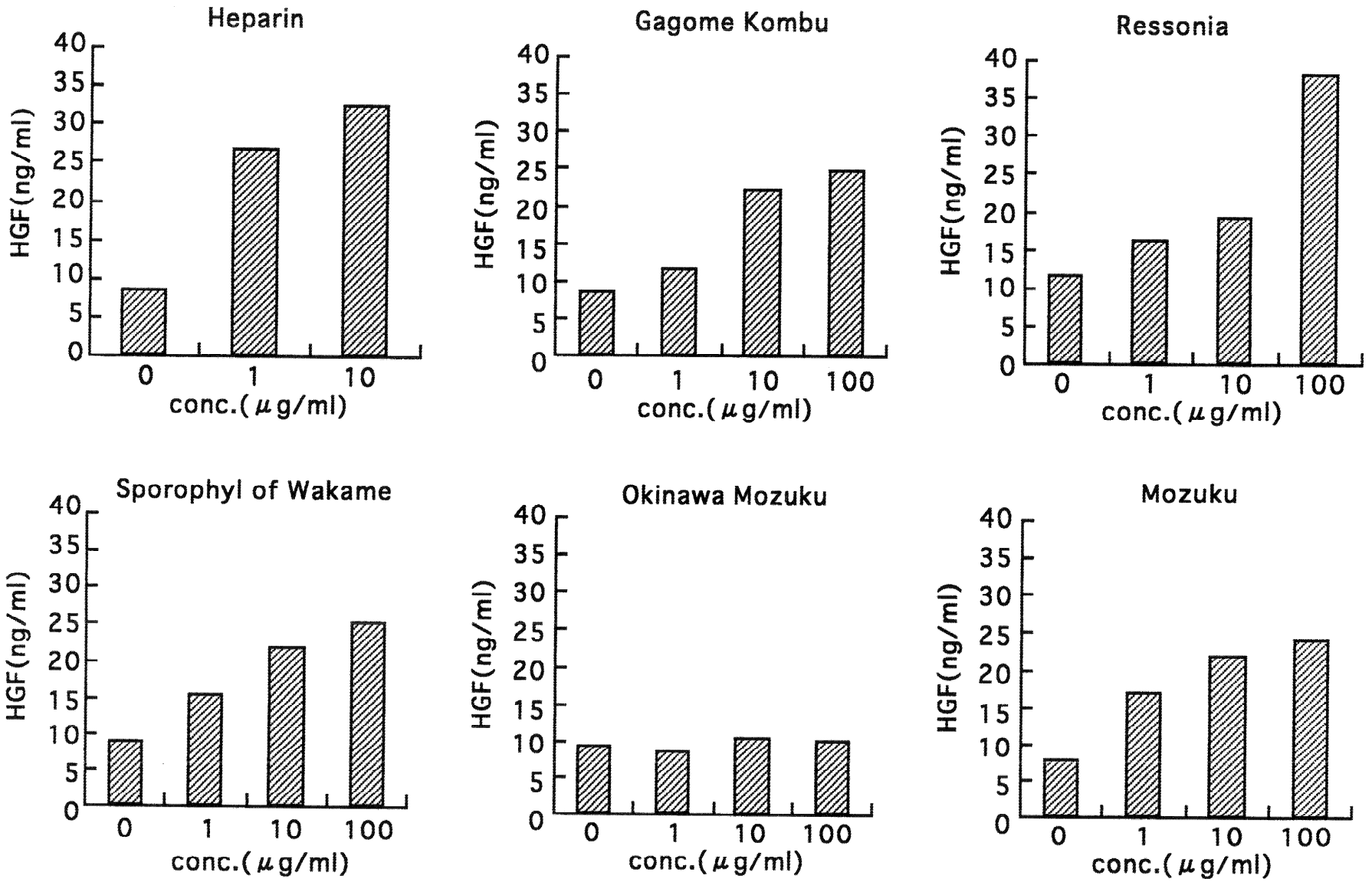

Heparin
Gagome Kombu
Ressonia
Sporophyl of Wakame
Okinawa Mozuku
Mozuku
HGF(ng/ml)
0
5
10
15
20
25
30
35
40
0
1
10
100
conc.(μg/ml)

