I The summary of BioBran

Overview of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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Background to the development of BioBran/MGN-3

1) Dietary fiber and immunomodulation

Many polysaccharides in microorganisms used in foods and fermentation have been shown to act as biological response modifiers (BRM) and enhance immunity. These include zymosan (β-1,3 glucan), a component of the cell wall of brewer's yeast; α-1,6 mannan, a component of the cell wall of kitchen and baker's yeast; and β-1,3 glucan, a component of the cell walls in shiitake and enokidake mushrooms and Grifola frondosa. Some of these are taken orally and exert a slight immunomodulatory effect after absorption in the digestive tract. Some are used as immunomodulatory agents in the treatment of cancer.

Most types of dietary fiber are polysaccharides, which are commonly ingested in foods. The ingredients above, on the other hand, are purified and used as pharmaceutical products by health professionals after obtaining manufacturing approval. In this case, they are categorized as drugs rather than food and treated accordingly. However, it is possible that some of the ingredients, taken as dietary fiber in a normal diet, are absorbed in the intestinal tract without changing their structure, and activate immune cells.

2) Immune responses to food ingredients

When foreign material enters the body, the host recognizes it as an antigen and produces antibodies that specifically react against the antigen to provide protection against injury. This is immunity, and it plays an important role in biological defense. The reaction against the antigen may be exaggerated in some cases and acute shock symptoms then appear. This hypersensitivity reaction is called an allergy. As food is a source of foreign matter for the body, it may be antigenic. As shown in the table (**Table 1**), many foodstuffs contain ingredients that can cause allergies.

Food allergies often occur as immediate reactions and mainly consist of type I reactions. These are typical allergic reactions in which IgE antibody plays an important role. However, some food allergies involve a type-IV delayed reaction. In this case, an antigen stimulates lymphocytes, and encourages them to activate, proliferate, and differentiate in to induce sensitized lymphocytes (T cells). When the antigen enters again, T cells react against it and release various lymphokines, proteins with a biological action, which may act on tissue cells and cause inflammation.

Although allergenic ingredients in foods are a negative factor, in that they induce adverse reactions, some of them, in terms of stimulation of immune system, especially those that induce type-IV reactions, and for which the reaction is mild, may enhance immunity, in other words, act as positive factors, as with BRMs. In fact, the cow's-milk protein casein, lysophospholipid, a kind of phospholipid which induces interferon, some fruit such as kiwi fruit and apples, and vegetables such as spinach, onions and carrots cause neutrophils to accumulate. It is fair to say that foods taken daily protect us from infections and malignant tumors.

If an immunomodulatory food that enhances immunity more actively can be conveniently ingested, it could become widely used in the maintenance and promotion of health and the prevention and treatment of disease.

Among the ingredients in immunomodulatory foods, there are those that exist in foods as intact components with immunomodulatory activity, those whose inactive parent compound is degraded into fragments in the digestion process and whose special fragments have an immunomodulatory function, and those that develop their function after undergoing processing. Food ingredients are very complex compounds and are digested into many substances after being taken into the body. In addition, foodstuffs are often processed to make them easier to eat and preserve, which changes their ingredients, resulting in a still wider variety.

Table 1

Foodstuffs that may be allergenic	
Animal and fish foods	Hens' eggs (the whole egg, white and yolk) and egg products, cows' milk and milk products, beef, pork, ham, sausage, bacon, mackerel, bonito, horse mackerel, sardine, saury, tuna, squid, octopus, shrimp, oyster, crab, and steamed fish paste
Plant foods	Soy beans and their products (miso paste, soy sauce, and tofu), sweetcorn, sugarcane, aroid, aubergines, tomatoes, spinach, bamboo shoots, buckwheat and its products, Japanese udon noodles, bread, konjac mannan, citrus fruits (such as satsumas), and cocoa
Others	Yeast (baker's yeast and brewer's yeast), beer, sake, whisky, wine, and chocolate

From Food Hygiene edited by Sawamura, Hamada, and Hayatsu, p. 96, Nankodo, Tokyo (1986)

3) The significance of and requirements for immunomodulatory food products

A number of pharmaceutical products that enhance immune function, such as *Bordetella pertussis* vaccine,

haemolytic streptococcal preparations, and basidiomycetous preparations (from the kawaratake, shiitake, and suehirotake mushrooms) have been developed and used for practical purposes, mainly in the treatment of cancer. Most components of these products are also present in food products but the methods of using foods and pharmaceutical products are very different, even where the same effect is generated. As the amount of food ingested depends on the mental and physical conditions of the individual, it is generally difficult to specify doses. It is therefore undesirable that the amount of active ingredients in foods exceeds a fixed level. In contrast, with pharmaceutical products a proper dose is specified and they are used under medical supervision because they may produce toxic effects if taken in excessive doses.

However, food products used to enhance immunity are different in their function from other foods. They are foods but work almost as medicines. Here, emphasis is placed on their tertiary function, their effect on the immune system, rather than taste or value as energy sources. Therefore, immunomodulatory foods should meet the following requirements:

① Their active ingredients should be known.

② The content and presence of the ingredient in the food should be known.

③ The mechanism of their action should be clear.

④ The food should be highly safe.

⑤ The food should be effective when taken orally.

Foods mentioned above will enable easy health maintenance by adults. We are surrounded with many immunocompromising factors such as stress, smoking, electromagnetic radiation, heat, chemotherapy, and inevitable aging. A compromised immune system lead to problems such as infections, malignant tumors, adult diseases and endocrine-related disorders and delays the return to health. Using immunomodulatory foods properly will make for easy health maintenance and increase our quality of life.

I-2

Production of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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Production of BioBran/MGN-3

1) Rice bran as an ingredient of immunomodulatory food

Rice bran consists mainly of components of the cell wall of the coat of rice seeds, which works as a strong barrier protecting the seed. It includes cellulose, hemicellulose, pectin, and lignin, all of which are polymeric. These components are all types of dietary fiber, of which rice bran is a good source. The recent recommendation to take unpolished rice in order to maintain and promote health is certainly correct from the aspect of dietary fiber intake.