Fig.3

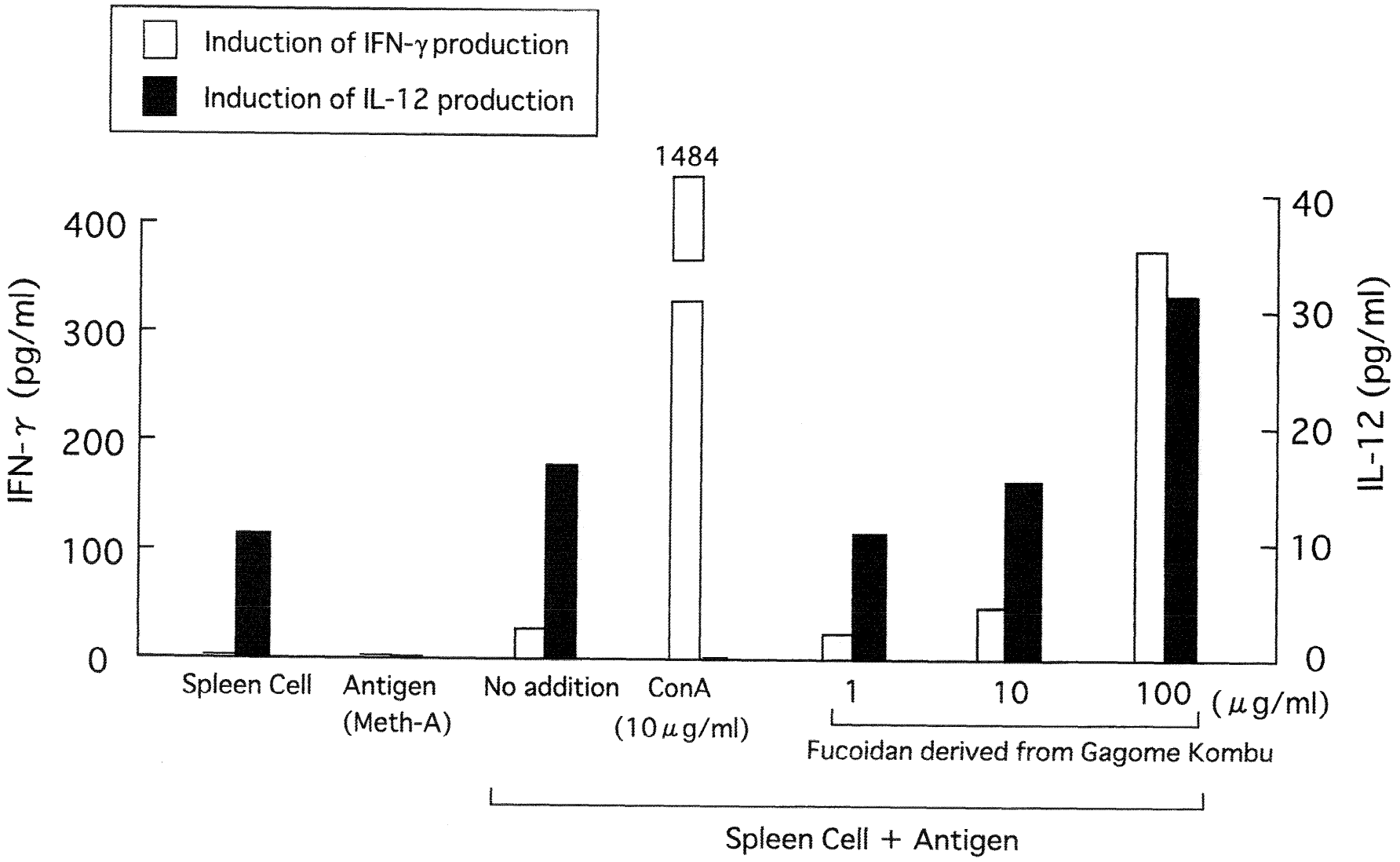

Induction of IFN-γ production
Induction of IL-12 production
1484
400
300
200
100
0
IFN-γ (pg/ml)
40
30
20
10
0
IL-12 (pg/ml)
Spleen Cell
Antigen (Meth-A)
No addition
ConA (10μg/ml)
1
10
100
(μg/ml)
Fucoidan derived from Gagome Kombu
Spleen Cell + Antigen