Hemicellulose, one of these components, is a heteroglycan comprising several monosaccharides and has a complex structure. Its molecular weight (MW) is lower than that of cellulose. If cellulose is likened to concrete blocks, hemicellulose can be said to play the role of the concrete that glues the blocks together.

Hemicellulose B, one of these types of hemicellulose, has attracted attention as a possible component of immunomodulatory foods. Hemicellulose B is water-soluble and is contained in rice bran in a ratio of 3-5 grams per kilogram.

Hemicellulose B also has a complex structure, consisting of a long xylose chain with arabinose branches,

each of which has further smaller branches such as galactose, glucose, or uronic acids. However, its MW is far lower than those of other hemicelluloses. It is not digested in the human gastrointestinal tract because of its chemical structure, but its size suggests the possibility that the molecule is absorbed without a change in structure. Hemicellulose B is a very interesting potential ingredient of immunomodulatory foods.

There are many reports on BRMs, also known as immunomodulatory substances, including many on dietary fiber BRMs. All these are polymeric. When they administered and absorbed into the blood, antigen-presenting cells recognize them as foreign, which activates the immune system. β-1,3-glucan in various mushrooms and α-1,6-mannan in baker's yeast produce high activity. However, many dietary fiber BRMs are not effective when taken orally, i.e. they are not absorbed from the gastrointestinal tract. This may be because of their MW and chemical structure. Even if they have high activity as BRMs, ingredients which cannot be absorbed cannot be used in immunomodulatory foods, but must be injected intravenously as purified molecule. In recent years, it has been reported that polymeric components are effective in stimulating the intestines but the causal relationship has not been shown quantitatively.

2) The preparation of a derivative of rice bran hemicellulose B by modification

Rice bran hemicellulose B has a complex sugar composition. Its main constituent sugars are arabinose and xylose and its other sugars include rhamnose, galactose, mannose, glucose and uronic acid. Hemicellulose B itself has no immunomodulatory action. We have tried to produce its derivatives by modification using a food processing technique. The constituent sugars, apart from arabinose and xylose, were hydrolyzed enzymatically to obtain a derivative of hemicellulose. As the carbohydrase allows reactions under very mild conditions including low temperature, a derivative can be produced without causing changes in the basic structure of complex hemicellulose.

Enzymes are widely used in food- processing. Representative examples are glucoamylase, used in the saccharification of starch in alcohol fermentation, pectin esterase, in the clarification of apple juice, and 5-phosphodiesterase and adenylic acid deaminase, in the production of nucleic acid flavourings. Although enzyme preparations are generally used in these products, in some cases a culture of enzyme-producing microorganisms is incorporated into the production process, and the culture filtrate then goes through a rough purification process and is used in production. Since rice bran hemicellulose B, with its complex structure, has a wide variety of hydrolysable linkages, we thought it better for efficient production to use an enzyme complex obtained from a culture fluid of microorganisms that produce enzymes which can degrade many types of plant tissue, rather than combined enzyme preparations.

The obvious choice for microorganisms that degrade plant tissue are basidiomycetes in the fungal group, in other words, mushrooms. Most basidiomycetes grow using wood as a source of nutrition. They hydrolyze polymeric fibers and lignin into lower-level molecular substances by means of a range of enzymes and use them as a source of nutrition. As a result, culture filtrates of asidiomycetes contain many carbohydrases. These work as an enzyme complex that catalyzes serial hydrolytic reactions changing polysaccharides to monosaccharides. The use of the enzyme complex accelerates the reaction, resulting in the efficient production of the final, desired product.

A shiitake fungus was fluid-cultured, the culture filtrate partially purified to obtain a carbohydrase complex, and the enzyme complex used to modify rice bran hemicellulose B. This enzyme complex contains α-glucosidase, β-glucosidase, and galactosidase, but no enzymes that catalyze hydrolysis of xylose

or arabinose-xylose linkages. The enzyme complex was reacted with rice bran hemicellulose B to obtain the rice bran hemicellulose derivative BioBran/MGN-3, which has an interesting immunomodulatory action.

Chemical Structure of the component Involved in Immunoregulation

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Background and Objective

BioBran/MGN-3 derived from rice bran is known to have various physiological functions including NK cell and macrophage activation and its immunomodulatory function is attracting attention. In addition, BioBran/MGN-3 has been reported to have antitumor activity and its clinical application as an antitumor agent is anticipated. Application of BioBran/MGN-3 requires analyses of the mechanisms of various physiological actions and the active ingredients. Although many studies on BioBran/MGN-3 have been reported, there are few detailed studies on the active component of BioBran/MGN-3 and the immunomodulatory ingredient is unknown.

This study analyzed the component of BioBran/MGN-3 involved in immunomodulatory activity using macrophage stimulation as the indicator as well as the chemical structure of the active ingredient.

This is a report of 2004 Annual Meeting of the Japanese Society of Applied Glycoscience.

Materials and Methods

1. Fractions of BioBran/MGN-3

1) BioBran/MGN-3 was dissolved in purified water and the soluble fraction was further separated into 50% (50 ppt), 66% (66 ppt), and 80% (80 ppt) methanol precipitation fractions and 80% methanol supernatant fraction (80 sup) by fractional alcohol precipitation.

2) The 80% methanol insoluble fraction was applied to a DEAE-Sephadex A-25 column equilibrated with 20 mM Na-acetate buffer (pH 5.0) and eluted stepwise with NaCl to obtain 5 fractions I to V. Fraction II, eluted with 0.2 M NaCl, was applied to a DEAE-Sephadex A-25 column again and subfractions II-1 to II-7 were obtained by elution using a linear concentration gradient of 0-0.5 M NaCl.