Fig.4

Table 1

	IgE antibody titer	
	Preventive administration	Therapeutic Administration
Control	8	8
	64	32
	16	32
	16	32
	32	32
Fucoidan 0.1%	8	16
	32	16
	32	32
	32	32
Fucoidan 1%	< 2	2
	< 2	2
	< 2	2
	4	4
	8	2

Publications of Study on Kombu Fucoidan (1992-2000)

	Academic society	Period	Place	No	Subject	Speakers
1	The 65th Meeting of the Japanese Biochemical Society	1992.10.9-11	Fukuoka	2268	Studies on fucoidan-degrading enzymes in the disgestive tract of *Anthocidaris crassipina.*	T.Sakai, H. Amarume, K. Sasaki, Y. Nakanishi , I. Kato and M. Endo
2	The 66th Meeting of the Japanese Biochemical Society	1993.10.2-4	Tokyo	3053	Studies on fucoidan-degrading enzymes in the digestive tract of *Strongylocentrotus nudus*(First report).	K. Sasaki, H. Amarume, T. Sakai. Y. Nakanishi and I. Kato
3	The 66th Meeting of the Japanese Biochemical Society	1993.10.2-4	Tokyo	3054	Studies on fucoidan-degrading enzymes in the digestive tract of *Strongylocentrotus nudus*(Second report).	T. Sakai, H. Amarume, K. Sasaki, Y. Nakanishi and I. Kato
4	The 67th Meeting of the Japanese Biochemical Society	1994.9.7-10	Osaka	2702	Preparation of novel oligosaccharides from fucoidan(First report).	S. Nakamura, K. Kojima, K. Ikai, T. Sakai, Y. Nakanishi and I. Kato
5	The 67th Meeting of the Japanese Biochemical Society	1994.9.7-10	Osaka	2703	Preparation of novel oligosaccharides from fucoidan(Second report).	K. Kojima, S. Nakayama, K. Ikai, T. Sakai, Y. Nakanishi and I. Kato
6	The 67th Meeting of the Japanese Biochemical Society	1994.9.7-10	Osaka	3703	Studies on fucoidan-degrading enzymes in the digestive tract of *Strongylocentrotus nudus*.	K. Sasaki, K. Kojima, S. Nakayama, T. Sakai, Y. Nakanishi and I. Kato
7	The Annual Meeting of the Japanese Society for Bioscience, Biotechnology, and Agrochemistry in 1995	1995.8.2-4	Sapporo	2Fa11	Purification and characterization of a novel endo-fucoidan-lyase;Elucidation of mechanism of enzymatic reaction and the structure of the side chain of fucoidan.	H. Kimura, T. Sakai, K. Kojima, Y. Nakanishi, K. Ikai and I. Kato
8	17th Japanese Carbohydrate Symposium	1995.7.18-20	Kyoto	D-19	Elucidation of structure of fucoidan by degradation using novel endo-fucoidan-lyase and acid.	T. Sakai, H. Kimura, K. Kojima, S. Nakayama, Y. Nakanishi, K. Ikai and I. Kato
9	XIIIth International Symposium on Glycoconjugates	1995.8.20-26	Seattle	S6	Novel sulfated trisaccharides produced from fucoidan by a novel bacterial endo-fucoidan-lyase degradation and elucidation of long side chain of fucoidan.	T. Sakai, H. Kimura, K. Kojima, Y. Nakanishi, K. Ikai and I. Kato
10	The 68th Meeting of the Japanese Biochemical Society	1995.9.16-18	Sendai	4205	Preparation of novel oligosaccharides from fucoidan(Third report).	S. Nakamura, K. Kojima, K. Ikai, T. Sakai, Y. Nakanishi and I. Kato
11	The 68th Meeting of the Japanese Biochemical Society	1995.9.16-18	Sendai	4206	Discovery of a novel endo-fucoidan-lyase produced by a fucoidan-utilizing bacterial strain(genus *Flavobacterium*).	T. Sakai, H. Kimura, K. Kojima, Y. Nakanishi, K. Ikai and I. Kato