2. Evaluation of macrophage activation

Female C57BL/6 mice aged 6-7 weeks were injected intraperitoneally with 4.05% thioglycolate medium (Difco) , the abdominal skin was removed after 4 days, and 5 ml of cold PBS was introduced into the peritoneal cavity to collect peritoneal exudate cells (PEC). Mac-1^+ cells (peritoneal macrophage) were separated using a magnetic bead cell sorter (Miltenyl Biotec) and cultured in the presence of BioBran/MGN-3 or each BioBran/MGN-3 fraction for 24 hours, and the culture supernatant was isolated. NO^{2-} in the culture supernatant was determined using the Griess method and tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) were determined using ELISA (Biosource).

3. Sugar analysis for each fraction

Analyses of sugar composition and linkages (methylation) and analysis of fragments with an enzyme were performed.

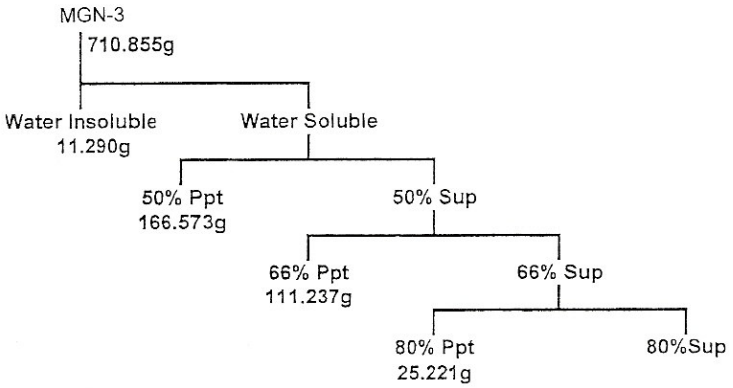

Fig. 1 Fractionation of MGN-3 by the graded methanol precipitation method

Results and Discussion

1. Search for an immunomodulatory component by alcohol fractional precipitation

BioBran/MGN-3 solution in purified water was subjected to stepwise alcohol fractional precipitation. As a result, 167 g of the 50% methanol precipitation fraction, 111 g of the 66% methanol precipitation fraction,

and 25 g of the 80% methanol precipitation fraction were obtained from about 710 g of BioBran/MGN-3 (**Fig. 1**). The yield by methanol fractional precipitation was 23.4% for 50 ppt, 15.6% for 66 ppt, and 3.5% for 80 ppt.

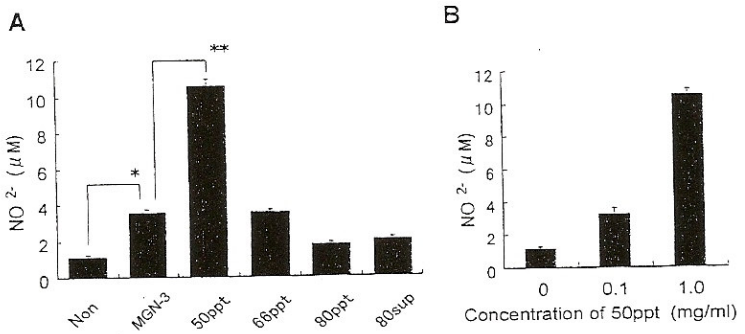

Fig. 2 Effect on NO^{2-} production of MGN-3 subfractions. A, comparison of the NO production in respectively stimulating the macrophage in MGN-3, 50%, 66%, 80% methanol precipitation fractions or, 80% methanol soluble fraction. B, dose dependency of 50%, 66%, 80% methanol precipitation fractions on NO^{2-} production. Each bar value represents the mean ± standard deviation based on four wells.

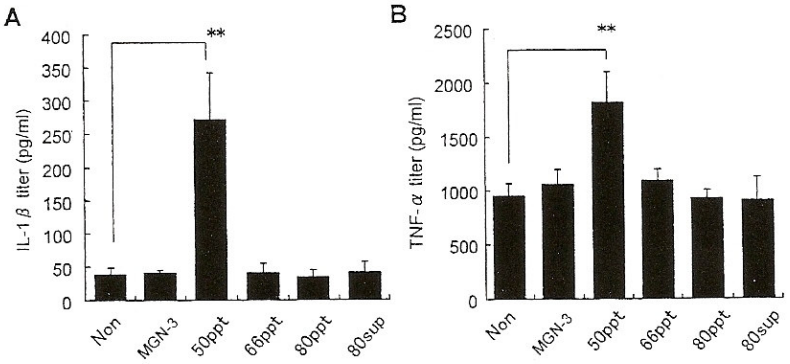

Fig. 3 Effect on cytokine production of MGN-3 subfractions, 50ppt, 60ppt, 80ppt, and 80sup. TNF-α (A) and IL-1β (B) production in culture supernatants after 24 h of incubation were estimated. Each bar value represents the mean ± standard deviation based on four wells.

Macrophages were stimulated *in vitro* with each precipitation fraction to compare their abilities to activate macrophages with BioBran/MGN-3. NO^{2-} production by machrophages was 3 times higher after stimulation with the 50 ppt fraction than after stimulation with unfractionated BioBran/MGN-3 (**Fig. 2A**). On the other hand, the 66% and 80% methanol precipitation fractions and 80% methanol soluble fraction induced production slightly but the NO^{2-} activities produced were equivalent to or lower than that of BioBran/MGN-3 (**Fig. 2A**). The NO^{2-} production of macrophages stimulated with the 50% methanol precipitation fraction was dose-dependent (**Fig. 2B**).

Next, the production of IL-1β and TNF-α were determined after stimulation with methanol precipitation fractions. BioBran/MGN-3 scarcely induced IL-1β, while the 50% methanol precipitation fraction induced it markedly (**Fig. 3A**). Similarly, the 50% methanol precipitation fraction markedly increased TNF-α

production (**Fig. 3B**). There are reports of the activation of immune cells with BioBran/MGN-3. Our study confirmed the activation of macrophages with BioBran/MGN-3. The ability to activate macrophages was 3-5 times higher for the 50% methanol precipitation fraction than for BioBran/MGN-3 itself and the 50% methanol precipitation fraction accounted for 23.4% of BioBran/MGN-3. From findings these, it is inferred that the immunomodulatory ingredient of BioBran/MGN-3 is present in this fraction.