	Academic society	Period	Place	No	Subject	Speakers
12	The 68th Meeting of the Japanese Biochemical Society	1995.9.16-18	Sendai	4207	Purification and characterization of a novel endo-fucoidan-lyase produced by a bacterial strain among genus *Flavobacterium* and large scale preparation of the products of the enzymatic reaction .	H. Kimura, T. Sakai, K. Kojima, Y. Nakanishi, K. Ikai and I. Kato
13	The 68th Meeting of the Japanese Biochemical Society	1995.9.16-18	Sendai	4208	Elucidation of the structure of the enzymatically produced oligosaccharides,reaction mechanism of a novel endo-fucoidan-lyase produced by a bacterial strain among genus *Flavobacterium*, and the structure of the side chain of fucoidan.	K. Kojima, H. Kimura, T. Sakai, Y. Nakanishi, K. Ikai and I. Kato
14	The 18th Japanese Carbohydrate Symposium	1996.8.19-21	Tokyo	A3-12	Apoptosis of human carcinoma cell lines induced by fucoidan(sulfated fucose-contaning polysaccharide) and its degraded fragments by fucoidanase and endo-fucoidan lyase.	F.Yu,H.Kitano,T.Sakai,K.Katayama, Y.Nakanishi,K.Ikai and I.Kato
15	The 18th Japanese Carbohydrate Symposium	1996.8.19-21	Tokyo	P-28	Two types of fucoidans(sulfated fucose-containning polysaccharides),two types of fucoidan-degrading enzymes,and their products.	T.Sakai,H.Kimura,S.Nakayama,K. Katayama,Y.Nakanishi and I.Kato
16	The 69th Meeting of the Japanese Biochemical Society	1996.8.26-30	Sapporo	1-P-0024	Purification of a novel endo-fucoidan-lyase produced by a bacterial strain among genus *Flavobacterium* and large scale preparation of the products of the enzymatic reaction(Second report).	H.Kimura,T.Sakai,K.Kojima,Y. Nakanishi,J.Akiyoshi,K.Ikai and I.Kato
17	The 69th Meeting of the Japanese Biochemical Society	1996.8.26-30	Sapporo	1-P-0025	Analysis of the structure of enzymatically produced oligosaccharides by endo-fucoidan -lyase from a bacterial strain among genus *Flavobacterium* and elucidation of the structure of glucuronic acid-containning fucoidan.	K.Kojima,H.Kimura,T.Sakai,Y. Nakanishi,J.Akiyoshi,K.Ikai and I.Kato
18	The 69th Meeting of the Japanese Biochemical Society	1996.8.26-30	Sapporo	1-P-0026	Purification and characteriztion of fucoidanase from a sea bacterial strain *Alteromonas sp.* SN-1009.	S.Nakayama,K.Kojima,H.Kimura,T. Sakai,K.Katayama,K.Shimanaka,K.Ik ai,Y.Nakanishi and I.Kato
19	The 69th Meeting of the Japanese Biochemical Society	1996.8.26-30	Sapporo	5-P-0677	Apoptosis of human gastric and colon carcinoma cell lines induced by fucoidan from *Kjellmaniella crassifolia* and its degraded products by enzymes.	F.Yu,H.Kitano,T.Sakai,K.Ikai,Y. Nakanishi,K.Katayama and I.Kato
20	The 69th Meeting of the Japanese Biochemical Society	1996.8.26-30	Sapporo	5-P-0678	Induction of apoptosis of HL-60 cells by fucoidan.	H.Kitano,F.Yu,T.Sakai,K.Katayama, Y.Nakanishi,K.Ikai and I.Kato
21	The 55th Annual Meeting of the Japanese Cancer Association	1996.10.10-12	Yokohama	225	Apoptosis of human gastric and colon carcinoma cell lines induced by fucoidan of *Kjellmaniella crassifolia* Miyabe and its degraded fragments by fucoidanase.	F.Yu,H.Kitano,T.Sakai,K.Ikai and I.Kato