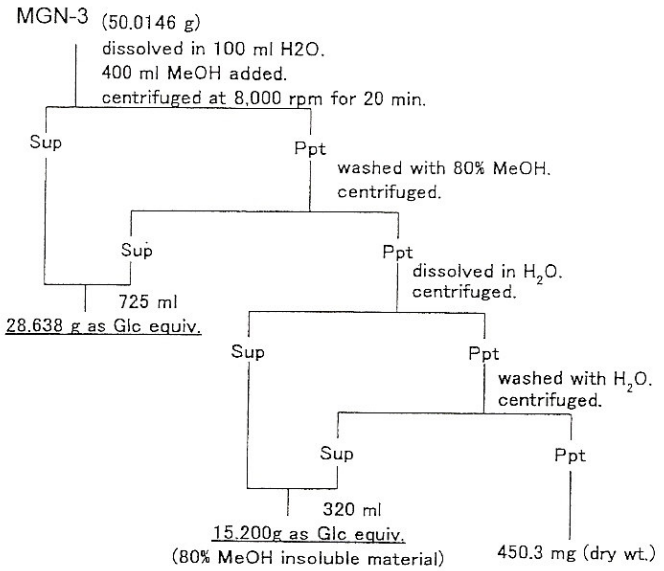

Fig. 4 A summary of preparation procedures of 80% MeOH insoluble material from MGN-3

Table 1 Neutral sugar composition of fraction I~V obtained from the 80% MeOH insoluble material by DEAE-Sephadex A-25 chromatography

Fraction	Yield (mg)	Neutral sugar composition (mol%)						
		Rha	Fuc	Ara	Xyl	Man	Glc	Gal
I	164. 7							
II	115. 0	1. 0	0. 2	23. 0	15. 1	2. 0	48. 1	10. 7
III	127. 2	1. 9	5. 9	15. 7	7. 5	1. 3	30. 1	37. 8
IV	57. 8	–	–	2. 7	1. 7	2. 4	89. 2	4. 0
V	38. 4							

80% MeOH insoluble material (14.49 g as Glc equiv./ 305ml) applied on a column (5×27cm) of DEAE-Sephadex A-25 equilibrated with 20mM Na-acetate buffer (pH 5.0) and eluted stepwise with 20mM Na-acetate buffer (fraction I), 0.2M NaCl in the same buffer (fraction II), 0.5M NaCl in the same buffer (fraction III), 1.0M NaCl in the same buffer (Fraction IV) and 0.5M NaOH (fraction V). Fractions II, III, IV and V were dialyzed against distilled water. Fraction I was treated with amylases, and then dialyzed against distilled water.

2. Search for an immunomodulatory component by ion-exchange chromatography

Methanol was added to the water soluble fraction of BioBran/MGN-3 to a final concentration of 80%, the precipitate was separated, washed with 80% methanol, and removed again, and after purification, 15 g of

the 80% methanol precipitation fraction (yield 30.4%) was obtained from 50 g of BioBran/MGN-3 (**Fig. 4**). This insoluble fraction was applied to a DEAE-Sephadex A-25 column equilibrated with a 20 mM Na-acetate buffer (pH 5.0) to obtain non-absorptive fraction I, fraction II eluted with 0.2 M NaCl, fraction III eluted with 0.5 M NaCl, fraction IV eluted with 1.0 M NaCl, and fraction V eluted with 0.5 M NaOH. Fractions II to V were dialyzed against water and fraction I was digested with amylase and there dialyzed against water. **Table 1** shows the yields and neutral sugar compositions of fractions II, III, and IV eluted with NaCl. Fraction II mainly contained arabinose, xylose, glucose, and galactose. Fraction III contained less arabinose and xylose but more galactose than fraction II. For fraction IV, glucose accounted for 89.2% of the neutral sugar while the contents of other neutral sugars were very low.

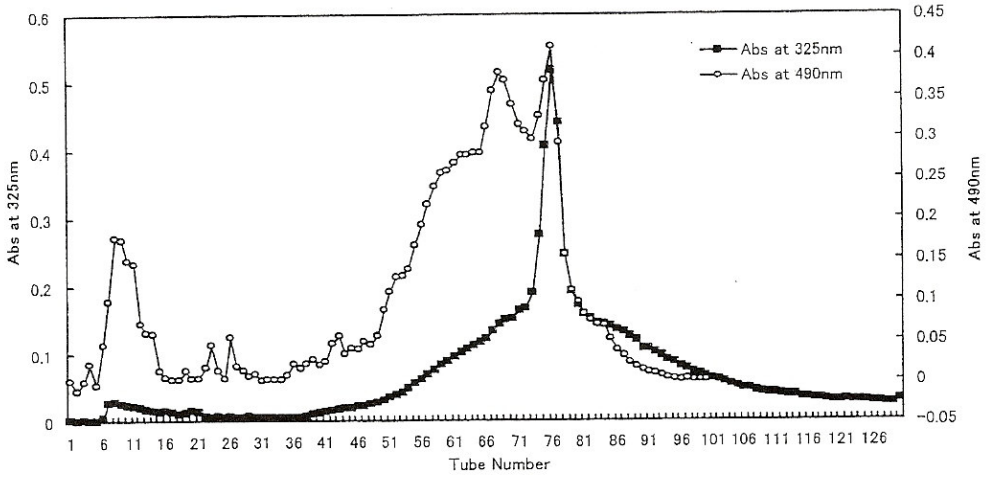

Fig. 5 **DEAE-Sephadex A-25 chromatography of Fr. II. Fraction II obtained from MGN-3 was applied to a column of DEAE-Sephadex A-25 equilibrated with 20 mM Na-acetate buffer, and then the column was washed with the same buffer. The adsorbed materials were eluted with the salt gradient (0~0.5 M NaCl in the Na-acetate buffer): 9.8ml-fractions were collected. Tubes 6-15 (Fr. II-1), 36-49 (II-2), 50-53 (II-3), 54-65 (II-4), 66-73 (II-5), 74-79 (II-6) and 80-90 (II-7) were separately combined and dialyzed against distilled water.**

Fraction II, with relatively high arabinose and xylose content, was applied to a DEAE-Sephadex A-25 column, for detailed fractionation by elution using a linear concentration gradient of 0-0.5 M NaCl. Based on absorption peaks of sugars, subfractions II-1 to II-7 were obtained (**Fig. 5**). In UV absorption, only subfraction II-6 showed a clear peak, suggesting that the other 6 subfractions contained almost no protein. Among the 7 subfractions, II-4, II-5, and II-6 were analyzed for macrophage activation and neutral sugar compositions. NO^{2-} production after stimulation with subfractions II-4 to II-6 was higher than that by the 50% methanol precipitation fraction and was 4-5 times higher than that with BioBran/MGN-3 (**Fig. 6A**). Lipopolysaccharide (LPS), Gram-negative outer membrane components, are known to play a priming role in the activation of machrophages. In our study, 10 μg/ml of LPS strongly stimulated macrophages. In a comparison of the subfractions with LPS, macrophages stimulated with the subfractions had NO production equivalent to or higher than those stimulated with LPS. Subfraction II-6 dose-dependently activated

machrophages and the activation inducing activity at 1/10th of the concentration was similar to that of the 50% methanol precipitation fraction (**Fig. 6B**). Among subfractions II-4 to II-6, only II-6 contained a UV absorbing component likely to be protein. Subfractions II-4 and II-5 were similar to II-6 in ability to activate macrophages, suggesting that the UV absorbing material plays no direct role in the activation of macrophages. Thus, it is inferred that polysaccharides in subfractions II-4, II-5, and II-6 have ability to activate macrophages.

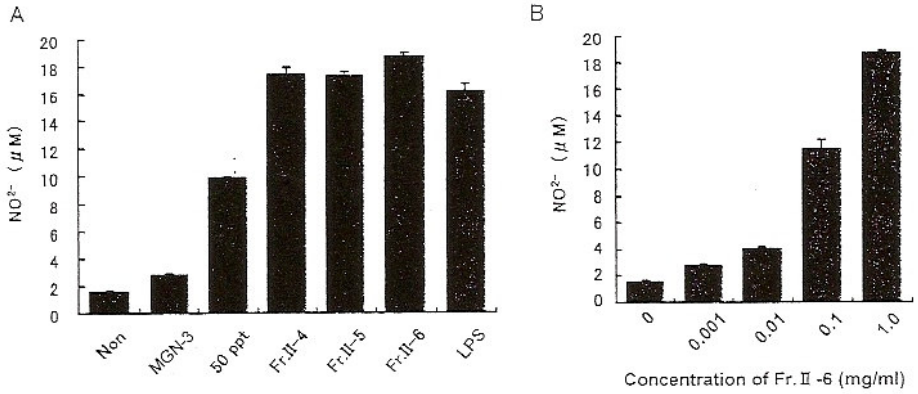

Fig. 6 **Effect on PEC activation of MGN-3 subfractions, Fr. II-4, Fr.II-5, and Fi. II-6. NO^{2-} production in culture supernatants after 24h of incubation was estimated. A, comparison of NO^{2-} production between crude MGN-3, 50% NeOH insoluble fraction (50ppt), and subfraction II eluted with 0.2M NaCl. B, dose dependency of the Fr. II-6 in NO^{2-} production from PEC.**

Table 2 **Neutral sugar composition of subfractions, II-1 ~ II-7 obtained from fraction II by DEAE-Sephadex A-25 chromatography (Fig. 5).**

Fraction (Tube Nos.)	Yield (mg)	Neutral sugar composition (mol%)						
		Rha	Fuc	Ara	Xyl	Man	Glc	Gal
II-1 (6-15)	1.6							
II-2 (36-49)	1.9							
II-3 (50-53)	1.5							
II-4 (54-65)	13.4	1.1	0.4	22.5	19.4	4.7	26.7	25.2
II-5 (66-73)	13.2	2.7	0.7	22.2	14.8	3.5	32.2	23.9
II-6 (74-79)	9.2	7.6	0.6	22.2	13.7	2.7	30.2	23.0
II-7 (80-90)	4.2							

3. Structural analysis of an immunomodulatory component

Subfraction II-6 with the highest macrophage stimulating activity was analyzed for molecular weight (MW) distribution by gel filtration using a Sepharose CL-6B (**Fig. 7**). It had a wide distribution of MW and comparison with a standard dextran suggested that the mean size was 10,000-20,000 Daltons. Methylation analysis was performed to study sugar linkages of the polysaccharides in II-6. Subfraction II-6 methylated by Hakomori's method was hydrolyzed and analyzed as alditol acetate by gas chromatography (**Fig. 8** and **Table 3**). As a result, the subfraction showed peaks of terminal non-reducing glucose, 5-linked arabinose, terminal non-reducing galactose, 4-linked xylose, 3,5-linked arabinose, 3-linked glucose, 4-linked glucose, 2,4-linked galactose, 4,6-linked glucose, and 3,6-linked galactose, showing that the main components are

arabinose, galactose, and glucose. Although it is difficult to estimate the structure of polysaccharides in II-6 from these methylated monosaccharides with various linkages, the approximate structure could be predicted based on the structures of many polysaccharides that compose the plant cell wall.