	Academic society	Period	Place	No	Subject	Speakers
22	The 55th Annual Meeting of the Japanese Cancer Association	1996.10.10-12	Yokohama	1183	Induction of apoptosis of HL-60 cells by sulfated fucose-containing polysaccharides(fucoidan).	T.Sakai,H.Kitano,F.Yu,K.Ikai and I.Kato
23	The Annual Meeting of the Marine Biotechnology in 1997	1997.5.31-6.1	Tokyo	SE104	Endo-fucoidan-lyase that degrades U-Fucoidan (Fucoglucuronomannan sulfate) and fucoidanase that degrades F-Fucoidan(Galactofucan sulfate).	T.Sakai and I.Kato
24	GYCOCONJUGATES AND MATRIX MOLECULES IN HEALTH AND DISEASE An International Conference	1997.8.20-22	Bethesda, Maryland	—	Apoptosis of human carcinoma cell lines induced by fucoidan	I. Kato, T. Sakai, F. Yu and K. Ikai
25	The 56th Annual Meeting of the Japanese Cancer Association	1997.9.25-27	Kyoto	DQ0-04	Relationship between the structure and apoptosis-inducing activity of U-fucoidan of *Kjellmaniella crassifolia* Miyabe.	F.Yu,H.Kitano,T.Sakai,N.Koyama,Y. Tatsumi,K.Ikai and I.Kato
26	The 20th Japanese Carbohydrate Symposium	1998.7.15-17	Sapporo	P(II)-09	Structures and substrate specificites of two enzymes capable of degrading of different fucoidan species.	M.Mitta,T.Sakai,H.Kimura,Y.Nomura, N.Koyama,K.Katayama and I.Kato
27	The 20th Japanese Carbohydrate Symposium	1998.7.15-17	Sapporo	B3-13	Determination of the structure of F-Fucoidan using newly discoverded fucoidanase.	T.Sakai,H.Kimura,K.Kojima,K.Katayama,K.Shimanaka,K.Ikai and I.Kato
28	The 49th Fourum for Protein Structure	1998.9.24-26	Nagaoka	C-15	Relationships between mechanisms and primary structures of two fucoidanases and two sulfated fucoglucuronomannan-lyases.	T.Sakai,M.Mitta,H.Kimura,N.Koyama, K.Katayama and I.Kato
29	The 57th Annual Meeting of the Japanese Cancer Association	1998.9.30-10.2	Yokohama	2233	Anti-tumor activity of fucoidan from *K. crassifolia* Miyabe against azoxymethaneinduced tumor and human colon cancer xenografts.	F.Yu,T.Sakai and I.Kato
30	XV International Symposium on Glycoconjugates	1999.8.22-8.7	Tokyo	2ap258	Three kinds of enzymes that degrade sulfated fucose-containing polysaccharide from brown seaweeds, fucoidanase, sulfated fucoglucuronomannan-lyase,and sulfated fucogalactanase.	T. Sakai, H. Kimura, K. Shimanura, K. Ikai and I. Kato
31	The 72th Meeting of the Japanese Biochemical Society	1999.10.7-9	Yokohama	2P-002	Hepatocyte growth factor inducing activity of brown seaweed fucoidan.	H.Sagawa,K.Akeyama,H.Onoki,T.Sakai and I.Kato
32	The 72th Meeting of the Japanese Biochemical Society	1999.10.7-9	Yokohama	2P-003	Structural analysis of fucoidan oligosaccharides using ion spray mass spctrometer	K.Kojima,H.Kimura,T.Sakai,K.Shimanaka,K.Ikai and I.Kato
33	The Autumn Symposium of the Japanese Phycology Society in 1999	1999.11.29	Tokyo		Decrease of the risk of cancer by dietary fiber from seaweed.	I.Kato and H.Sagawa
34	The Annual Meeting of the Japan Society for Bioscience,Biotechnology,and	2000.3.31-4.2	Tokyo	2E139 β	Inducing activity of IL-12 dependent IFN-γ by *Kjellmaniella crassifolia* fucoidan under the stimulation with the antigen.	T.Tominaga,S.Mizutani,T.Sakai,H.Sagawa and I.Kato