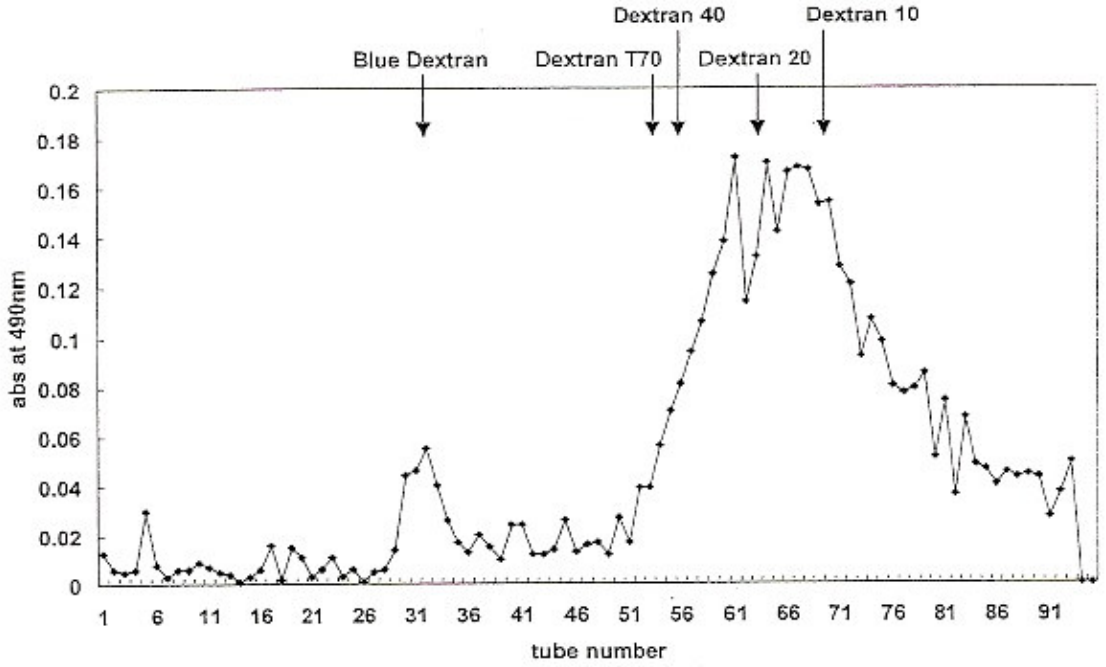

Fig. 7 Molecular weight distribution of fr.II-6

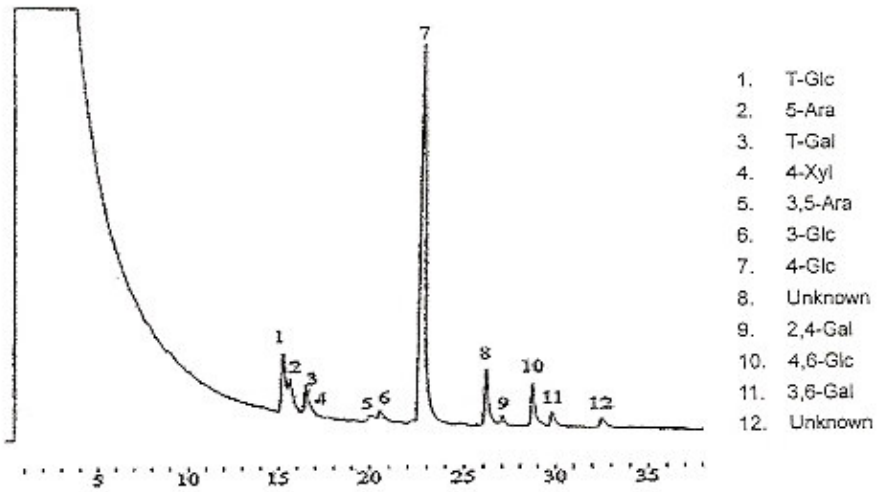

Fig. 8 Glycosidec linkage analysis of fr.II-6

Table 3 Sugar linkage composition of fr.II-6

Peak No.	Methylated sugar linkage	Deduced glycosidic linkage*	Amount**	Peak No.	Methylated sugar linkage	Deduced glycosidic linkage*	Amount**
1	2,3,4-Me3-Ara***	T-Ara	13.9	17	2,3,6-Me3-Glc	4-Glc	12.1
2	2,3,4-Me3-Xyl	T-Xyl	0.6	18	Ara	2,3,5-Ara	3.3
3	Unidentified		0.1	19	Unidentified		0.9
4	Unidentified		0.6	20	Unidentified		0.9
5	3,4-Me2-Rha	2-Rha	1.8	21	Unidentifie		1.3
6	2,3,4,6-Me4-Glc and/or	T-Glc and/or	3.5	22	3,6-Me2-Gal	2,4-Gal	3.7
	2,3,4,6-Me4-Man	T-Man		23	Unidentified		0
7	2,3-Me2-Ara	5-Ara	13.1	24	2,3-Me2-Glc	4,6-Glc	0.3
8	2,3,4,6-Me4-Gal	T-Gal	4.6	25	Unidentified		2.9
9	2,3-Me2-Xyl and/or	4-Xyl and/or	6.2	26	2,4-Me2-Gal	3,6-Gal	6.1
	3,4-Me2-Xyl	2-Xyl		27	Unidentified		0.6
10	Unidentified		0.3	28	Unidentified		0.9
11	Unidentified		0.4	29	Unidentified		0.1
12	Unidentified		0.3	30	Unidentified		0.3
13	Unidentified		0.7	31	Unidentified		0.2
14	2,4,6-Me3-Glc		3.6	32	Unidentified		2.5
15	2,4,6-Me3-Gal and/or		2.4	33	Unidentified		2.3
	2,3,6-Me3-Man						
16	2,3,6-Me3-Gal,		9.2				
	3,4,6-Me3-Gal and/or						
	2,3,4,-Me3-Glc						

*The numerical prefixes represesnt the carbon atoms involved in glycosidic linkages in the original polysaccahrides. Prefix T indicates sugaras linked through C(O)-1 only.
**% total area.
***2,3,5-Me_3-Ara = 2,3,5-tri-O-methyl-1,4-di-O-acetyl-arabinitol, etc.

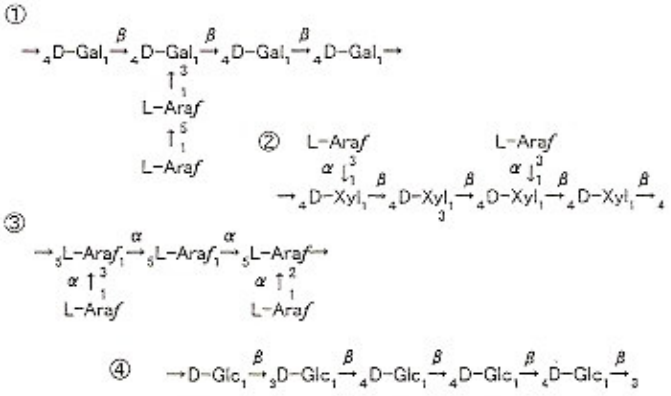

Fig. 9 Possible structures for the polysaccharide present in fr.II-6

The results of methylation analysis suggested the following polysaccharide structures (**Fig. 9**) (1)

arabinogalactan with a main chain of 1,4-β-galactan and side chains of arabinose, (2) arabinoxylan with a main chain of 1,4-β-xylan and side chains of arabinose, (3) arabinan with the main chain of 1,5-α-arabinan and side chains of arabinose, and (4) β-1,3:1,4-glucan. Further analysis is needed to determine whether these polysaccharides are mixed or present as parts of one molecule. However, the immunomodulatory ingredient of BioBran/MGN-3 is estimated to be a heteropolysaccharide of complex structure.

I-4

The Safety of Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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Introduction

The ingredients in BioBran/MGN-3 are rice bran and shiitake-fungus culture fluid. Rice bran is classified as a grain food and listed as Food Number 1-57 in the 5th edition of the Japan Food Composition Table. The method of extracting constituents from rice bran in the production process for BioBran/MGN-3 is performed with lukewarm water and no organic solvents are used. The extracted polysaccharides, which consist mainly of hemicellulose, are modified with carbohydrase, derived from the shiitake mushroom. The shiitake mushroom has been used in food since ancient times in Japan. A carbohydrase complex is produced from the secondary mycelium of shiitake mushrooms. Carbohydrase preparations are often produced by biosynthesis using one of any number of species of fungi and purified. Based on the result of a study into cost and safety, we chose to use shiitake-fungus culture fluid, being an enzyme preparation which is sufficiently active even after rough purification, and has no safety problems even if present in the final food product. In short, BioBran/MGN-3 has no safety problems because the production process uses no materials that might raise doubts about food safety. However, since the rice bran ingredient is

concentrated in BioBran/MGN-3 and the hemicellulose is partially modified, ingesting BioBran/MGN-3 is slightly different from taking wholegrain rice or rice bran. For this reason, we have conducted experiments to verify the basic safety of BioBran/MGN-3 as a food.

1. Acute oral toxicity

AMA LABORATORIES, INC.(NY, USA)

(1) Animals

Healthy, young adult Wistar derived aibino rats weighing between 150 to 300 grams were obtained from ACE Animals, Inc., Boyertown, Pennsylvania. 5 male and 5 female rats were selected for each dose level chosen for this study.

(2) Procedure

18 to 24 hours prior to dosing, the rats were fasted. During the fast period water was allowed *ad libitum*. After fasting rats were individually weighed. All body weights were recorded and individual doses calculated based on these weights. The test material was then made available at dose levels chosen to achieve test groups with sufficient mortality rates to permit calculation of the LD_{50}. Once the material had been ingested completely, feed and water were provided *ad libitum*. The rats were individually caged and observed for mortality or other signs of gross toxicity for 14 days. At the end of the test period, all surviving animals were weighed.

(3) Conclusions

The above material (AMA Lab No.:B-4881, Client No.:DAI-001 BioBran/MGN-3) when tested as indicated herein may be regarded as NON-TOXIC according to the reference. $LD_{50} > 36.0$ g/kg.

2. Ames salmonella mutagenicity test

Product Safety Laboratries (NJ, USA)

(1) Assey objective

The purpose of this protocol is to evaluate the ability of a chemical, formulation or extract to induce a mutagenic response in 4 different strains of *Salmonella typhimurium*, namely TA98, TA100, TA1535, TA1537 and one strain of *Escherichia coli* WP2 uvrA (pKM101). Test materials are screened at different dose levels by plating them with the tester strains both directly and along with Aroclor™1254 induced rat liver microsomes (S9). Mutagenic materials cause an increase in revertant colonies above the spontaneous background (i.e. no test material) level.

(2) Overview

The *S. typhimurium* reversion assay is widely used to evaluate the mutagenic properities of chemicals. The test is based on the work of Dr. Bruce Ames and his coworkers and is generally referred to as the Ames Test. Their studies involved the development of select histidine auxotrophs of *S. typhimurium* which are normally growth arrested due to mutations in a gene needed to produce the essential amino acid histidine. In the absence of an external histidine source, the cells cannot grow to form colonies unless a reversion of the mutation occurs which allows the production of histidine to be resumed. The *E.coli* WP2 uvrA (pKM101) is also used to add a DNA repair-proficient strain; it has an AT base pair at the critical mutation site within the *trpE* gene, which is excision proficient (and thus will detect cross-linking agents) and carries the pKM101 plasmid to enhance error-prone repair. As might be expected, spontaneous reversions occur with each of the strains, usually at a low level (with the exception of strain TA 100).

However, chemical agents can induce a mutagenic response so that the number of revertant colonies is substantially higher than the spontaneous background reversion level. The test involves the analysis of the number of revertant colonies that are obtained with each strain in the presence and absence of the test chemical. Since the mutagenic response of a formulation could vary with the concentration, test materials are routinely dosed over an appropriate concentration range. Samples can also be plated in duplicate or triplicate, at the discretion of the sponsor. In this protocol, a complete set of positive and negative controls is included with each assay, and is plated routinely with all of the tester strains. Aroclor™1254 induced rat liver microsomes are included to mimic the *in vivo* activity of the liver enzymes in activating some pro-mutagens to mutagenic status.