	Academic society	Period	Place	No	Subject	Speakers
35	The Annual Meeting of the Japan Society for Bioscience,Biotechnology,and Agrochemistry in 2000	2000.3.31-4.2	Tokyo	2E201 α	Increase of production of Hepatocyte growth factor by 7-12SFd-F.	H.Sagawa,K.Akeyama,H.Onoki,M. Shraga,T.Sakai and I.Kato
36	The Annual Meeting of the Japan Society for Bioscience,Biotechnology,and Agrochemistry in 2000	2000.3.31-4.2	Tokyo	3C029 α	Large scale preparation of sulfated trisaccharide and hexasaccharide by using two kind of recombinant sulfated fucoglucuronomannan lyase.	K.Miyatake,H.Kimura,K.Kojima,M. Takayama,T.Sakai and I.Kato
37	The Annual Meeting of the Japan Society for Bioscience,Biotechnology,and Agrochemistry in 2000	2000.3.31-4.2	Tokyo	3C030 α	Structural analysis of sulfated fucogalactan using a novel sulfated fucogalactanase(First report).	H.Kimura,K.Kojima,T.Sakai,K. Shimanaka,K.Ikai and I.Kato
38	The Annual Meeting of the Japan Society for Bioscience,Biotechnology,and Agrochemistry in 2000	2000.3.31-4.2	Tokyo	3C031 α	Structural analysis of sulfated fucogalactan using a novel sulfated fucogalactanase(Second report).	K.Shimanaka,H.Kimura,K.Kojima,T. Sakai,K.Ikai and I.Kato
39	The Annual Meeting of the Marine Biotechnology in 2000	2000.5.20-21	Kagawa	B2	Increase of HGF production by *Kjellmaniella crassifolia* fucoidan and hair restoration.	K.Akeyama,S.Deguchi,S.Mizutani,H. Sagawa and I.Kato
40	The 21th Japanese Carbohydrate Symposium	2000.7.27-29	Nagaoka	B3-02	Two nowel enzymes from a sea bacterium, α-D-glucuronidase and endo-α-L-fucosidase and their use for the analysis of the structure of fucoidan from *Cladosiphon okamuranus.*	T.Sakai,K.Ishizuka,K.Kojima,K. Shimanaka,K.Ikai and I.Kato
41	The 59th Annual Meeting of the Japanese Cancer Association	2000.10.4～6	Yokohama	3620	Antigen presenting cell(APC)-dependent IFN-γ-inducing effect of fucoidan from *K.crassifolia* Miyabe in splenic lymphocyte from tumor sensitized mice.	T.Tominaga,E.Nshiyama,T.Sakai,H. Sagawa and I.Kato
42	The 73th Meeting of the Japanese Biochemical Society	2000.10.12-14	Yokohama	1P-001	Cosmetic effect of *K.crassifolia* fucoidan and its fractionated substances on contraction of collagen by skin fibroblast cells.	H.Hua-Kang,M.Yasuda,T.Sakai and I.Kato
43	The 73th Meeting of the Japanese Biochemical Society	2000.10.12-14	Yokohama	1P-002	Study on the hair restoration by *Kjellmaniella crassifolia* fucoidan.	S.Deguchi,K.Fujii,E.Nishiyama,S. Mizutani,K.Akiyama,H.Sagawa and I.Kato
44	The Biotechnology Seminar promoted by Osaka Kouken Society	2000.10.13	Osaka		Characterization and utilization of maltifunctional saccharides from seaweed.	T.Sakai and I.Kato