(3) Conclusion

The results show that the test strains are sensitive to the positive control mutagens and had a spontaneous reversion rate well within the accepted values of each strain, indicating that under the test conditions, the strains were sensitive to the detection of potentially genotoxic agents. The metabolic activation using the S9 activation mixture shows an active microsomal preparation. Using the same test conditions, there was no detectable genotoxic activity associated with the sample under testing BioBran/MGN-3 Lot # DAI-001, neither in the presence or absence of the S9 enzyme activation, at the following concentrations: 100,000, 50,000, 10,000, 5,000 and 1,000 μ g/mL.

3. Subchronic toxicity study (90-day dietary study in rats)

Product Safety Laboratries (NJ, USA)

(1) Purpose

To determine the oral toxicity and health hazards likely to arise from continuous exposure to BioBran/MGN-3 in the diet over a 90-day period. The assessment of toxicity was limited to the toxicological endpoints evaluated in this study. A No-Observed-Adverse Effect Level (NOAEL) was also sought for each sex based on these endpoints.

(2) Summary

80 Sprague-Dawley rats (40 males and 40 females) were selected for the test and equally distributed into 8 groups (10 per group, males or females only). Dietary levels of 0, 200, 2,000, and 20,000 ppm of the test article were selected for the test. An appropriate amount of the test article was administered in the diet to each rat for 91 consecutive days. The animals were observed for mortality, signs of gross toxicity, and behavioral changes at least once daily the study. Body weights were recorded once during the acclimation period, prior to test initiation (Day0) and weekly thereafter. Individual food consumption was also recorded weekly. Blood was collected from randomly selected animals (3 per group for all groups except for the male control group in which there were 4) on Day 92 prior to necropsy, and analyzed for a series of blood chemistry and hematology parameters. Necropsies were performed on the decedent and all euthanized animals. Tissues and organs of 5 randomly selected animals from each control and high dietary level group were evaluated histologically.

The average dietary intake of Hydrolysate of BioBran/MGN-3 in male rats administered diets at nominal concentrations of 0, 200, 2,000 or 20,000 ppm was 0, 13, 134, and 1,301 mg/kg/day, respectively. The dietary intake of female rats at the same nominal dietary concentrations was 0, 15, 150 and 1562 mg/kg/day, respectively.

(3) Conclusion

There were no significant effects in rats of either sex after 91 consecutive days of BioBran/MGN-3 presented in the diet. Based on the parameters evaluated (which were limited in scope), the No-Observed-Adverse Effect Level (NOALE) for this study is considered to be 20,000 ppm (1,301 mg/kg/day in males and 1,562 mg/kg/day in females).

4. Guinea Pig Antigenicity Study

Product Safety Laboratries (NJ, USA)

(1) Procedure

The first two group of animals were exposed to the test or control articles over a 3-week sensitization period. During this time, each animal was injected intra-peritoneally (IP) with 10 mg/kg of the test or control article 3 days per week for 3 successive weeks. The test and positive control articles were administered as 1% w/w mixtures in physiological saline. The doses were adjusted weekly based on the most recent body weight. After the last IP injection, the animals were allowed to rest for 17 days. Following the rest period, the guinea pigs were challenged with 2 mg/kg of the test or control articles by intravenous (IV) injection. The test or positive articles were administered as 0.2% w/w mixtures in physiological saline.

In addition to the test and positive control animals, ten native control guinea pigs (5 test and 5 positive control) from the same shipment were maintained under identical environmental conditions and treated with the test or control article at challenge only.

During the challenge injections and the 15 minutes thereafter, all animals (test and control) were observed for the following positive reactions:

i licking the nose or rubbing the nose with forefeet
ii rubbing of the fur
iii abored breathing
iv sneezing or coughing (3 or more times)
v retching

The test article would be considered antigenic if any of the challenged test animals show more than two of the listed symptoms or show rales, convulsions, prostration or die. If the corresponding naïve animals exhibit similar signs, the test is considered inconclusive. In order for the test to be considered valid, at least 3 of the positive control animals need to show at least two of the above listed symptoms.

(2) Results

All test substance, test substance naive control, positive control and positive naive control animals survived following the challenge phase injection.

Group1 Test Substance

All test animals appeared active and healthy throughout the sensitization phase. Apart from chewing noted in 8 of 10 test animals 3 to 15 minutes after challenge, no other positive reactions were observed. Overall, this was considered a negative response.

Group2 Positive Control

All positive control animals appeared active and healthy throughout the sensitization phase. Following challenge, all positive control animals exhibited an antigenic response. Positive reactions including chewing, retching, licking, rubbing, coughing, labored breathing, prone posture and/or ataxia were observed in all

animals after challenge.

Group3 Test Substance Naive Control

No positive reactions were noted in any of the test naive control animals following the challenge injection.

Group4 Positive Naive Control

No positive reactions were noted in any of the positive naive control animals following the challenge injection.

(3) Conclusion

Under the conditions of this study, BioBran/MGN-3 is not considered to be antigenic. The positive response in the positive control group to Egg Albumin, a known antigenic agent, validates the system used.

Summary

The safety of BioBran/MGN-3 has been verified by conducting acute oral toxicity, mutagenicity, subacute toxicity, and antigenicity studies. The safety of BioBran/MGN-3 in humans has also been verified by a 6-month ingestion study in cancer patients (Hiroshi Tsunekawa: Effect of Long-term Administration of Immunomodulatory Food on Cancer Patients Completing Conventional Treatments. Clinical Pharmacology and Therapy 14(3): 295-302, 2004). From the results of the studies, BioBran/MGN-3 has been judged to be a highly safe food.